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Generation of a Tumorigenic Milk-Borne Mouse Mammary Tumor Virus by Recombination between Endogenous and Exogenous Viruses

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Two novel exogenous mouse mammary tumor viruses (MMTV), BALB2 and BALB14, that encode superantigens (Sags) with Vβ2$^+$ and Vβ14$^+$ specificities, respectively, were found in the BALB/cT mouse strain. BALB/cT females were crossed with AKR/J males to generate F_1 females. Foster nursing of BALB/cT mice on (BALB/cT × AKR/J)F_1 mothers resulted in the generation of a new mouse strain, BALB/cLA, that had acquired a new exogenous MMTV (hereafter called LA) with a Vβ6$^+$/Vβ8.1$^+$-T-cell-specific Sag. Sequence analysis of the long terminal repeats of the BALB2, BALB14, and LA viruses indicated that LA virus resulted from recombination between BALB14 and the endogenous *Mtv-7* provirus. *Mtv-7* is expressed only in lymphoid tissues but not the mammary glands of *Mtv-7*-containing mouse strains such as AKR. In contrast, LA virus was highly expressed in the mammary gland, although it had the *sag*-specific region from *Mtv-7*. The LA virus, as well as different recombinant viruses expressed in the mammary glands of (BALB/cT × AKR/J)F_1 mice, acquired a specific DNA sequence from BALB14 virus that is required for the mammary-gland-specific expression of MMTV. Since the Sag encoded by LA virus strongly stimulated cognate T cells in vivo, selection for recombinant virus with the *Mtv-7 sag* most likely occurred because the increased T-cell proliferation resulted in greater lymphoid and mammary gland cell infection. As a result of the higher virus titer, 80% of BALB/cLA females developed mammary gland tumors, although the incidence was only 40% in BALB/cT mice.

Exogenous mouse mammary tumor virus (MMTV) is transmitted from infected mice to newborns by milk and causes mammary carcinomas in susceptible animals (33). Since MMTV does not encode an oncogene, mammary tumorigenesis takes place after proviral DNA insertion near and activation of specific cellular proto-oncogenes (37). Because retroviral integration is not sequence specific, the more virus produced, the more likely it is that proviral DNA will integrate near a proto-oncogene. Indeed, there is a high degree of correlation between virus titer in milk and mammary tumor incidence within a given mouse strain (32). In addition to containing exogenous MMTVs, all common strains of laboratory mice contain endogenous MMTVs (27). Because of mutations in either their transcriptional regulatory or their coding regions, the majority of endogenous MMTVs do not produce infectious viral particles (27).

Each exogenous and endogenous MMTVs has an open reading frame in its long terminal repeat (LTR) that encodes a superantigen (Sag) (1, 7). In contrast to conventional antigens, Sags stimulate profound T-cell responses, since they are recognized by T cells that express a particular T-cell receptor (TCR) Vβ chain (28). Although the LTR sequences of different MMTVs are highly conserved, the region encoding the C-terminal part of Sag shows less homology and is known as the hypervariable region (1, 3, 7, 39). The amino acid sequence of this region contacts the Vβ chain of the TCR and thus determines which T cells are affected (4, 52). Sags stimulate either specific Vβ$^+$ T-cell proliferation when they are recognized as foreign or the deletion during the shaping of the immune repertoire of this same subset of lymphocytes when they are present in the germ line.

Sags play an important role in the MMTV life cycle. Cells of the immune system, particularly B cells, are the first targets of this virus (20). The infected B cells present viral Sags in the context of major histocompatibility complex class II to T cells, leading to the stimulation and consequent proliferation of specific Vβ-bearing T cells. As a result of the T-cell activation, bystander cells also proliferate (6, 21). Because MMTV most likely infects only activated cells, as do most other retroviruses (12, 48), these events result in viral amplification and subsequent virus transport to the mammary glands. Sag function is indispensable to the MMTV life cycle, since mice that lack Sag-cognate T cells due to the expression of transgenes (14) or endogenous proviruses (21) cannot be infected with exogenous viruses bearing Sags of the same Vβ specificity. In addition, viruses without functional Sags cannot be propagated in vivo (13, 17a).

After integration of the provirus into the chromosome, proviral expression is transcriptionally regulated by DNA sequences in the LTRs. For example, there are sequences located in the LTR that cause increased transcription of the proviral RNAs in response to glucocorticoid receptor/steroid hormone complexes (for a review, see reference 51). In addition, it has been shown that sequences within the LTRs of exogenous viruses such as MMTV(C3H) determine expression in mammary gland and lymphoid tissue (8, 22, 31, 43). Many of the *Mtv* loci are also transcribed. However, whereas all exogenous and some endogenous MMTVs are expressed in both

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mammary gland and lymphoid tissues, loci such as *Mtv-7* and *-9* are expressed only in the latter tissue (17, 42).

The LTRs of the different endogenous MMTVs are remarkably homologous to each other outside the Sag hypervariable coding region (4; see below). Indeed, the promoters and hormone regulatory elements among the different MMTVs are almost identical (4). However, there is a consensus sequence upstream from the transcription start site in the LTR (bp 520 to 526) that determines whether the different MMTVs are expressed in lymphoid or mammary gland tissue (39a, 41).

