Genetics of Mouse Mammary Tumor Virus-Induced Mammary Tumors: Linkage of Tumor Induction to the gag Gene

LAUREN M. HOOK,1 YELENA AGAFONOVA,1 SUSAN R. ROSS,2 STEPHANIE J. TURNER,1 AND TATYANA V. GOLOVKINA1*

The Jackson Laboratory, Bar Harbor, Maine 04609,1 and Department of Microbiology/Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania 191042

Received 14 April 2000/Accepted 11 July 2000

Retroviruses are believed to induce tumors by acting as insertional mutagens that activate expression of cellular proto-oncogenes. Indeed, almost 90% of mouse mammary tumor virus (MMTV)-induced mammary tumors in C3H/He mice show upregulation of Int proto-oncogenes. We have analyzed three different MMTV variants [MMTV(C3H), MMTV(HeJ), and a genetically engineered MMTV hybrid provirus (HP)] for tumorigenicity in mice from two distinct genetic backgrounds. All three viruses were tumor causing in BALB/c mice. However, only MMTV(C3H), but not MMTV(HeJ) or HP, induced mammary tumors in C3H/He mice. Thus, only MMTV(C3H) induced mammary tumors in C3H/He as well as in C3H/HeJ mice and integrated into the germ line of C3H/HeJ mice.

MMTV(C3H), which caused tumors in C3H/He as well as in C3H/HeJ mice and integrated into the germ line of C3H/HeJ mice, was found to be a genetic recombinant between endogenous Mtv1 provirus and exogenous MMTV(C3H). Sequence comparison of MMTV variants linked the tumorigenicity of MMTV(C3H) to the gag region of the retrovirus.

Many retroviruses carry oncogenes (v-onc) and induce tumors after a short latency period (33). For viruses lacking v-onc genes, tumors arise after an extended latency period and provirus integration near a cellular proto-oncogene (33). Most tumors induced by retroviruses that lack oncogenes cause hematopoietic malignancies, although a few of these viruses induce carcinomas (33). Mouse mammary tumor virus (MMTV) is a B-type retrovirus that does not have an oncogene but induces mammary carcinomas and, more rarely, T-cell lymphomas (for a review, see reference 35).

Exogenous MMTV is spread via the milk of infected females and is acquired by suckling pups (27). On rare occasions, an exogenous MMTV provirus is inserted into germ or early embryonic cells, thereby becoming a stably inherited endogenous provirus (2, 9). The primary targets for exogenous MMTV are T and B cells located in Peyer's patches of the gastrointestinal tract of neonatally infected pups (3, 19). MMTV gains access to these cells by traveling through M cells located in the follicle-associated epithelium of the Peyer's patches (18). Both endogenous and exogenous MMTVs encode a superantigen (Sag) in their 3' long terminal repeat (LTR) (7). In contrast to conventional antigens, Sags stimulate profound T-cell responses, because they are recognized by all T cells that express a particular T-cell receptor VB chain (22, 25). Since proliferation increases the number of T cells and because dividing cells are susceptible to retroviral infection, the rate of infection is increased (41). Infection rates of the T and B cells remain high sufficiently long to infect the mammary gland cells when they begin to divide at about 3 to 4 weeks after birth. Overall, the LTR sequences of different MMTVs are highly conserved (4). However, the region encoding the C-terminal segment of Sag is more diverse and is known as the hypervariable region. The amino acid sequence of this region contacts the VB chain of the T-cell receptor and thus determines which T cells are affected (45). When recognized as foreign, Sags stimulate specific VB+ T-cell proliferation (22, 25) whereas Sags present in the germ line stimulate deletion of the VB+ T-cell subset during formation of the immune repertoire (1, 10, 11, 43). The Sag function is dispensable to the MMTV life cycle, because mice that lack Sag-cognate T cells, due to the expression of transgenes (15) or endogenous proviruses (20), cannot be infected with exogenous viruses bearing Sags of the same VB specificity. In addition, viruses without functional Sags cannot propagate in vivo (16).

