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Coexpression of Exogenous and Endogenous Mouse Mammary Tumor Virus RNA In Vivo Results in Viral Recombination and Broadens the Virus Host Range

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Mouse mammary tumor virus is a replication-competent B-type murine retrovirus responsible for mammary gland tumorigenesis in some strains of laboratory mice. Mouse mammary tumor virus is transmitted horizontally through the milk (exogenous or milk-borne virus) to susceptible offspring or vertically through the germ line (endogenous provirus). Exogenously acquired and some endogenous mouse mammary tumor viruses are expressed at high levels in lactating mammary glands. We show here that there is packaging of the endogenous *Mtv-1* virus, which is expressed at high levels in the lactating mammary glands of C3H/HeN mice, by the virions of exogenous C3H mouse mammary tumor virus [MMTV(C3H)]. The mammary tumors induced in C3H/HeN mice infected with exogenous MMTV(C3H) virus contained integrated copies of recombinant virus containing a region of the *env* gene from an endogenous virus. This finding indicates that there was copackaging of the *Mtv-1* and MMTV(C3H) RNAs in the same virions. Moreover, because *Mtv-1* encodes a superantigen protein with a Vβ specificity different from that encoded by the exogenous virus, the packaging of *Mtv-1* results in an infectious virus with a broader host range than MMTV(C3H).

Retroviruses are subject to recombination with other endogenous or exogenous retroviral genomes present in the same cell because the RNA-dependent DNA polymerase, reverse transcriptase, can switch templates during replication (16, 20). Retroviruses have dimeric genomes; two homologous or heterologous positive-strand RNA molecules are held together at or close to their 5′ ends in virus particles (8). High-frequency retroviral recombination due to strand switching occurs following infection of cells by virus particles containing two different RNA strands (19). Such heterozygous viruses result either from infection of a given cell expressing endogenous proviruses by exogenous viruses or from coexpression of two different endogenous or exogenous sequences (6, 19, 36). As a result of the recombination events, new infectious retroviruses with altered biological activities can be generated (7).

Mouse mammary tumor virus (MMTV) is an endogenous virus in mice that is expressed at high levels in the lactating mammary gland (5, 18), in part as a result of a tissue-specific enhancer localized in the long terminal repeat (LTR) (27). MMTV can also be acquired as an infectious agent, when newborn mice suckle on viremic females that shed virus particles in milk (28, 29). This virus, termed exogenous MMTV, infects cells of the mammary gland, leading to mammary tumors when it integrates near cellular oncogenes (31). The majority of endogenous *Mtv* proviruses are noninfectious and are not associated with mammary gland tumors (24).

MMTV has an open reading frame in its LTR that encodes a superantigen (Sag) protein (1, 3). The Sag proteins encoded by endogenous MMTV proviruses stimulate the proliferation of T cells with specific Vβ chains of the T-cell receptor in mixed cultures of spleen cells from mice of different genetic backgrounds that are matched at the major histocompatibility complex locus (3, 32) and cause T-cell deletion during shaping of the immune repertoire in vivo (11, 12, 40). Variations in the amino acid sequence of the C-terminal end of the Sag proteins encoded by the different endogenous proviruses result in the stimulation or deletion of different Vβ-bearing T cells (2, 4). It has recently been shown that the Sag stimulation of T cells is a requisite step in the MMTV infection pathway (13, 17). Furthermore, mice that express endogenous Sag proteins and delete cognate T cells are resistant to infection by exogenous MMTVs encoding Sag proteins with the same Vβ specificity (13, 17).

Because exogenous MMTV infects epithelial cells of the mammary gland, endogenous MMTV proviruses that are highly expressed in this tissue could be copackaged with exogenous virus and result in the formation of recombinant virus. We show here that the endogenous *Mtv-1* locus is highly transcribed in the lactating mammary gland of C3H/HeN mice, although little or none of this viral RNA is packaged into virions. In contrast, in C3H/HeN mice infected with the exogenous virus, MMTV(C3H), the *Mtv-1* RNA is packaged into virions that are shed into milk. Because the Sag protein encoded by *Mtv-1* stimulates T cells containing a different T-cell receptor Vβ chain, this virus can infect different mice than virus that contains only exogenous virus RNA. We also show that MMTV(C3H)-induced mammary tumors contain integrated recombinant proviruses that have part of their *env* genes derived from an endogenous virus, indicating that the *Mtv-1* and MMTV(C3H) RNAs are copackaged. These results indicate that recombination could play a role in MMTV-induced mammary tumorigenesis.

MATERIALS AND METHODS

Mice. Female and male C3H/HeN MMTV$^-$, C3H/HeN MMTV$^+$, AKR/NCr, and BALB/c mice from colonies of germ-free-derived, defined-flora animals were purchased from the National Institutes of Health Frederick Cancer Research Facility, Frederick, Md.