Exogenous MMTV infects lymphoid and mammary gland cells that express endogenous, nonpathogenic MMTVs. This coexpression can lead to copackaging of two different viral RNAs into the same particles and viral recombination (16). A number of different exogenous MMTVs were recently discovered, and it has been suggested that at least some resulted from recombination between exogenous and endogenous MMTVs (4, 46). The products of viral recombination will be selected in vivo if they have a selective advantage over the parental viruses in terms of the level of either infectivity or pathogenicity. For example, novel viruses with strong Sag activity might be selected if Sag stimulation of responsive T cells results in increased viral titer in lymphoid cells and consequent mammary gland transmission.

We show here that new MMTVs do arise through recombination between endogenous and exogenous viruses. We identified two novel exogenous MMTVs in the BALB/cT mouse strain, BALB2 and BALB14, with Vβ2- and Vβ14-specific Sags, respectively. When BALB/cT mice were foster nursed on (BALB/cT × AKR/J)F_1 females, a novel Vβ6- and Vβ8.1-T-cell-deleting virus was transmitted (38). These subsets of T cells are normally deleted in mice containing the endogenous *Mtv-7* locus (11). We isolated a substrain of BALB/cT mice, BALB/cLA, that carried this novel MMTV. We found that the virus isolated from these mice, termed LA virus, was generated by recombination between BALB14 and the *Mtv-7* provirus present in AKR/J mice. Unlike *Mtv-7*, which is expressed only in lymphoid tissues, LA virus was highly expressed in the mammary gland. Sequence analysis of the LA virus showed that it had the *Mtv-7 sag* and the mammary gland transcription element (bp 520 to 526) from BALB14. We also demonstrated that the recombination event that generated LA virus was not unique and that all (BALB/cT × AKR/J)F_1 females tested shed recombinant viruses that retained the *Mtv-7 sag* and a BALB14-like mammary gland element. The LA virus had greater T-cell-stimulating activity than the BALB2 and BALB14 viruses. Therefore, selection of this new recombinant virus most likely occurred both because of its ability to be expressed in mammary gland and because of its strong Sag activity; the combination of these two features resulted in better virus transmission and increased virus titer. As a result, BALB/cLA females have a dramatically higher incidence of mammary gland tumors than do BALB/cT mice.

MATERIALS AND METHODS

Mice. Female and male C57BL/6, DBA/2, and AKR/NCr mice from colonies of germfree-derived, defined-flora animals were purchased from the National Institutes of Health Frederick Cancer Research Facility, Frederick, Md. Different sublines of BALB/cT (sublines 9, 10, and 7), BALB/cLA, MMTV-negative BALB/c, AKR/J, (BALB/cT × AKR/J)F_1, and (AKR/J × BALB/cT)F_1 mice were raised in the animal facility of the Division Medicina Experimental, Academia Nacional de Medicina de Buenos Aires, Buenos Aires, Argentina. The BALB/cLA substrain was generated from a single BALB/cT female nursed on a (BALB/cT [subline 7] × AKR/J)F_1 mother and maintained by sister-brother mating.

Foster nursing was performed as previously described (34). In brief, full-term pregnant BALB/cT females were placed in special cages to avoid any suckling by their litters; immediately after birth, mice in the litter were nursed on (BALB/cT × AKR)F_1 mothers which had been mated with BALB/cT males.

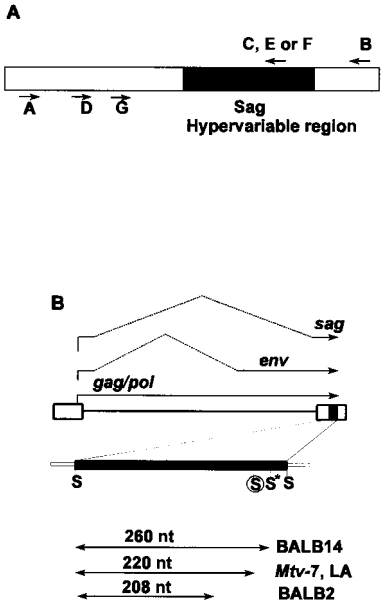


FIG. 1. (A) Diagram of the MMTV LTR with the primers used for PCR and RT-PCR. Primers A, B, D, and G hybridize to all known MMTVs, while primers C, E, and F are specific for the hypervariable regions (solid box) of *Mtv-7*/LA, BALB14, and BALB2, respectively. (B) Diagram of the MMTV provirus, viral transcripts, and probes used for RNase T_1 protection assays. The *Sau*3A-*Sau*3A fragments subcloned from plasmids containing the LTRs of the different MMTVs are shown as solid boxes, while the open boxes represent plasmid sequences. The BALB/cV LTR (23) was used to generate the BALB2-specific probe. However, because BALB2 has an 18-bp deletion upstream of the 3′ *Sau*3A site (S) (nt 1083) as well as a stretch of sequence dissimilarities with BALB/cV upstream of this deletion, the size of the protected fragment was 208 nt. Abbreviations; S, the *Sau*3A site at nt 1083 in BALB/cV; S*, the *Sau*3A site at nt 1003 in BALB14; Ⓢ, the *Sau*3A site at nt 963 in *Mtv-7*.

Fluorescence-activated cell sorting (FACS) analysis. Lymph node cells or mononuclear peripheral blood cells (10^6) were stained with fluoresceinated anti-TCR Vβ-specific monoclonal antibodies: anti-Vβ6 (RR4-7), anti-Vβ2 (B20.6), anti-Vβ14 (MR14-2), or anti-Vβ10b (B21.5) (Pharmingen, San Diego, Calif.). Phycoerythrin-coupled anti-CD4 (GK 1.5) antibodies were used in the second dimension. Leukocytes were recovered from a heparinized blood sample by centrifugation through a Ficoll cushion. Lymph node or peripheral blood mononuclear cells (10^4) were analyzed by FACScan using the Lysis II program (Becton Dickinson). Dead cells were gated by forward- and side-scatter analyses.