Once proviral DNA is integrated into a chromosome, its expression is regulated by specific sequences within the LTR that cause increased viral transcription in response to glucocorticoid receptor-steroid hormone complexes (44). The increased virion production that occurs during lactation results in a greater number of infected mammary gland cells and more proviral integrations into the genome. MMTV does not encode an oncogene, so mammary tumorigenesis takes place after proviral insertion near specific cellular proto-oncogenes, thereby up-regulating their transcription. Because retroviral integration into the host chromosome occurs at random locations, the more viruses that are produced, the more likely it is that integration near cellular proto-oncogenes will occur. The large majority of MMTV integrations in mammary tumors result in activation of proto-oncogenes that are not normally expressed in the mammary gland (28). Thus, it has been postulated that MMTV-induced mammary tumors result from the expression of proto-oncogenes controlling cellular growth.

It has been shown that exogenous MMTV carried by C3H/He mice [MMTV(HeJ)] cannot efficiently induce mammary tumors in C3H/HeJ mice (30). We have extended these findings and shown that besides MMTV(HeJ), another highly infectious genetically engineered MMTV hybrid provirus (HP) was incapable of efficiently inducing tumors in C3H/He mice. In contrast, both of these viruses were tumorigenic in BALB/c mice and integrated into the Wnt-1 and Int-2/Fgf3 loci with high frequency. By comparing the sequences of these viruses to MMTV(C3H), which caused tumors in C3H/He as well as in BALB/c mice, we found that a determinant of oncogenicity mapped to the gag gene.

* Corresponding author. Mailing address: The Jackson Laboratory, 600 Main St., Bar Harbor, ME 04609. Phone: (207) 288-6287. Fax: (207) 288-6078. E-mail: tvg@aretha.jax.org.
Two or three independently isolated clones of each plasmid were sequenced.

Southern blot analyses. Mammary gland tumors were excised from the surrounding normal tissue, and DNA was isolated as previously described (17). Twenty micrograms of each DNA sample was digested with indicated restriction enzymes and electrophoresed on 0.8% agarose gels. After transfer to nylon, the blots were hybridized with 32P-labeled probe (see Fig. 3A), washed, and exposed to Kodak XAR-5 film using Cronex Lightning-Plus intensifying screens.

RNA isolation and Northern blot analysis. RNA was isolated from mammary gland tumors or normal mammary glands in accordance with a protocol published elsewhere (6). Twenty micrograms of total RNA was subjected to electrophoresis on a 1% formaldehyde gel, transferred to a nylon membrane, and hybridized with a Wnt-1 (29) or Int-2/8 (32) probe.

RESULTS

Tumor occurrence in C3H substrains. Approximately 50 years ago, the high-tumor-incidence, MMTV-infected C3H mouse strain was divided between the National Institutes of Health (C3H/HeN) and The Jackson Laboratory (C3H/HeJ). In 1973, it was reported that C3H/HeJ mice demonstrated a drastic decrease in mammary tumor incidence compared to the infected C3H/HeN strain (30; D. M. Richardson, JAX Notes 41:1–3, 1973). We have repeated these experiments and confirmed the results. Indeed, 50% of MMTV-infected C3H/HeJ mice developed tumors after approximately 250 days whereas only 10% of the C3H/HeJ mice developed tumors after 350 days (Fig. 2E). Exogenous MMTV was present in C3H/HeJ mice, since they showed deletion of Sag-cognate Vp14+ T cells characteristic of MMTV(C3H) infection. Three- to four-month-old C3H/HeJ mice had only about 3.5% CD4+ Vp14+ T cells among peripheral T cells, in contrast to 7.5% in virus-free C3H/HeJ mice. Furthermore, RNA isolated from the C3H/HeJ mammary tumors contained large amounts of virus-specific RNA (data not shown). Quantitative analysis of the viral transcripts in the mammary glands of MMTV-infected C3H/HeN and C3H/HeJ mice ruled out differences in the virus load between these two strains; if anything, there was a slight increase in MMTV(HeJ) expression compared to MMTV(C3H) expression (a factor of 1.2) (Fig. 2A; expression of endogenous Mtv1 provirus was used as an internal control).