Fluorescence-activated cell sorting analysis. Peripheral

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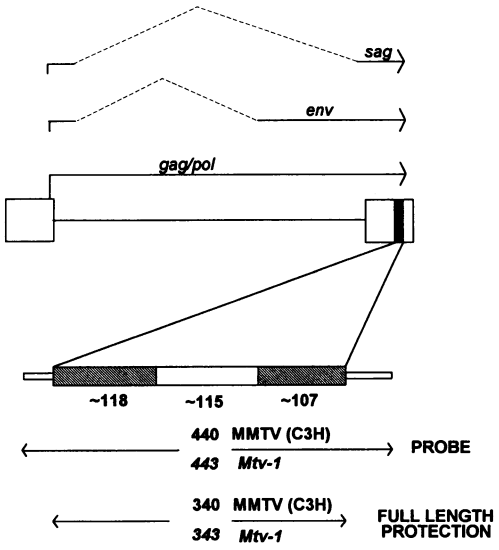

FIG. 1. Diagram of the MMTV provirus, the three transcripts generated from it, and the probes used for RNase T_1 protection analysis. The 115-bp hypervariable region of the LTRs of different MMTVs is shown as an open box of 115 bp, while the hatched boxes (118 and 107 bp) flanking this area represent regions of high homology. The probes produced from the MMTV(C3H) and *Mtv-1* plasmids were 440 and 443 nt long, respectively. RNA transcribed from the MMTV(C3H)- and *Mtv-1*-like proviruses produced protected fragments of 340 and 343 nt, respectively.

blood leukocytes were isolated as described previously (13). Cells were stained for 45 min at 4°C with titrated amounts of antibodies (rat anti-CD4 labeled with phycoerythrin [Gibco/BRL, Gaithersburg, Md.] and fluoresceinated rat anti-Vβ14 [PharMingen, Inc., San Diego, Calif.] [25] or fluoresceinated rat anti-Vβ3 [PharMingen]), washed twice, and analyzed on an electronically programmable individual cell sorter (Coulter Electronics Inc., Hialeah, Fla.). Dead cells were gated out by propidium iodide staining.

RNase T_1 protection assays. The regions containing the 3′ LTR sequences present in all three viral transcripts (filled box in Fig. 1) were isolated as *Sau*3A-*Sau*3A fragments of 340 bp [MMTV(C3H)] and 343 bp (*Mtv-1*) [map positions bp 744 to 1083 relative to the MMTV(C3H) LTR (2)] and cloned into the *Bam*HI site of the pBluescript vector (Stratagene, Inc.) (narrow open boxes in Fig. 1). For the generation of probes, pBluescript carrying either of the inserts was cut with *Xho*I, and T3 RNA polymerase was used to create [^{32}P]UTP-labeled antisense RNA. Forty micrograms of total RNA isolated from the lactating mammary glands of different strains of mice was used for RNase T_1 protection analysis as previously described (14).

Isolation of viral RNA from milk. Solidified milk was isolated from the stomachs of four to five nursing pups (1 to 2 days old), diluted in 10 volumes of phosphate-buffered saline containing 1 mM EDTA, and centrifuged at 2,000 × *g* for 15 min, and the cream and pellet were discarded. The skim milk was centrifuged at 15,000 × *g* for 30 min, the pellet was discarded, and the supernatant was recentrifuged at 95,000 × *g* for 1 h. RNA was isolated from the pellets and subjected to RNase T_1 protection analysis. The relative amounts of endogenous and exogenous viral RNA packaged were estimated by densitometric analysis of X-ray films, using the Scan Analysis program from Biosoft, Inc., and a Hewlett-Packard Scanjet Plus scanner with a Macintosh II computer.

Southern blot analyses. Mammary gland tumors were excised from the surrounding normal tissue, and DNA was isolated by proteinase K-sodium dodecyl sulfate digestion followed by phenol-chloroform extraction and ethanol precipitation. Twenty micrograms of each DNA was digested with the indicated restriction enzymes as instructed by the manufacturer (Gibco/BRL) and electrophoresed on 0.8% agarose gels. After transfer to nitrocellulose, the blots were hybridized with the ^{32}P-labeled probe diagrammed in Fig. 5B, washed, and exposed to Kodak XAR5 film, using Cronex Lightening Plus intensifying screens. Densitometric scanning and analysis were performed as described for the RNase protection autoradiograms.

RESULTS

The *Mtv-1* and *Mtv-6* proviruses are expressed in the lactating mammary glands of C3H/HeN mice. Five endogenous MMTV proviruses (*Mtv-1*, *Mtv-6*, *Mtv-8*, *Mtv-11*, and *Mtv-14*) are present in the genome of C3H/HeN mice (24). All of these loci except *Mtv-6* (which lacks the *gag* and *pol* genes and most of the *env* gene) have complete proviral genomes (24). To determine which of these *Mtv* loci were expressed in the lactating mammary glands of C3H/HeN mice, RNase T_1 protection analysis was performed with probes that were specific to the *Mtv-1* virus or MMTV(C3H) LTR (Fig. 1). The probes spanned the region encoding the C terminus of the MMTV Sag protein; this region shows the least homology among different LTRs (2). The sequences of the *Mtv-1* and *Mtv-6* LTRs in this region are similar to each other but are different from those of other sequenced endogenous and exogenous MMTV LTRs (Fig. 2) (2, 9); RNAs transcribed from the *Mtv-1* and *Mtv-6* loci are indistinguishable by this analysis. Similarly, RNA transcribed from MMTV(C3H) is indistinguishable from that expressed from the *Mtv-11*, *Mtv-8*, and *Mtv-9* endogenous viruses, because their sequences in this region are highly homologous (Fig. 2) (2). Thus, using the two probes, RNAs transcribed from the two different groups of retroviruses in several inbred mouse strains, *Mtv-1*-like versus MMTV(C3H)-like, could be distinguished.