Cloning. Viral cDNA was prepared by using SuperScript II reverse transcriptase in the buffer supplied by the manufacturer (GIBCO/BRL, Gaithersburg, Md.) and a $(dT)_{15}$ primer (Promega Biotec, Inc., Madison, Wis.). The BALB14 and BALB2 cDNAs were prepared by using RNA isolated from mammary gland tumors that developed in BALB/cT sublines 10 and 9, respectively, while LA cDNA was synthesized from RNA isolated from the milk of BALB/cLA females. Recombinant virus cDNAs from (BALB/cT × AKR/J)F_1 females were prepared with RNA isolated either from their mammary glands or their milk. The RNA samples were treated with RNase-free DNase (Boehringer Mannheim Corp., Indianapolis, Ind.) prior to the reverse transcription reaction.

The BALB2 and BALB14 cDNAs were amplified by PCR with oligonucleotide primers that hybridize to all MMTV LTR sequences: 5′TCCCGAGAGTGTCC TACAC3′ (nucleotides [nt] 31 to 49 in the MMTV LTR) (forward primer A, Fig. 1A) and 5′GGACTGTTGCAAGTTTACTC3′ (nt 1203 to 1184) (reverse primer B, Fig. 1A). The LA and recombinant (BALB/cT × AKR/J)F_1 viral LTRs were cloned from cDNA by using primer A and a *Mtv-7 sag*-specific reverse primer 5′CCCCCATGAGTATATTTGA3′ (nt 944 to 926) (primer C, Fig. 1A). The positions of all the primers are according to the numbering of Brandt-Carlson et al. (4). The PCR products were size fractionated on 1% agarose gels, purified, blunt ended, and cloned into the *Sma*I site of pBluescript (Stratagene, Inc., La Jolla, Calif.).

At least two independently isolated clones of each plasmid were sequenced. Nucleotide sequences have been submitted to the Genbank Nucleotide Sequence Data Base and have been assigned accession numbers U71271 (BALB2), U71270 (BALB14), and U71272 (LA).

RNase T_1 protection assays. RNA was isolated from peripheral lymph nodes, spleens, milk, and lactating mammary glands of the indicated mice as previously described (15, 16). The 3′ LTR sequences used as probes (Fig. 1B) were isolated as *Sau*3A-*Sau*3A fragments either from a plasmid containing the BALB/cV LTR (a Vβ2-deleting MMTV [23, 26]) (319 bp; nt 741 to 1060) or from the BALB14 LTR-containing plasmid (260 bp; nt 742 to 1002) and cloned into the *Bam*HI site of pBluescript (diagrammed in Fig. 1B). The probe specific for the *Mtv-7 sag* was described elsewhere (17). To generate probes, the plasmids were cut with *Xho*I or *Xba*I, and T_3 or T_7 RNA polymerase was used to create [^{32}P]UTP-labeled antisense RNAs. Ten micrograms of total RNA isolated from the milk and mammary gland tumors and 40 μg of RNA isolated from the lymphoid organs and lactating mammary glands were used for RNase T_1 protection analysis, as previously described (15, 16).

Virus injection. Milk (50 μl) from BALB/cLA mice was injected into the footpads of BALB/c mice. After 4 days, the percentage of the $CD4^+$ T cells expressing the indicated TCR Vβ chain in the draining popliteal lymph nodes was determined by FACS analysis.

Genomic DNA isolation and PCR. High-molecular-weight DNA was isolated from spleens of 3- to 5-week-old to 5-month-old BALB/cT, BALB/cLA, BALB/c, and AKR/J mice, and 0.25 μg was amplified by PCR. All of the primers used are diagrammed in Fig. 1A. Amplification of all endogenous MMTV DNAs was accomplished by using forward primer G 5′GAGACGCTCAACCTCAATTG A3′ (nt 507 to 527) and reverse primer B. Primers D ((5′AATTCGGAGAA CTCGACCTTCC3′, nt 268 to 289) and C were used to detect the LA provirus, primers D and E (5′CTTTCCCCACAAGCTCATCAT3′, nt 879 to 859) were used to detect the BALB14 provirus, and primers D and F (5′CAAGGGCAA TGCCTTAATACTA3′, nt 939 to 918) were used to detect the BALB2 provirus. Semiquantitative PCR was carried out by 31 cycles of 1 min at 55°C, 1 min at 72°C, and 1 min at 94°C. To show that the PCR assay was in the linear range, the same reactions were performed for 31 cycles using known copy numbers of plasmid DNA containing the BALB2, BALB14, or BALB LA viral LTRs. At 31 cycles, the amount of PCR products increased in a linear fashion between 3,000 and 300,000 copies of plasmid DNA. The PCR products were size fractionated on 1.2% agarose gels, stained with ethidium bromide, and visualized under UV light. Relative copy numbers were estimated by comparing the amplified bands in the genomic DNA to those of the plasmid standards.