It has been suggested that such a radical decrease in tumor incidence in C3H/HeJ mice is due to mutations in MMTV itself resulting in attenuation of the virus [MMTV(HeJ)] in C3H/HeJ mice versus MMTV(C3H) in C3H/HeN mice] (30). To confirm this, we fostered MMTV-negative C3H/HeN mice on MMTV-infected C3H/HeN milk and monitored them for mammary gland tumors. C3H/HeN mice became infected with MMTV(HeJ), since they secreted a large amount of this virus on MMTV-infected C3H/HeJ milk and monitored them for Mammary gland tumorigenesis. Mammary gland tumor incidence was monitored by weekly palpation of the animals. Tumor-bearing mice were sacrificed, and DNA isolated from a portion of each tumor was subjected to Southern blot analysis to confirm the viral etiology (17, 23).

The Sag hypervariable region contains sequences that are complementary to the Sag insert of MMTV(HeJ) in the Sag hypervariable region located in the U3 LTR (not present in RNA), high-molecular-weight DNA, and high-molecular-weight DNA. In order to compare virus load in MMTV-infected C3H/HeJ and C3H/HeN mice, the ratios of exogenous to endogenous viral RNA expression levels were quantified using a phorosphorimager (Bio-Imaging analyzer BAS 1000 MacBac; Fuji Photo Film Co., Ltd.).

Mammary gland tumorigenesis. Mammary gland tumor incidence was monitored by weekly palpation of the animals. Tumor-bearing mice were sacrificed, and DNA isolated from a portion of each tumor was subjected to Southern blot analysis to confirm the viral etiology (17, 23).

Nucleotide sequence accession numbers. Nucleotide sequences have been submitted to the GenBank nucleotide sequence database and have been assigned accession numbers AF228552 for MMTV(C3H) provirus, AF228553 for MMTV(HeJ) provirus, and AF228550 for Mtv1 provirus.

Materials and methods.

Mice. All of the mice used in this study were bred and maintained at the animal facility of The Jackson Laboratory, Bar Harbor, Maine. C3H/HeJ MMTV+ and BALB/cJ mice were obtained from The Jackson Laboratory. Please note that in 1999, C3H/HeJ MMTV+ mice were rederived to improve the overall health status of the distribution colonies, resulting in elimination of endogenous virus (JAX Notes 48:86, 2000). Thus, MMTV-infected C3H/HeJ mice are no longer available from The Jackson Laboratory. MMTV-negative C3H/HeN and MMTV-infected C3H/HeN mice were originally obtained from the National Cancer Institute, Frederick Cancer Research Facility, Frederick, Md. MMTV HP transgenic mice were made on a C3H/HeJ genetic background (14).

Cloning and sequencing. Sequences of exogenous MMTV(C3H) and MMTV(HeJ) and endogenous Mtv1 proviruses were obtained using a panel of overlapping plasmids (Fig. 1). Except for pAB, all plasmids were cloned from viral RNA templates. The viral RNA templates were isolated from milk-filled stomachs of MMTV-infected C3H/HeJ, C3H/HeN, or MMTV-negative C3H/HeN pups as previously described (17). Viral cDNA was prepared using SuperScript II reverse transcriptase in the buffer supplied by the manufacturer (GIBCO/BRL, Gaithersburg, Md.) and a (dT)x primer. The amplified DNA was cloned into a vector using the PCRScript cloning kit (Stratagene, La Jolla, Calif.) and subsequently sequenced. A single-copy pACYC177 vector (New England Biolabs, Beverly, Mass.) was used to clone MMTV(C3H) gag (pGAG3) to avoid problems with the poison sequences (5). Since primer A was specific for the Sag hypervariable region located in the U3 LTR to the 5′ LTR (Fig. 1), these plasmids were sequenced using primers A, 5′GAGACTGGCTGACTAATAAGAACATT3′ (nucleotides [nt] 897 to 922, according to the numbering system of Brandt-Carlson et al. [4]); reverse gag-specific primer B, 5′CTCTCCITCTTGCGGAAACAGG (nt 151 to 181 from the start codon in gag); forward gag-specific primer C, 5′ATGGGCTGCTCGGCTCAAAGGG (nt 1 to 24 from the start codon in gag); reverse gag-specific primer D, 5′GGAGTCTGCCCCCTTAACAGTTTGAATG (nt 1888 to 1762 from the start codon in gag); forward gag-specific primer E, 5′ATGGGATCTACAGCTCTCC (nt 1720 to 1740 from the start codon in gag); reverse env-specific primer F, 5′GACCCAGATTGGTGTTTCGGCAT3′ (nt 5999 to 6019 according to BR6 provirus); and reverse LTR-specific primer G, 5′GAAGCTTGTAGTTAGTGAGTTGAG3′ (nt 27 to 49 according to the numbering system of Brandt-Carlson et al. [4]); forward LTR-specific primer H, 5′GGACCTCAGAGCTTGGTCTCGAGACCT3′ (nt 1104 to 1124 according to the numbering system of Brandt-Carlson et al. [4]); and reverse LTR-specific primer K, 5′GACTGTTGCAGTTCATGACCTGCT3′ (nt 1204 to 1185 according to the numbering system of Brandt-Carlson et al. [4]). Primer A and H are specific for the hypervariable region of MMTV(C3H) gag, whereas primer K is specific for the hypervariable region of Mtv1 gag.