Figure 3 represents the results of RNase T_1 protection analysis performed with total RNA isolated from the lactating mammary glands of mouse strains that have different endogenous *Mtv* loci: C3H/HeN (*Mtv-1*, *-6*, *-8*, *-11*, and *14*), AKR/NCr (*Mtv-7*, *-8*, *-9*, *-17*, *-23*, and *30*), and BALB/c (*Mtv-6*, *-8*, and *-9*) (24). With the *Mtv-1*-specific probe, a band of 343 nucleotides (nt) corresponding to full-length protection of the probe was seen with RNA from BALB/c (*Mtv-6*) (Fig. 3A, lane 2) and C3H/HeN (*Mtv-1* and *Mtv-6*) (lanes 3 and 4) but not AKR/NCr (no *Mtv-1* or *Mtv-6*) (lane 1) mice. The bands of 118 and 107 nt observed with the RNA from the lactating mammary glands of C3H/HeN MMTV$^+$ and AKR/NCr mice correspond to MMTV(C3H) and endogenous *Mtv* viruses, respectively, which have incomplete homology to the probe used (Fig. 1).

When the MMTV(C3H)-specific probe was used for similar analysis, a 340-nt band corresponding to full-length protection was seen only with the RNA from the infected mammary glands of C3H/HeN MMTV$^+$ mice (Fig. 3B, lane 3) but not uninfected C3H/HeN, BALB/c, or AKR/NCr mice (lane 4, 2, or 1, respectively). The smaller bands of 118 and 107 nt observed with RNA from the infected and uninfected lactating mammary glands of C3H/HeN and BALB/c mice corresponded to the *Mtv-1* and *Mtv-6* loci. The bands observed with RNA isolated from AKR/NCr mice represented expression from the *Mtv-17* locus (data not shown).

These experiments showed that there are at most two

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MMTV (C3H)   CACTTTTGGGGAAAGATTTTCCAT---ACCAAGGAGGGGACAGTGGCTGGACTAATAGAACATTATTCT---GCAAAAACTTATGGCATGAGTTATTATGAA---TAG
Mtv-8        A..G.....T...TAT.....G.....A...A.T.....CAT....---...G.TG...T.....C...A...G.---..A
Mtv-9,11     A..G.....T...TAT.....G.....A...A.T.....CAT....---...G.T....T.....C...A...G.---..A
Mtv-7        A..G.....AA.....T.G.TGT.T.....G.....A...A.T.....CAT....---..GG.T..C.T..A...A..G...A..A..---..A
Mtv-17       A....C.....A..TG.CTAC...G.A.....AG.TA.A..AAA.A.TT..T.TA..ATGAAATATA.TC.TGGGGG.C..G.TG.C.TCG..CCCTTT..A
Mtv-1        ---A.ACATT.G..AG.A..TT..AAC.GT.GA....A.G.CAAAAGACATA.....AT.AAG---..TTTGC.C.TA.CTT.C----------..A
Mtv-6        ---A.ACATT.G..AG.A..TT..AAC.GT.GA....A.G.CAAAAGACATA.....AT.AAG---...TTGC.C.TA.CTT.C----------..A
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FIG. 2. Sequences of the 115-bp hypervariable regions of the LTRs of MMTV(C3H) and the endogenous *Mtv* viruses described in this report (taken from reference 2).

endogenous *Mtv* loci expressed at high levels in the lactating mammary glands of uninfected C3H/HeN mice, *Mtv-1* and *Mtv-6*. Surprisingly, none of the other *Mtv* loci were expressed at detectable levels in C3H/HeN mammary glands; *Mtv-9*, *Mtv-11*, or *Mtv-14* RNA (the *Mtv-8* locus is transcriptionally silent [40]) was detected, however, in the spleens of these mice (data not shown).

Coexpression of *Mtv-1* and exogenous MMTV(C3H) results in packaging of *Mtv-1* viral RNA. *Mtv-1* was a possible candidate for packaging into MMTV(C3H) particles in C3H/HeN MMTV$^+$ mice, since it is one of two loci expressed at high levels in mammary gland cells and it has a complete proviral genome (the *Mtv-6* provirus contains primarily two LTRs) (24). To determine if *Mtv-1* RNA was packaged into virus particles, RNA was isolated from the milk of infected and uninfected C3H/HeN females and used for RNase T_1 protection analysis. Figure 4 represents the results of the analysis performed with this RNA as well as with RNA isolated from the lactating mammary gland tissue of infected and uninfected C3H/HeN females, using the *Mtv-1*-specific probe.