RESULTS

TCR Vβ expression in BALB/cT mice. Comparison of the TCR Vβ repertoire in virus-positive (BALB/cT) and virus-negative (BALB/c) adult mice demonstrated that the former had profound and selective deletion of Vβ2- and Vβ14-expressing $CD4^+$ T cells (BALB/cT, subline 7; Table 1). Foster nursing of these BALB/cT mice on (C57BL/6 × BALB/c)F_1 mothers restored $V\beta14^+$ and $V\beta2^+$ T-cell numbers to normal levels (37a), suggesting the presence of at least one exogenous MMTV. We were also able to establish sublines of BALB/cT mice that had deletions of $V\beta2^+$ (subline 9, Table 1) or $V\beta14^+$ (subline 10, Table 1) T cells alone. Thus, BALB/cT mice carried two different exogenous MMTVs, BALB14 and BALB2, which interacted with Vβ14- and Vβ2-bearing T cells, respectively. Unless otherwise indicated, all the experiments described here were performed with BALB/cT mice bearing both BALB2 and BALB14 exogenous viruses.

Sequence analysis of the BALB2 and BALB14 LTRs. To determine the nucleotide sequences of the BALB2 and BALB14 LTRs, RNAs isolated from the mammary gland tumors of two different BALB/cT sublines, one carrying the BALB2 (subline 9) and the other carrying the BALB14 (subline 10) virus, were used for reverse transcription-PCR (RT-PCR) and the resultant products were cloned and sequenced. Two primers common to all MMTVs (A and B in Fig. 1A) were used to amplify the cDNAs; these primers also amplified endogenous viruses expressed in the tumors. Since the levels of exogenous viral RNA produced in these tumors were much higher than those of endogenous viral RNA, however, the majority of the cloned products were from the former (not shown). The nucleotide sequences of the *sag* regions of BALB2 and BALB14 are shown in Fig. 2A and B. The LTR sequences of both viruses are very similar to those of previously described MMTVs (4). The predicted amino acid sequence of the BALB2 hypervariable region (Fig. 2C) was highly homologous to this region in other Vβ2-deleting viruses, such as BALB/cV (26), II-TES2 (2), Mtv-DDO (25), and MMTV(C4) (46). Moreover, BALB2 was also similar to MMTV(C3H-K), a provirus isolated from a kidney tumor of BALB/cf/Cd (10) (Fig. 2C); the Sag encoded in the MMTV(C3H-K) LTR has been reported to be nonfunctional (1). The BALB14 hypervariable region was similar to those of MMTV(C3H), BR6, and *Mtv-2*, all of which encode Vβ14-specific Sags (4).

Deletion of $V\beta2^+$, $V\beta14^+$, and $V\beta6^+$ T cells in BALB/cLA mice correlates with the presence of three exogenous MMTVs. Mice infected with exogenous MMTV show a slow deletion of Sag-cognate T cells following their activation (29). We reported previously that BALB/cT mice foster nursed on (BALB/cT × AKR/J)F_1 but not on (AKR/J × BALB/cT)F_1 mothers lacked $V\beta6^+$ and $V\beta8.1^+$ T cells as adults (38). Deletion of these T-cell subsets is associated with the *Mtv-7* locus found in several mouse strains, including AKR/J (11). This suggested that new exogenous viruses with Sag specificities different from those of BALB2 and BALB14 were generated in the (BALB/cT × AKR/J)F_1 mothers and passed to the foster-nursed BALB/cT pups. We generated a substrain of BALB/cT mice from a single animal that nursed on a (BALB/cT × AKR/J)F_1 mother; this substrain we called BALB/cLA. This female exhibited early and progressive deletion of her $V\beta14^+$ and $V\beta2^+$ T cells, whereas no deletion of $V\beta6^+$ T cells was detected until 24 weeks of age. From this point onward, a progressive decrease in the percentage of this subset of T cells was detected. This female was bred, and in her offspring, the deletion of $V\beta6^+$ T cells had an earlier onset (37a). After three

TABLE 1. TCR Vβ repertoire and MMTVs present in BALB/cT and BALB/cLA mice

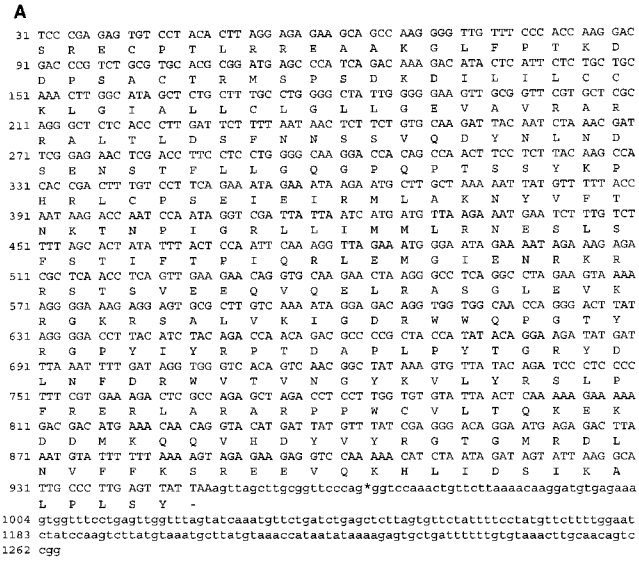
Mouse line	% Indicated $V\beta^+/CD4^+$ T cells[a]			MMTVs present		
	Vβ2	Vβ14	Vβ6	BALB2	BALB14	LA/*Mty-7*
BALB/c	7.3 ± 0.08	9.9 ± 0.17	10.2 ± 0.18	−	−	−
AKR	7.8 ± 0.07	9.4 ± 0.10	0.3 ± 0.09	−	−	+(*Mtv-7*)
BALB/cT[b]	1.6 ± 0.10	0.6 ± 0.08	10.2 ± 0.16	+	+	−
BALB/cT[c]	7.8 ± 0.08	0.5 ± 0.07	11.4 ± 0.12	−	+	−
BALB/cT[d]	1.2 ± 0.10	11.2 ± 0.10	10.6 ± 0.17	+	−	−
(BALB/cT × AKR)F_1	1.2 ± 0.10	0.8 ± 0.12	0.2 ± 0.08	+	+	+(*Mtv-7*)
BALB/cLA	1.2 ± 0.17	0.4 ± 0.06	0.3 ± 0.06	+	+	+(LA)