FIG. 1. Diagram of the MMTV provirus with the primers used (see Materials and Methods) to PCR amplify different regions of MMTV(HeJ), HP, and MMTV(C3H). Solid box, Sag hypervariable region. R, EcoRI.

The Sag hypervariable region contains sequences that are complementary to the Sag insert of MMTV(HeJ) in the Sag hypervariable region located in the U3 LTR (not present in RNA), high-molecular-weight DNA, and high-molecular-weight DNA. In order to compare virus load in MMTV-infected C3H/HeJ and C3H/HeN mice, the ratios of exogenous to endogenous viral RNA expression levels were quantified using a phosphorimager (Bio-Imaging analyzer BAS 1000 MacBac; Fuji Photo Film Co., Ltd.).

Mammary gland tumorigenesis. Mammary gland tumor incidence was monitored by weekly palpation of the animals. Tumor-bearing mice were sacrificed, and DNA isolated from a portion of each tumor was subjected to Southern blot analysis to confirm the viral etiology (17, 23).

Nucleotide sequence accession numbers. Nucleotide sequences have been submitted to the GenBank nucleotide sequence database and have been assigned accession numbers AF228552 for MMTV(C3H) provirus, AF228553 for MMTV(HeJ) provirus, and AF228550 for Mtv1 provirus.
fore, MMTV(HeJ) did not cause tumors in tumor-susceptible C3H/HeJ mice, at least within 350 days (Fig. 2E).

In reciprocal experiments, MMTV-free C3H/HeJ mice were foster nursed by C3H/HeN MMTV females. These MMTV(C3H)-infected C3H/HeJ mice (generation G1) then were mated with C3H/HeJ males to produce infected offspring (generation G2). All of the animals from both the G1 and G2 generations became MMTV infected and produced MMTV in their milk (Fig. 2C and D). The generation G1 and G2 females were bred and observed for mammary tumors. MMTV(C3H)-infected C3H/HeJ mice in both generations were even more susceptible to MMTV(C3H)-induced tumors than were C3H/HeN mice. Fifty percent of MMTV(C3H)-infected C3H/HeJ mice developed mammary tumors by 183 days, whereas 50% of C3H/HeN females infected with the same virus developed mammary tumors by 250 day (Fig. 2E). Therefore, C3H/HeJ mice are genetically susceptible to MMTV-induced mammary tumors and the change in tumor incidence and latency in this substrain must be due to the occurrence of a new MMTV.

MMTV-induced mammary tumors in C3H/HeJ mice contain recombinant virus. In addition to endogenous loci, MMTV-induced mammary tumors always demonstrate newly
acquired exogenous proviruses (8). We sought to determine whether the newly integrated exogenous MMTV proviruses present in MMTV-infected C3H/HeJ mammary tumors differ from those found in C3H/HeN MMTV+ tumors. Tumor DNA was isolated and subjected to Southern blot restriction fragment length polymorphism analysis and compared with splenic DNA (Fig. 3A) (17, 37). Splenic DNA digested with BglII and PstI endonucleases and hybridized with the env probe depicted in panel A. The 6.7-, 5.8-, and 4.3-kb fragments correspond to exogenous Mtv proviruses present in C3H/HeJ mice. Arrows show newly integrated exogenous MMTVs.