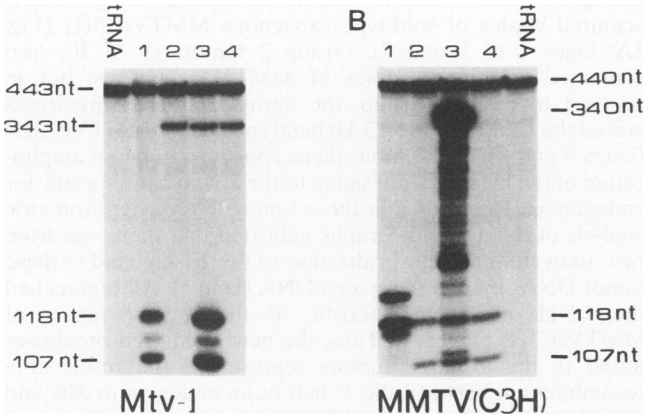

FIG. 3. Expression of endogenous *Mtv* loci in the mammary glands of different mouse strains. RNA isolated from the lactating mammary glands of AKR/NC (lane 1), BALB/c (lane 2), C3H/HeN MMTV$^+$ (lane 3), and C3H/HeN MMTV$^-$ (lane 4) mice was used for RNase T_1 protection analysis. A probe specific for the LTR of *Mtv-1* (A) or an MMTV(C3H) LTR-specific probe (B) was used. In lane 3 of panel B, in addition to the bands corresponding to exogenous virus, there are multiple bands between 118 and 343 nt, most likely corresponding to premature termination of viral RNA transcription, as previously reported (13).

RNA isolated from the milk of nonviremic females contained no detectable *Mtv-1*-specific sequences (Fig. 4, lane 1), although this RNA was abundant in their mammary glands (lane 4). This result indicates that packaging of the *Mtv-1* RNA does not occur in uninfected females and that this provirus must be defective. In contrast, both the milk (lane 2) and mammary glands (lane 3) of infected females contained large amounts of both MMTV(C3H)- and *Mtv-1*-specific RNA (at about a 10:1 ratio, as determined by densitometric analysis of

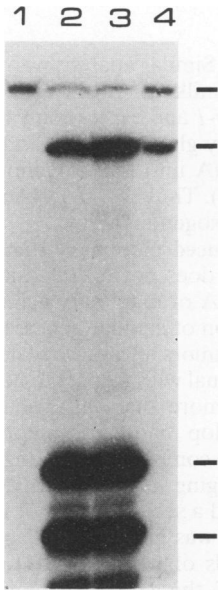

FIG. 4. Packaging of *Mtv-1* RNA into MMTV(C3H) viral particles. RNA was isolated from high-speed pellets (see Materials and Methods) of the milk of infected and uninfected C3H/HeN females and subjected to RNase T_1 protection analysis using the *Mtv-1*-specific probe. Lane 1, RNA isolated from the milk of C3H/HeN MMTV$^-$ mice; lane 2, RNA isolated from the milk of C3H/HeN MMTV$^+$ mice; lane 3, RNA from the lactating mammary glands of C3H/HeN MMTV$^+$ mice; lane 4, RNA from the lactating mammary glands of C3H/HeN MMTV$^-$ mice. Forty micrograms of total RNA isolated from the lactating mammary glands of C3H/HeN$^-$ MMTV and MMTV$^+$ mice and 1 μg of RNA isolated from the milk were used in this experiment. The 343-nt protected band corresponds to *Mtv-1* RNA; 118- and 107-nt protected bands correspond to MMTV(C3H) RNA.