[a] Leukocytes were isolated from heparinized blood by Ficoll density centrifugation and stained with a mixture of fluorescein isothiocyanate-labeled anti-Vβ2, anti-Vβ6, or anti-Vβ14 and phycoerythrin-coupled anti-CD4 antibodies. The data are expressed as mean and standard deviation for each group ($n = 6$). All mice were 12 weeks old.

[b] BALB/cT, subline 7.

[c] BALB/cT, subline 10.

[d] BALB/cT, subline 9.



C

Vβ2-deleting MMTVs

Mtv-DDO	LIHLKVFFNSREEVKKHLIESIKALPLAY
MMTV(C4)	MRD-NL--K-----Q----D-------S-
MMTV(C3H-K)*	MRD-N---K-----Q----D-------S-
BALB/CV	MRD-N---K-----Q----D-------S-
MMTV(CS)	M---EA--K-----QR--M--------S-
II-TES2	M---EA--K-----QR--M--------S-
BALB2	MRD-N---K-----Q----D-------S-
Mtv-6 **	M--W---Y-----A-R-I--H-------F

Vβ14-deleting MMTVs

MMTV(C3H)	M.HFWGKIFHTKEGTVAGLIEHYSAKTYGMSYYE
BR6x	-SSI---------R---A---------------D
II-TES14	-SSI-------------A---------------D
Mtv-2	-.-----V------A-----------------D
BALB14	-MSL-----R-----MTA---------------D

FIG. 2. (A) Partial nucleotide and predicted amino acid sequences of the BALB2 Sag; *, the deleted region, relative to other MMTV LTRs. (B) Nucleotide and predicted amino acid sequence of the BALB14 Sag; the 18-bp region deleted in BALB2 is doubly underlined. (C) Comparison of the amino acid sequences of the BALB2 and BALB14 hypervariable regions (amino acids 287 to 315) with those of other MMTVs that encode Vβ2- and Vβ14-interacting Sags. Identity is indicated by a hyphen, and a period indicates deleted amino acids; *, MMTV(C3H-K) virus encodes a nonfunctional Sag; **, *Mtv-6* Sag interacts with Vβ3-, -5-, and -17-bearing T cells.

generations, the decrease in the percentage of $CD4^+/V\beta6^+$ T cells was detected in 15-day-old BALB/cLA mice (5.5% ± 0.1% versus 10.2% ± 0.18% in BALB/cT mice). As can be seen in Table 1, by 12 weeks of age, BALB/cLA mice showed almost complete deletion of this subset of T cells.

To determine which viruses were expressed in and produced by the lactating mammary glands of BALB/cT, (BALB/cT × AKR/J)F_1, and BALB/cLA mice, we performed RNase T_1 protection assays with probes specific for the BALB2, BALB14, and *Mtv-7* viruses. The probes were derived from the hypervariable regions of the Sags encoded by these or similar viruses (see Materials and Methods). Figure 3A shows the results of such analysis performed with total RNA isolated from the lactating mammary glands and milk of the different mice. When probes specific for BALB2 and BALB14 were used, full-length protection corresponding to the expression of both viruses was seen with RNA isolated from the mammary glands and milk of BALB/cT, (BALB/cT × AKR/J)F_1, and BALB/cLA mice (Fig. 3A). Neither (AKR/J × BALB/cT)F_1 (Fig. 3A) nor BALB/c (not shown) mice expressed any of these viruses in mammary gland or shed exogenous virus into milk. With the *Mtv-7*-specific probe, full-length protection was seen only with RNA isolated from the mammary glands and milk of BALB/cLA mice but not with RNA from the other mice (Fig. 3A, lower panel). Although *Mtv-7* is present in several strains of mice, it is not expressed in their mammary glands [Fig. 3A, (AXB)F_1 mice; Fig. 3B, AKR/J and DBA/2 mice (17, 42)]. Expression of this provirus was easily detected in the lymphoid organs of the *Mtv-7*-positive mice, however (Fig. 3B). Therefore, a new *Mtv-7*-like exogenous virus was expressed in the BALB/cLA mammary gland, in contrast to its endogenous counterpart, which was silent in this tissue.

Sequence analysis of the *sags* of the new exogenous MMTVs in BALB/cLA and (BALB/cT × AKR/J)F_1 mice. To clone the *sag* coding region of the new exogenous *Mtv-7*-like virus present in BALB/cLA mice, RNA isolated from the virus fraction of their milk was used for RT-PCR with primers A and C (Fig. 1A; see Material and Methods), and the product was cloned and sequenced. The partial nucleotide sequence of the cloned cDNA is shown in Fig. 4. As can be seen, the new exogenous virus present in BALB/cLA mice, termed LA virus, resulted from recombination between the exogenous BALB14 and the endogenous *Mtv-7* viruses. The LTR sequence upstream of the hypervariable *sag* region was derived from BALB14, and the *sag* hypervariable region was derived from *Mtv-7*. The breakpoint of recombination occurred between nt 721 and 789, since nt 720 is BALB14 specific and nt 790 is *Mtv-7* specific.