FIG. 3. Mammary tumors of MMTV(HeJ)-infected C3H/HeJ mice contain a recombinant MMTV. (A) Map of endogenous (endo) proviruses present in C3H/He and BALB/c mice. Also shown are the maps of exogenous (exo) MMTV (C3H) and HP. The presence of the 2.3- or 2.3- and 1.5-kb fragments is characteristic of newly integrated copies of HP or MMTV(C3H), respectively. Abbreviations: E, EcoRI; P, PstI; B, BglII; P+, PstI site present in Mtv9 provirus inherited by BALB/c but not C3H/HeJ mice; filled bars, two probes used for hybridization. (B) Mammary tumors of C3H/HeJ mice are not induced by wild-type exogenous MMTV(C3H). Top panel, high-molecular-weight DNAs from mammary gland tumors of MMTV(HeJ)-infected C3H/HeJ mice and from the spleen of a C3H/HeJ mouse were digested with PstI and BglII and subjected to Southern blot analysis with the hybridization gag-pol probe depicted in panel A. The 6.7-, 5.8-, and 4.3-kb fragments correspond to endogenous Mtv proviruses present in C3H/HeJ mice. Arrows show newly integrated exogenous MMTVs. SP, splenic DNA of a C3H/HeJ mouse.

derived from the few endogenous Mtv proviruses present in C3H/He mice (8, 9). As a hybridization probe, we used a 1.8-kb PstI fragment of cloned MMTV DNA that contains the viral env gene and detects only EcoRI fragments derived from the 3′ portion of MMTV proviruses (Fig. 3A). This probe also detects three EcoRI fragments (6.7, 5.8, and 4.5 kb) derived from endogenous Mtv proviruses present in C3H/He mice (23). Almost all of the MMTV-infected C3H/HeJ mammary tumors exhibited multiple additional proviruses located at different sites in the host genome (Fig. 3B, bottom). Based on these results, we concluded that mammary tumors in C3H/HeJ mice were induced by exogenous MMTV; however, this virus [MMTV(HeJ)] was different from MMTV(C3H).

Genetically engineered MMTV HP is not tumorigenic in tumor-susceptible C3H/HeN mice. Previously, we produced transgenic mice on a C3H/HeN genetic background with a genetically engineered MMTV HP (37) of which the 5′ half was derived from the endogenous Mtv1 provirus and the 3′ half was derived from exogenous MMTV(C3H) (Fig. 3A) (14). Transgenic females shed virus into the milk, and nontransgenic mice foster nursed by them became infected with the virus, suggesting that HP was infectious (14). We have also tested whether HP amplification within the mammary gland was similar to that of exogenous wild-type MMTV(C3H) by analyzing viral RNA production in the mammary gland after each subsequent pregnancy. HP reached the same level of amplification as wild-type MMTV(C3H) in the mammary glands of infected females (Fig. 4A; all RNA samples were normalized by expression of endogenous Mtv1/Mtv6 proviruses). However, when we monitored mice infected with HP for mammary gland tumors, we discovered that this virus did not cause tumors on a C3H/HeN background. Whereas 100% of C3H/HeN females infected with MMTV(C3H) developed tumors after approximately 350 days, C3H/HeN mice infected with HP developed no tumors during this period (Fig. 4B).