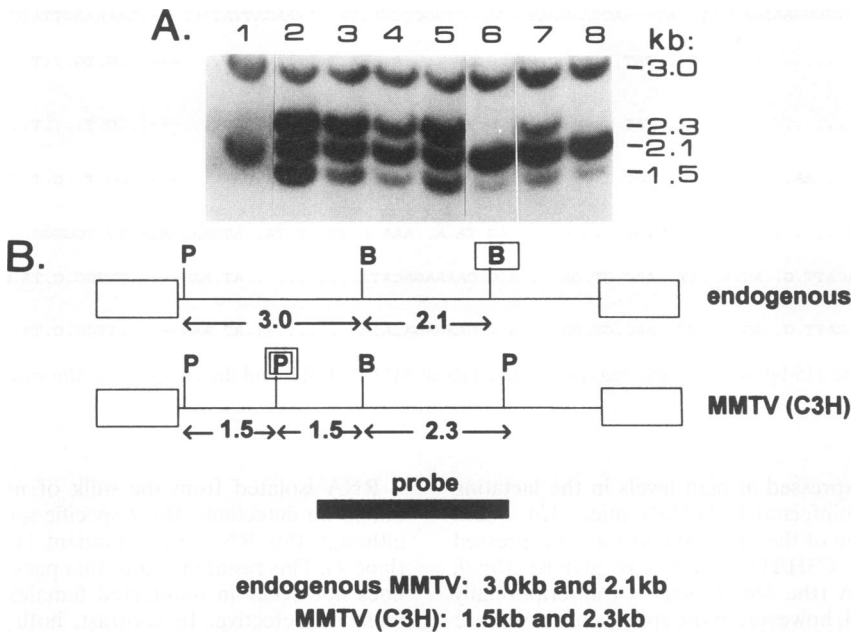

FIG. 5. Analysis of the structure of the newly integrated recombinant MMTV found in the mammary gland tumors of C3H/HeN MMTV$^+$ mice. (A) High-molecular-weight DNAs from mammary gland tumors (lane 2 to 8) and from the spleen of an uninfected C3H/HeN mouse (lane 1) were digested with *Pst*I and *Bgl*II and subjected to Southern blot analysis with the hybridization probe depicted in panel B. (B) Map of the endogenous proviruses present in C3H/HeN mice and MMTV(C3H) provirus. The presence of the 2.3- and 1.5-kb fragments is characteristic of newly integrated copies of MMTV(C3H). Abbreviations: P, *Pst*I; B, *Bgl*II.

the autoradiogram). Similar analysis was performed with RNA isolated from the milk of MMTV(C3H)-infected BALB/c mice, which lack *Mtv-1* and express only the *Mtv-6* endogenous provirus in mammary gland (Fig. 3), and no packaging of this incomplete viral RNA into the exogenous virus particles was detected (not shown). Thus, *Mtv-1* RNA is efficiently packaged into the virions of exogenous virus.

MMTV(C3H)-induced mammary tumors contain recombinant virus. MMTV does not encode an oncogene, and integration into the DNA of mammary gland cells after infection leads to the activation of cellular genes, termed *int* genes (31). The virus-induced tumors always carry new proviruses and are oligoclonal or uniclonal with respect to acquired proviruses. By 10 months of age, more than 90% of force-bred C3H/HeN MMTV$^+$ mice develop mammary tumors (15).

It is known that recombination among retroviruses appears to require copackaging of two genetically different RNA species in virions and a second round of infection (8). Because we found that there was efficient packaging of *Mtv-1* RNA in the mammary glands of MMTV(C3H)-infected mice, it was likely that some of the virions contained both *Mtv-1* and MMTV(C3H) genomic RNAs. Infection of the mammary gland with such heterologous virions could lead to viral recombination and integration of such recombinant viruses into the genome of mammary gland cells.

To determine whether recombinant MMTV was present in the mammary gland tumors of C3H/HeN MMTV$^+$ mice, DNA was extracted from 30 independent tumors that developed by 10 months of age and examined by Southern blot analysis for additional copies of exogenous or endogenous MMTV. The tumor DNA was digested with *Pst*I and *Bgl*II and hybridized to the probe diagrammed in Fig. 5B. This analysis allowed distinction of MMTV(C3H) from the five endogenous MMTV proviruses (*Mtv-1*, *Mtv-6*, *Mtv-8*, *Mtv-11*, and *Mtv-14*) present in C3H/HeN genome. The endogenous *Mtv* loci (except *Mtv-6*, which does not hybridize to this probe because it lacks the *gag* and *pol* genes and most of the *env* gene [24]) yielded fragments of 3.0 kb (*gag-pol*) and 2.1 kb (*pol-env*), while digestion of integrated MMTV(C3H) proviruses yielded fragments of 2.3 kb (*pol-env*) and 1.5 kb (*gag-pol*) (Fig. 5B).

By this analysis, the tumors could be divided into two groups. Group 1 represented the majority of tumors (21 of 30), which contained bands of both 1.5 and 2.3 kb, corresponding to newly acquired copies of wild-type exogenous MMTV(C3H) (Fig. 5A, lanes 2 to 5 and 7). Group 2 tumors (9 of 30) also contained additional copies of MMTV proviruses, but in contrast to the first group, the newly integrated proviruses lacked the characteristic 2.3-kb band specific for MMTV(C3H) (lanes 6 and 8). In addition, there appeared to be an amplification of the hybridization signal to the 2.1-kb band specific for endogenous *Mtv* viruses in these tumor DNAs; densitometric analysis of the autoradiographs indicated that there was from two- to fivefold more hybridization to the 2.1-kb band in these tumor DNAs than in the spleen DNA (lane 1). All tumors had the 1.5-kb band characteristic of the *gag-pol* region of MMTV(C3H) (Fig. 5A). Thus, the newly acquired proviruses found in the group 2 tumors represented the result of a recombination between the 3′ half of an endogenous *Mtv* and the 5′ half (including 5′ LTR, *gag*, and *pol* regions) of MMTV(C3H). Some of the DNAs in group 1 also appeared to have increased hybridization to the 2.1-kb band (Fig. 5A, lane 7), suggesting that these tumors had recombinant viruses in addition to MMTV(C3H).