As we previously reported, 52% of BALB/cT mice nursed on (BALB/cT × AKR/J)F_1 females showed significant deletion of their $V\beta6^+$ and $V\beta8.1^+$ T cells between the ages of 10 and 36 weeks (38). This result indicated that LA virus was not unique and that recombinant viruses were generated in many (BALB/cT × AKR/J)F_1 females. Although no *Mtv-7*-like viruses were detected in the mammary glands of (BALB/cT × AKR/J)F_1 females by RNase protection assays (Fig. 3A, bottom panel), it was possible that such recombinant viruses were present at low levels. To determine whether new exogenous *Mtv-7*-like MMTVs appeared in (BALB/cT × AKR/J)F_1 females and had

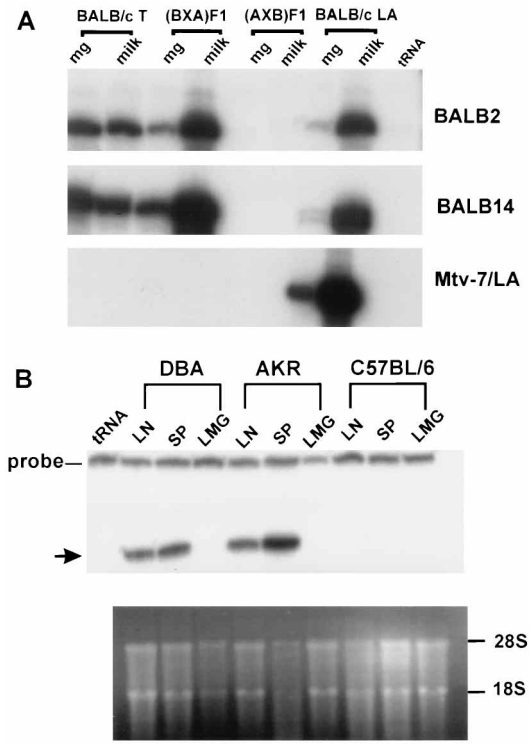


FIG. 3. (A) Expression and production of different exogenous MMTVs by the mammary glands of BALB/cT, (BALB/cT × AKR/J)F_1, (AKR/J × BALB/cT)F_1, and BALB/cLA mice. Forty micrograms of lactating mammary gland and 10 μg of milk RNA isolated from the indicated mice were subjected to RNase T_1 protection analysis with probes specific for BALB2, BALB14, and *Mtv-7*. Abbreviations: (BXA)F1, viremic (BALB/cT × AKR/J)F_1 mice; (AXB)F1, nonviremic (AKR/J × BALB/cT)F_1 mice. (B) *Mtv-7* is expressed only in lymphoid cells. Total RNA was isolated from the lymph nodes (LN), spleen (SP), or lactating mammary gland (LMG) of *Mtv-7*-positive DBA/2 and AKR/NCr mice or *Mtv-7*-negative C57BL/6 mice, and 40 μg was subjected to RNase T_1 protection analysis with the *Mtv-7*-specific probe. (Bottom) Twenty micrograms of the same RNA was electrophoresed on a 1% formaldehyde gel and stained with ethidium bromide to verify the quality of the RNA. The migrations of the 18S and 28S ribosomal bands are indicated.

recombination breakpoints similar to that of LA virus, the same strategy was used to PCR amplify and clone such viruses from the mammary glands or milk of these mice. RNA isolated from four different (BALB/cT × AKR/J)F_1 females was used for this analysis.

We isolated several distinct *Mtv-7*-like exogenous viruses that were expressed in the mammary glands of these mice (Fig. 4). All of them resulted from recombination between the BALB14 exogenous and *Mtv-7* endogenous viruses; none of the novel viruses contained BALB2-specific sequences (Fig. 4). The viruses had recombination breakpoints scattered throughout the region between nt 524 and 861; this latter site is just upstream of the *Mtv-7* Sag hypervariable region. These viruses were found multiple times in different mammary gland samples (Fig. 4), indicating that they were selected and not the products of random recombination. Importantly, no recombinants were found with breakpoints upstream of nt 524, indicating that the sequences from this point to the 5′ end of the BALB14 LTR were selectively retained.

Mammary gland tumorigenesis in BALB/cT and BALB/cLA mice. The recombinant virus acquired by BALB/cLA mice had the *Mtv-7* Sag and was expressed in mammary glands. To determine whether this virus was also tumorigenic, we continuously bred BALB/cT and BALB/cLA mice and monitored mammary gland tumor incidence. Multiparous BALB/cT females containing both BALB2 and BALB14 had a mammary gland tumor incidence of about 40% by 1 year of age (Table 2). In contrast, 80% of BALB/cLA mice developed mammary gland tumors within the same period (Table 2). Insufficient numbers of BALB/cT sublines 9 (BALB2 alone) and 10 (BALB14 alone) mice are currently available for similar analysis. Southern blot analysis of DNA isolated from a number of BALB/cT and BALB/cLA tumors revealed that they all contained newly acquired copies of integrated exogenous MMTVs (not shown).