To ensure that the HP can cause tumors, a tumor-susceptible strain of mice, BALB/cJ, was foster nursed on HP-containing milk, bred, and monitored for mammary tumors. Fifty percent of BALB/cJ mice infected with HP developed mammary tumors by 267 days (Fig. 5A and reference 37). To confirm that these tumors were induced by HP, we isolated their DNA and subjected it to Southern blot analysis, which allows distinction between endogenous and exogenous MMTVs as described above. The endogenous BALB/cJ loci (except for Mtv9) yielded fragments of 3.0, 2.7, and 2.1 kb, while digestion and hybridization of integrated HP yielded fragment of 2.3 kb (Fig. 3A). BALB/cJ mammary tumors were induced by HP, since all of the tumors contained the 2.3-kb fragment characteristic of integrated HP (Fig. 5B). We also fostered BALB/cJ mice on C3H/HeJ milk and monitored them for mammary tumors. Fifty percent of BALB/cJ females infected with MMTV(HeJ) developed mammary tumors by 292 days (Fig. 5A), and all of the tumors were induced by MMTV(HeJ) (data not shown). Thus, both MMTV(HeJ) and HP were capable of causing mammary tumors in BALB/cJ mice but not in tumor-susceptible C3H/HeN mice.

Both MMTV(HeJ) and HP are capable of up-regulating expression of Int genes. In contrast to MMTV(C3H), the two other viruses, MMTV(HeJ) and HP, did not cause tumors on the C3H/HeN background even though both were infectious. In over 80% of C3H/He MMTV-induced mammary tumors, at least one of the additional MMTV genomes is integrated near the cellular Wnt-1 gene (29) and in approximately 10% of the tumors integration occurs near the Int-2/Fgf-3 locus (12, 31). Proviral integrations are found at several locations near these genes, but insertions always leave the protein-coding domain
Based on our Southern blot data, we hypothesized that MMTV(HeJ) is a recombinant virus. Of five different endogenous MMTVs present in the C3H/HeN genome, Mtv1 is the only one that can be copackaged with exogenous MMTV in the mammary gland to give rise to a new recombinant (17). Thus, we have cloned and sequenced Mtv1, MMTV(C3H), and MMTV(HeJ). MMTV (HeJ) was found to be a recombinant between Mtv1 and MMTV(C3H) (Fig. 7). Interestingly, almost the entire genome of the virus was derived from Mtv1, except for the Sag hyper-variable region and the first 360 bp of the gag region, which were from MMTV(C3H). Knowing the sequences of different viruses, it was possible to compare the phenotypes they produced and to deduce which gene might be responsible for tumor resistance in C3H/He mice. The presence of the MMTV (C3H) gag gene in the context of the provirus was found to correlate with tumorigenicity in the mammary glands of C3H/He mice. Although the entire pro and pol genes in MMTV(HeJ) and pro and part of the pol gene (until the EcoRI site) in HP were of Mtv1 origin, we think that it is unlikely that these genes can contribute to tumorigenesis. First, the majority of amino acid changes in the pro and pol genes of Mtv1 were conservative relative to those of pro and pol of MMTV(C3H). Second, the nonconservative differences in the pol gene of Mtv1 relative to the pol gene of MMTV(HP) were found downstream of the EcoRI site (after amino acid [aa] 571), where the pol gene in nontumorigenic HP is of MMTV(C3H) origin.

The Gag protein is the precursor to the internal structural proteins of all retroviruses. All Gags are organized in the same order, from the amino terminus to the carboxyl terminus, with domains that are cleaved inside the viral particle to yield the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The MA protein is located beneath the viral membrane and initiates virus assembly, the CA protein forms the mature intact. In most tumors, the transcriptional orientation of the proviruses is directed away from the Int genes, an indication that the proviral DNA enhancers up-regulate expression of the oncogenes. The induction of Int transcription by MMTV provirus insertion is believed to be an early step in the transformation process.

It had been shown previously that HP-induced mammary tumors in BALB/c mice exhibit induced or altered expression of different int genes (24, 36), suggesting that HP is capable of up-regulating int gene expression. We also analyzed RNA isolated from tumors induced by HP or MMTV(HeJ) for the expression of Wnt-1 and Int-2/Fgf-3 transcripts. Expression of Wnt-1 was detected in 71% of MMTV(HeJ)-infected C3H/HeJ mammary tumors and in 58% of HP-infected BALB/c mouse mammary tumors (Fig. 6). Expression of Int-2/Fgf-3 was detected in 25% of C3H/HeJ tumors and in 16% of BALB/c mouse mammary tumors induced by HP (data not shown). Thus, both HP and MMTV(HeJ) are capable of integrating next to and up-regulating the expression of Int genes.