These results indicated that recombination between exogenous and endogenous viruses had occurred, such that tumors of group 2 had the 5′ half of MMTV(C3H) and the 3′ half of an endogenous virus, most likely *Mtv-1*. To determine whether the entire 3′ half of the recombinant virus was derived from

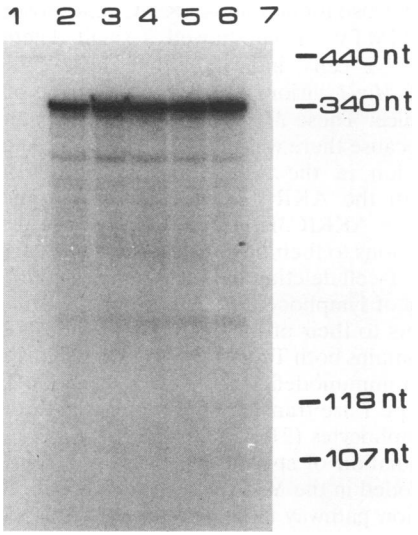

FIG. 6. RNase T_1 protection analysis with RNA isolated from mammary gland tumors. RNA was isolated from individual mammary tumors and subjected to RNase T_1 protection analysis using the MMTV(C3H)-specific probe. Lane 1, yeast tRNA; lanes 2 to 4, RNA isolated from group 1 tumors; lanes 5 and 6, RNA isolated from group 2 tumors; lane 7, RNA from the lactating mammary glands of C3H/HeN MMTV$^-$ mice. Ten micrograms of total RNA and the MMTV(C3H)-specific probe were used in this assay. Because of the high level of expression of RNA from newly integrated MMTV(C3H) proviruses, the gel was exposed for only 10 min. Upon longer exposure, the fragments corresponding to expression of the endogenous *Mtv-1*-specific fragments produced in the lactating mammary glands of C3H/HeN MMTV$^-$ mice were seen (not shown).

Mtv-1, RNA was isolated from several group 1 (Fig. 6, lanes 2 to 4) and group 2 (lanes 5 and 6) tumors and analyzed by RNase T_1 protection analysis using the MMTV(C3H)-specific 3′ LTR probe diagrammed in Fig. 1. All of the newly integrated proviruses in the tumors produced RNA that protected full-length probe. Therefore, although group 2 tumors contained integrated recombinant virus with a *Mtv-1 env* gene, the 3′ end of its transcripts, including the *sag* gene, were derived from MMTV(C3H).

Packaging of *Mtv-1* and MMTV(C3H) broadens the host range of the virus. The MMTV Sag protein causes the stimulation of cognate Vβ-bearing T cells, and this function is required for virus infection (13, 17). As a result, only mice that retain Sag-cognate T cells in their immune repertoire can be infected with virus. The Sags encoded in the LTRs of the *Mtv-1* and *Mtv-6* endogenous loci cause the deletion of Vβ3-bearing T cells during the perinatal period of development (38). Although there are no endogenous *sag* genes that cause the deletion of Vβ14$^+$ T cells during negative selection, transgenic mice that express the MMTV(C3H) Sag delete these cells (13) and mice infected neonatally with MMTV(C3H) virus show a slow deletion of their Vβ14-bearing T cells (22, 26). If *Mtv-1* RNA was packaged into the virions of exogenous MMTV, mice nursed on milk containing such viral particles should exhibit slow deletion of their Vβ14$^+$ and Vβ3$^+$ T cells.

To test whether the packaging of *Mtv-1* RNA into MMTV(C3H) resulted in virus with an altered host range (i.e., infection via Vβ3$^+$ T cells), newborn AKR/NCr mice were foster nursed on C3H/HeN MMTV$^+$ milk, and the percentages of their CD4$^+$ Vβ3$^+$ and CD4$^+$ Vβ14$^+$ T cells in the periphery were analyzed at different ages. Unlike C3H/HeN mice, which have both the *Mtv-1* and *Mtv-6* endogenous proviruses and thus no Vβ$^+$3 T cells, AKR/NCr mice contain both Vβ3$^+$ and Vβ14$^+$ T cells (38). As a control, a second group of newborn AKR/NCr mice was foster nursed on C3H/HeN MMTV$^-$ mothers. As seen in Fig. 7, AKR/NCr mice nursed on viremic mothers (AKR/NCrfC3H/HeN MMTV$^+$) showed deletion of both Vβ3$^+$ and Vβ14$^+$ T cells, with similar kinetics. This result showed that *Mtv-1* RNA is packaged into the virions of MMTV(C3H) and that this virus is apparently coamplified in the infected offspring, since the Vβ3$^+$ T-cell deletion increased with time.