If the LA virus was more infectious and as a result more tumorigenic than BALB2 and BALB14, it should be both expressed at higher levels in normal mammary gland tissues and found amplified in a greater percentage of mammary tumors. We used RNase protection analysis with the virus-specific probes to determine the relative levels of expression of the different viruses in healthy tissues and in tumors. We found more LA-specific than BALB2- and BALB14-specific RNA in BALB/cLA mammary gland and milk (Fig. 3A). When a similar analysis was done with RNA isolated from 11 independent BALB/cLA mammary gland tumors, 6 different tumors expressed only LA virus (Fig. 5, lanes 1, 2, 4, 6, 9, and 10), and 2 tumors each expressed LA together with BALB2 (lanes 7 and 11) or BALB14 (lanes 3 and 8). BALB14 viral RNA was expressed alone in only one tumor (tumor 5), and no tumors expressed only BALB2. Thus, although *Mtv-7*-like exogenous viruses are produced only at low levels in (BALB/cT × AKR/J)F_1 mothers, LA virus was highly infectious, was expressed at high levels in infected BALB/cT mice that acquired this virus through nursing, and was associated with more mammary gland tumors.

LA virus is highly infectious for lymphoid cells and has a strong Sag. The data described in the preceding sections demonstrated that LA virus was present at higher titer in mammary glands and therefore was associated with more tumors in BALB/cLA mice than the BALB2 and BALB14 viruses were. To determine whether LA virus also infected lymphoid cells at high levels, we examined the relative levels of viral DNA in the lymphocytes of BALB/cLA mice. Semiquantitative PCR analysis was carried out with primers specific for BALB2, BALB14, and LA virus. When the same DNA samples from spleens from two different 3- to 5-week-old BALB/cLA mice were subjected to this analysis, only LA virus could be detected (Fig. 6A). In 5-month-old BALB/cLA mice, there were approximately 120,000 copies of LA virus per μg of genomic DNA, while only 12,000 copies/μg were found of both BALB14 and BALB2. Thus, lymphoid tissue was more highly infected with LA virus than with the other two exogenous viruses.

Because LA virus encoded an *Mtv-7*-like Sag, the high levels of infection of lymphoid tissue could be due to its strong cognate T-cell-stimulatory activity. The ability of LA virus to stimulate T cells was tested in vivo by injecting BALB/cLA milk into the footpads of adult BALB/c mice. This route of infection induces a vigorous localized immune response in which the number of Sag-reactive T cells increases in the drain-

TABLE 2. BALB/c LA mice have a higher tumor incidence than BALB/c T mice

Mouse line	Viruses	Tumor incidence[a]
BALB/cT	BALB2, BALB14	68/171 (39.7%)
BALB/cLA	BALB2, BALB14, LA	24/30 (80%)

[a] Tumor incidence at 1 year. Number of mice with tumors/total number of mice. $P < 0.01$ by Fisher's exact test.