MMTV(HeJ) is a genetic recombinant between endogenous Mtv1 and exogenous MMTV(C3H). Based on our Southern
virion core, and the NC protein (small basic protein) coats the viral RNA in a sequence-independent manner. We found three regions of nonconservative amino acid changes; two are located within putative bipartite nuclear localization domains of the MA protein (aa 174 to 191 and 231 to 248), and one lies inside the Zn\(^{2+}\) binding finger motif CX\(_2\)CX\(_4\)HX\(_4\)C of the NC protein (aa 527 to 540) (Fig. 8).

**DISCUSSION**

Our studies provide an explanation for the major change in mammary tumor incidence and latency occurring in the MMTV-infected C3H/HeJ mouse strain maintained at The Jackson Laboratory (30; D. M. Richardson, JAX Notes 413: 1–3, 1973) (Fig. 2E). Only 30% of breeding MMTV-infected C3H/HeJ females developed tumors by 500 days, in contrast to the 97% of MMTV\(^{+}\) C3H/HeN mice that developing tumors by 290 days (Fig. 2E). However, C3H/HeJ mice are genetically susceptible to MMTV-induced tumors because they developed high-incidence mammary tumors in BALB/cJ mice (Fig. 5A). Nucleotide sequence comparison of tumor-inducing MMTV(C3H) and non-tumorogenic MMTV(HeJ) and HP made it possible to map the tumor attenuation region to gag of Mtv1.

The change in tumor incidence and latency in C3H/HeJ MMTV\(^{+}\) mice was investigated by Outzen et al. in 1985 (30). According to them, only 37% of the C3H/HeJ breeding females had detectable MMTV antigens in their milk samples during the first lactation and the percentage of positive milk samples increased with parity to 63% in the second lactation and to 74% in the third lactation (30). In addition, Outzen et al. demonstrated that C3H/HeJ mice fostered by C3H/HeOuJ MMTV\(^{+}\) females (a high tumor incidence strain of C3H/He mice similar to the C3H/HeN substrain) had lower transmission of exogenous MMTV(OuJ) through milk since the percentage of exogenous MMTV antigen-positive milk samples at the third parity declined from more than 83% in the first generation to less than 67% in the third generation (30). Based on these results, the authors concluded that the C3H/HeJ host inhibited the milk-borne transmission of exogenous MMTV. Although we have not analyzed as many mice for milk production as did Outzen et al., we have consistently seen the

**FIG. 6.** Both HP and MMTV(HeJ) are capable of upregulating expression of the Wnt-1 gene. RNA was isolated from mammary tumors induced by MMTV (C3H) (top panel), MMTV(HeJ) (middle panel), and HP (bottom panel) in MMTV(C3H)-infected C3H/HeN (C3H/HeN MMTV\(^{+}\)), MMTV(HeJ)-infected C3H/HeJ (C3H/HeJ MMTV\(^{+}\)), and HP-infected BALB/cJ (BALB/cJ HP) mice, respectively, and used for Northern blot analysis with a Wnt-1-specific probe (29).

**FIG. 7.** Structure and tumorigenic features of different MMTVs. Exogenous MMTV(C3H), MMTV(HeJ), and HP and endogenous Mtv1 proviruses were cloned and sequenced as described in Materials and Methods. The HP pol gene is chimeric; its first 570 aa were derived from Mtv1, and the rest are from exogenous MMTV(C3H). The recombination break point in MMTV(HeJ) occurred in the gag gene between nt 1845 and 1869 [nt 1845 is specific for MMTV(C3H), whereas nt 1869 is specific for Mtv1] and in LTR between nt 9333 and 9361 [nt 9333 is specific for Mtv1, whereas nt 9361 is specific for MMTV(C3H)]. The hypervariable region of sag is nt 9430 to 9530. As a result, the first 120 aa of the gag gene and the hypervariable region of the sag gene in MMTV(HeJ) are derived from MMTV(C3H). Although the original DNA construct used to make HP transgenic mice has a 5' LTR derived from Mtv1 and a 3' LTR derived from MMTV(C3H), upon infection of cells with the resulting virus, MMTV(C3H)-specific information in the U3 region of the 3' LTR is present in the newly synthesized 5' LTR. TCR, T-cell receptor.
same level of virus production by MMTV-infected C3H/HeJ and C3H/HeN mice. The most likely explanation for the discrepancy between their and our results is the sensitivity of the assays used to detect virus production. Outzen et al. tested milk samples by means of an immunodiffusion assay (IDA) for the presence of MMTV antigen (30). Because the IDA is relatively insensitive, it usually detects only large quantities of the antigen (30). Indeed, only 89% of MMTV-infected mice and that the level of MMTV antigen-positive milk as determined by IDA (30). Nevertheless, all of them developed mammary tumors by 300 days (30 and our own data). We have used the much more sensitive RNase T1 protection assay to detect virus in the milk of infected mice. Using this approach, we have shown that there is always virus produced by C3H/HeN MMTV+ or C3H/HeJ MMTV+ mice and that the level of production does not show dramatic differences between the different age- and pregnancy-matched mice of these two strains (Fig. 2 and 4).