In contrast to the AKR/NCr mice foster nursed on C3H/HeN MMTV$^+$ mothers, those nursed on uninfected females (AKR/NCrfC3H/HeN MMTV$^-$) showed a small deletion of their CD4$^+$ Vβ3$^+$ T cells that did not increase with time (Fig. 7B). To determine whether this represented packaging of *Mtv-1* viral RNA in the absence of exogenous virus, the AKR/NCrfC3H/HeN MMTV$^-$ mice were bred and offspring

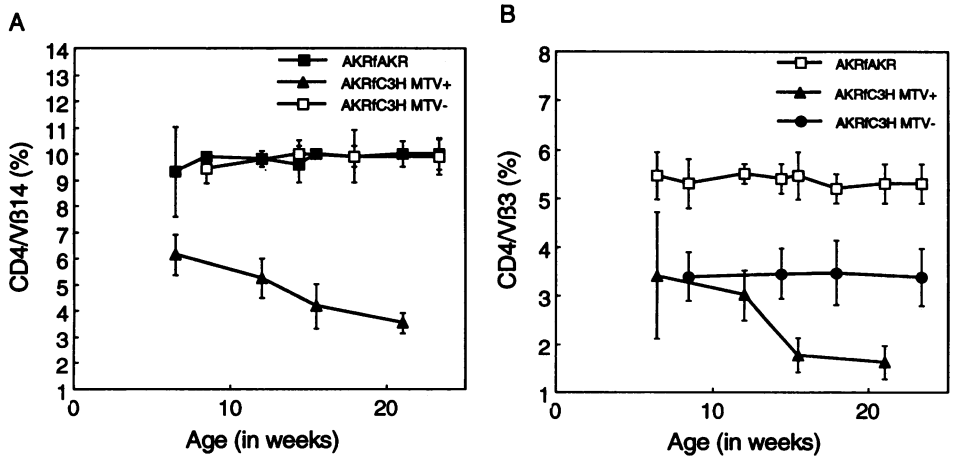

FIG. 7. Deletion of CD4$^+$ Vβ14$^+$ and CD4$^+$ Vβ3$^+$ T cells in AKR/NCr mice foster nursed on C3H/HeN MMTV$^+$ females. AKR/NCr mice were foster nursed on C3H/HeN MMTV$^+$ or C3H/HeN MMTV$^-$ mice. T cells were isolated from the peripheral blood of mice at the ages indicated and analyzed for the percentages of CD4$^+$ Vβ14$^+$ (A) and CD4$^+$ Vβ3$^+$ (B) T cells as described previously (13). AKR/NCr mice foster nursed on AKR/NCr mothers served as controls. Each datum point represents the average of results for three to four different mice.

TABLE 1. *Mtv-1* is transmitted as an infectious virus only in the presence of MMTV(C3H)

Mice	%[a] of CD4$^+$ T cells bearing:	
	Vβ3	Vβ14
AKRfAKR	4.9 ± 0.42	10
AKRfC3H/HeN MMTV$^+$	3.0 ± 0.01 (61)	5.4 ± 0.06 (54)
AKRfC3H/HeN MMTV$^+$ offspring	2.9 ± 0.35 (59)	5.6 ± 0.07 (56)
AKRfC3H/HeN MMTV$^-$	3.3 ± 0.04 (67)	9.9 ± 0.02 (99)
AKRfC3H/HeN MMTV$^-$ offspring	5.1 ± 0.60 (104)	10 ± 0.03 (100)

[a] T cells were isolated from the blood of the mice indicated, stained, and analyzed as described in Materials and Methods. All of the mice analyzed were 10 weeks old. Values are means of two to four mice ± standard errors. Numbers in parentheses are percentages of Vβ3$^+$ and Vβ14$^+$ T cells relative to the level found in uninfected AKR/NCr mice.

were produced. These offspring were then examined for deletion of Vβ3$^+$ T cells; if their mothers produced an *Mtv-1*-containing virus, the offspring would exhibit a slow deletion of this class of T cells. As seen in Table 1, no deletion of Vβ3$^+$ T cells was seen in these mice (AKR/NCrfC3H/HeN MMTV$^-$ offspring). In contrast, the offspring of AKR/NCrfC3H/HeN MMTV$^+$ females did show such a deletion (Table 1), indicating that an *Mtv-1*-containing virus could be propagated only in the presence of MMTV(C3H).

DISCUSSION

We have demonstrated that there is packaging of a highly expressed endogenous MMTV, *Mtv-1*, by the exogenous MMTV(C3H) in the mammary glands of C3H/HeN MMTV$^+$ mice. This endogenous provirus is apparently defective and is not efficiently packaged in the absence of exogenous virus infection. The defect probably lies within the 3′ half of the provirus, since a recombinant virus containing the 5′ half of *Mtv-1* and the 3′ half of MMTV(C3H) has been shown to be infectious (35). As a result of infection of C3H/HeN mammary glands by exogenous virus, the endogenous virus is rescued, leading to an infectious virus with a different host range. This occurs because virions containing *Mtv-1* genomic RNA are able to infect mice with different T-cell Vβ repertoires than MMTV(C3H).

Previous work with C3H/HeN MMTV$^-$ mice indicated that the endogenous *Mtv-1* provirus was capable of causing tumors in situ with a long latency (39), implying that some virus was produced. We also found that AKR/NCr mice foster nursed on C3H/HeN MMTV$^-$ mice showed a small deletion of their CD4$^+$ Vβ3$^+$ T cells, indicating that there could be small amounts of *Mtv-1* virions shed into the milk of C3H/HeN MMTV$^-$ mice. These *Mtv-1* virions must be nonreplicating, however, because there was no increase with time of the Vβ3$^+$ T-cell deletion in the AKRfC3H/HeN MMTV$^-$ mice, in contrast with the AKRfC3H/HeN MMTV$^+$ mice (Fig. 7). Moreover, the AKRfC3H/HeN MMTV$^-$ mice did not transmit *Mtv-1* virions to their offspring (Table 1). Alternatively, the small Vβ3$^+$ T-cell deletion in these offspring could result from the transfer of lymphocytes that present the *Mtv-1* Sag from C3H mothers to their offspring. It has previously been shown that milk contains both T and B cells (23), and it is thought that both human immunodeficiency virus and human T-cell leukemia virus type I are transferred to newborns through milk by infected lymphocytes (21, 33, 34, 43).

The stimulation of specific Vβ-bearing T cells by the Sag protein encoded in the MMTV genome is a critical step in the virus infection pathway (13, 17). However, this stimulation is limited to a specific subset of T cells, determined by the C-terminal amino acids of Sag. When a mouse has an endogenous provirus that encodes a Sag with the same Vβ specificity as an exogenous virus, the particular Vβ-bearing T cells are missing from its immune repertoire and thus the animal is protected from infection (13, 17). When exogenous virus infects an inbred mouse strain and packages an endogenous virus, the endogenous virus cannot be amplified in mice of the same inbred background, since all of the T cells capable of interacting with the Sag from the endogenous virus are absent. Outbred mice, however, have different endogenous *Mtv* loci that encode Sag proteins with a variety of Vβ specificities. This means that viruses containing RNAs encoding different Sag proteins would be able to infect many more mice than an exogenous virus with a homodimeric RNA. Moreover, if recombination within the sequences encoding the C terminus of the Sag protein occurred, novel proteins with new Vβ specificities might also be produced, thus increasing the host range of the virus.

A second consequence of the packaging of *Mtv-1* RNA was that recombinant virus that could be detected in MMTV-induced mammary tumors was produced. Recombination probably occurred as a result of copackaging of endogenous *Mtv-1* and MMTV(C3H) RNAs in the same particle. Although viral recombination associated with tumor induction has been shown to occur for a large number of retroviruses, it has not

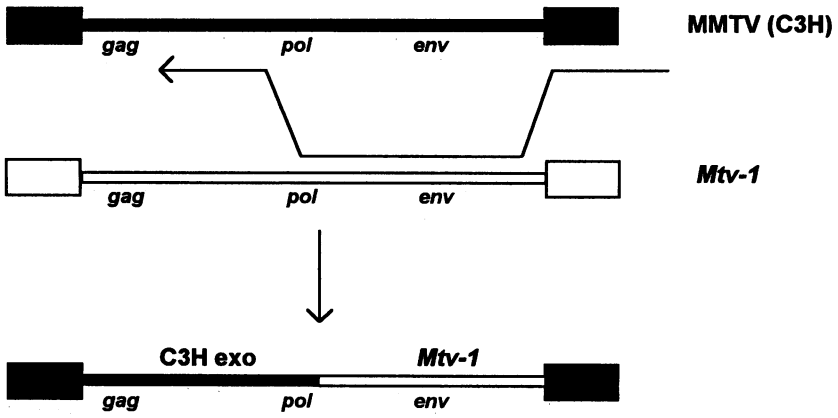

FIG. 8. Model for the generation of the recombinant MMTV found in mammary tumors.

previously been seen in MMTV-induced mammary tumors, most likely because detection of these recombinants requires a specific combination of restriction enzymes and probes. We have not precisely defined the breakpoints of the recombination for the tumor-associated viruses. However, it appears that the recombinant provirus derives its *gag* and *pol* genes and LTRs from MMTV(C3H), while at least part of its *env* gene comes from an endogenous virus, most likely *Mtv-1*. This implies that a minimum of two strand-switching events by the reverse transcriptase must have occurred to create this recombinant MMTV (Fig. 8). A similar type of recombination has been seen with feline leukemia viruses (30).

Almost 30% of the tumors analyzed here had exclusively recombinant virus, as their newly integrated provirus and a large percentage of tumors appeared to have both MMTV(C3H) and recombinant virus. That there were tumors containing only recombinant virus implies that it may play a role in either the virus life cycle or the induction of mammary tumors. In the case of polytropic murine leukemia viruses, it has been shown that recombination between different viruses is required to generate a transforming virus (10, 36), through the acquisition both of enhancer sequences that direct high-level expression in the target T cells (42) and of Env proteins that most likely allow the recombinant virus to bind to different cellular receptors than the parental viruses from which they were derived (36). Moreover, it is thought that recombination plays an important role in the generation of pathogenic variants of human immunodeficiency virus (6). Whether recombination between endogenous and exogenous MMTVs results in a virus with increased pathogenicity or tumor-inducing ability can be determined by examination of the precise structure of the recombinant virus and the ability of such viruses to infect mice and cause mammary tumors.

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