In a majority of mammary tumors, the MMTV proviruses are integrated into the host's genome near one of the Int protooncogenes activating their expression (29, 31). The oncogetic properties of the Wnt-1 gene were proven in transgenic mice, where expression of this gene in the mammary epithelium induced mammary adenocarcinoma development in both males and females (40). However, the median latency of mammary tumor formation in female Wnt-1 transgenic mice was 5 months of age, with >80% of mice developing tumors by 7 months (40). In addition, transgenic females rarely developed more than one tumor per mouse and never developed more than three tumors per mouse (36, 40). This relatively long latency period before tumor development and the stochastic nature of mammary tumors in Wnt-1 transgenic mice argue that Wnt-1 contributes to, but is not sufficient for, tumorigenesis in these mice. Interestingly, Wnt-1 transgenic mice infected with exogenous MMTV demonstrated a dramatic increase in the number of mammary tumors (36). At 4 months of age, infected female breeders showed >5 mammary tumors per mouse, with some animals developing 10 tumors (36). Analysis of provirus-containing tumors for induced or altered expression of known Wnt genes showed activation of Int-2/Fgf-3 in 39% and Hst/Fgf-4 in 3% of the MMTV-infected Wnt-1 transgenic tumors (36). Therefore, it was suggested that cooperation between different oncogenes is an important step in mammary gland tumor induction. Our data indicate that in addition to cooperation between oncogenes, viral genes also contribute to mammary tumorigenesis.

The original virus in the progenitor C3H/He strain was highly tumorigenic. Even if there were two different viruses in the original C3H/He stock, why is it that the attenuated MMTV(HeJ) was selected or became prominent in the C3H/HeJ substrain? Because mammary tumors are not required for the virus life cycle, selection for a tumor-attenuated MMTV would be unusual unless a less tumorigenic virus had a competitive advantage over the more tumorigenic ones. Tumor-causing MMTV(HeJ) is infectious in C3H/HeJ mice (Fig. 2C and D), suggesting that the selection and retention of a less tumorigenic MMTV(HeJ) required the presence of a heritable, selective pressure operating over many generations. It could be related to some mutation, such as Thra+/+, which occurred during the same time period when mammary tumor incidence changed in the C3H/HeJ substrain (34, 38, 39, 42). The Thra+/+ mutation is carried homozygously in the C3H/HeJ substrain. This gene was originally named for its ability to increase resistance to lipopolysaccharide toxicity (Lps) and was recently shown to be the Toll-like receptor 4 gene (Tlr4), a member of the neonate I-I/Toll receptor family (21, 26). Studies are ongoing to determine why and how the new recombinant tumor-attenuated virus was selected in C3H/HeJ mice.

ACKNOWLEDGMENTS
L.M.H. and Y.A. contributed equally to this work.

This work was supported by PHS grants CA65795 to T.V.G. and CA45954 to S.R.R. and by a grant from The Jackson Laboratory to T.G. This work was also supported by a grant (CA34196) from the National Cancer Institute to The Jackson Laboratory.

We are thankful to A. Chervonsky and D. Roopenian for helpful discussion.

REFERENCES