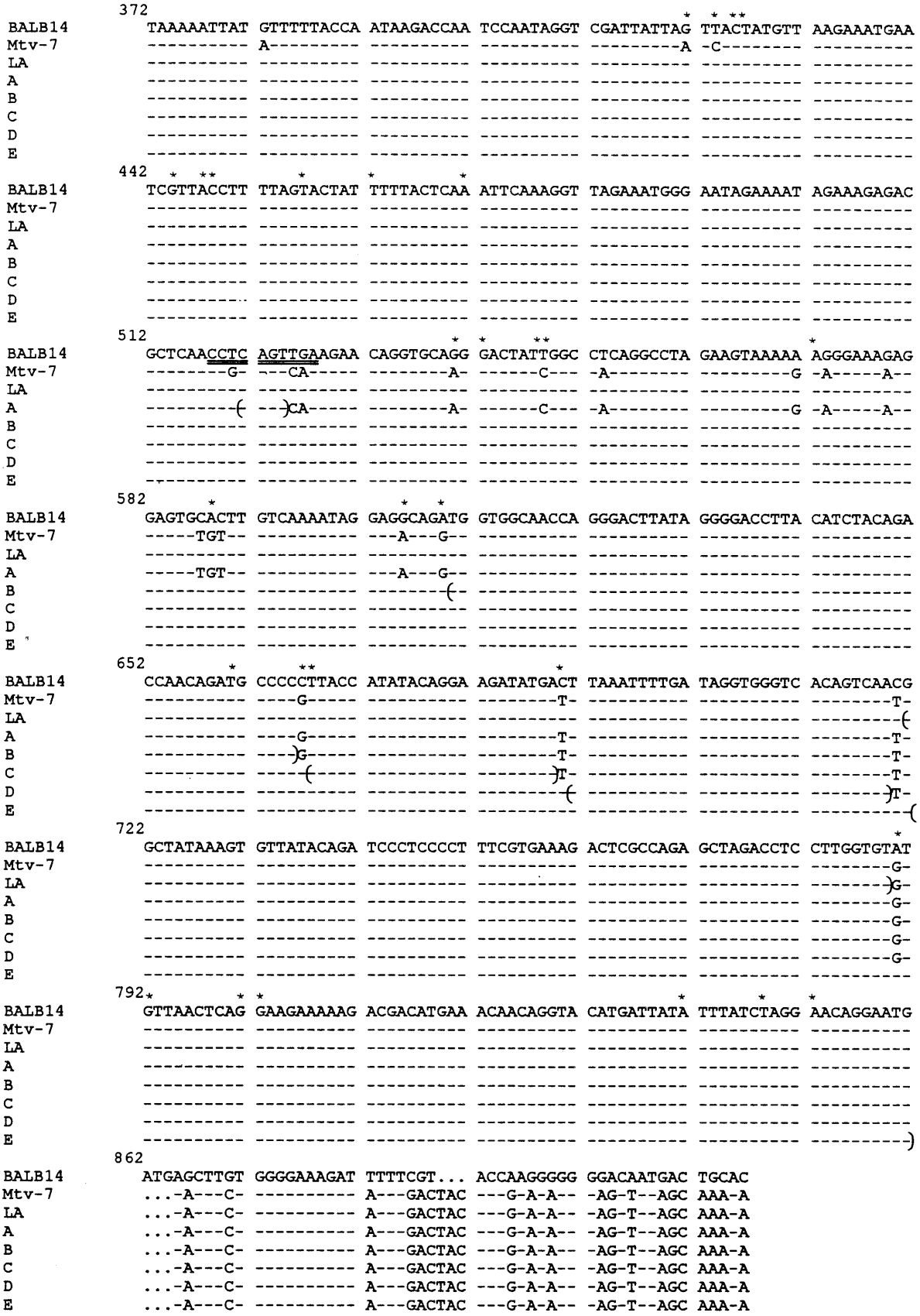


FIG. 4. Sequence comparison of the recombinant *Mtv-7*-like MMTVs cloned from the milk or mammary glands of (BALB/cT × AKR/J)F_1 and BALB/cLA females. Nine recombinant viruses from four different (BALB/cT × AKR/J)F_1 animals were sequenced. Five different recombination breakpoints (A through E) were found in these viruses. Parentheses show the breakpoints of recombination. The viruses were found as follows: mouse 1, two A and two E viruses; mouse 2, one C virus; mouse 3, two B and one D virus; mouse 4, one B virus. The stars above the nucleotides indicate nucleotide differences between BALB14 and BALB2 (see Fig. 2). Nucleotide sequences identical to BALB14 are indicated by hyphens; periods indicate missing nucleotides. The STAT consensus sequence is doubly underlined.

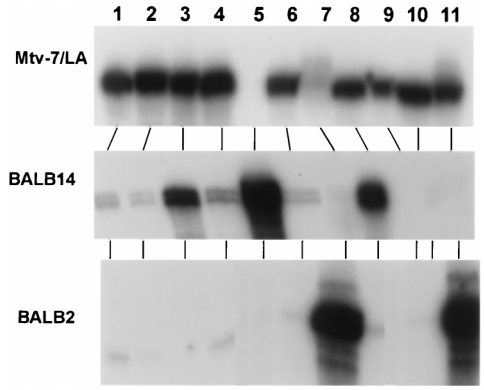


FIG. 5. LA virus is highly expressed in mammary gland tumors. RNA was isolated from 11 different BALB/cLA mammary gland tumors and subjected to RNase T_1 protection analysis with probes specific to *Mtv-7*/LA, BALB2, and BALB14. Ten micrograms of total RNA from each tumor was used for this analysis. The doublet seen with the BALB14 probe is probably due to incomplete digestion by RNase T_1.

ing popliteal lymph nodes (19). Four days after injection, vigorous expansion of $CD4^+/V\beta6^+$ T cells in the draining popliteal lymph nodes in BALB/c mice was evident when BALB/cLA but not BALB/c milk was injected (Fig. 6B). In contrast, only a small expansion of $CD4^+/V\beta14^+$ and $CD4^+/V\beta2^+$ T cells induced by BALB14 and BALB2, respectively, was seen (Fig. 6B). Thus, the ability of LA virus to infect lymphoid cells was paralleled by its stimulation of cognate T cells.

DISCUSSION

A unique feature of retroviruses is that two RNA genomes are packaged in one virion, and thus some virus particles produced by cells expressing both endogenous and exogenous viruses contain heterogenous RNA molecules (9). Recombination between the exogenous and endogenous genomes often occurs after infection by strand switching during cDNA synthesis and is responsible for the generation of highly tumorigenic variants of murine (47) and feline (36) leukemia viruses. Exogenous MMTV also infects cells in which endogenous MMTVs are highly expressed, and novel exogenous viruses generated through recombination and with the ability to infect different strains of mice have been detected in laboratory settings (16).

Such viruses may naturally arise in mice bearing exogenous MMTVs and thus could be the source of novel MMTVs (4, 46). We found two new exogenous MMTVs of unknown origin in BALB/cT mice, BALB2 and BALB14, which encode Vβ2- and Vβ14-specific Sags, respectively. Both viruses are highly homologous to the known endogenous and exogenous MMTVs. For example, the sequence of the BALB2 Sag, as well as the sequences of the other Vβ2-deleting viruses, is strikingly similar to that of *Mtv-6*, which interacts with Vβ3-, -5, and -17-bearing T cells. Only 3 of the 288 amino-terminal amino acids (not shown) and 14 of the carboxyl-terminal amino acids (Fig. 2C) differ between these two viruses. Thus, *Mtv-6*, which is a defective provirus, may represent a germ line insertion of a BALB2-like virus. Alternatively, all of the Vβ2-like viruses may have arisen from an *Mtv-6*-like virus and then diverged through recombination and mutation events.

The BALB2 Sag is also nearly identical to that of BALB/cV, which also arose in BALB/c mice and induces mammary tumors (10, 23) (Fig. 2C). In contrast, there are several amino acid sequence differences in the BALB2 and BALB/cV Sags

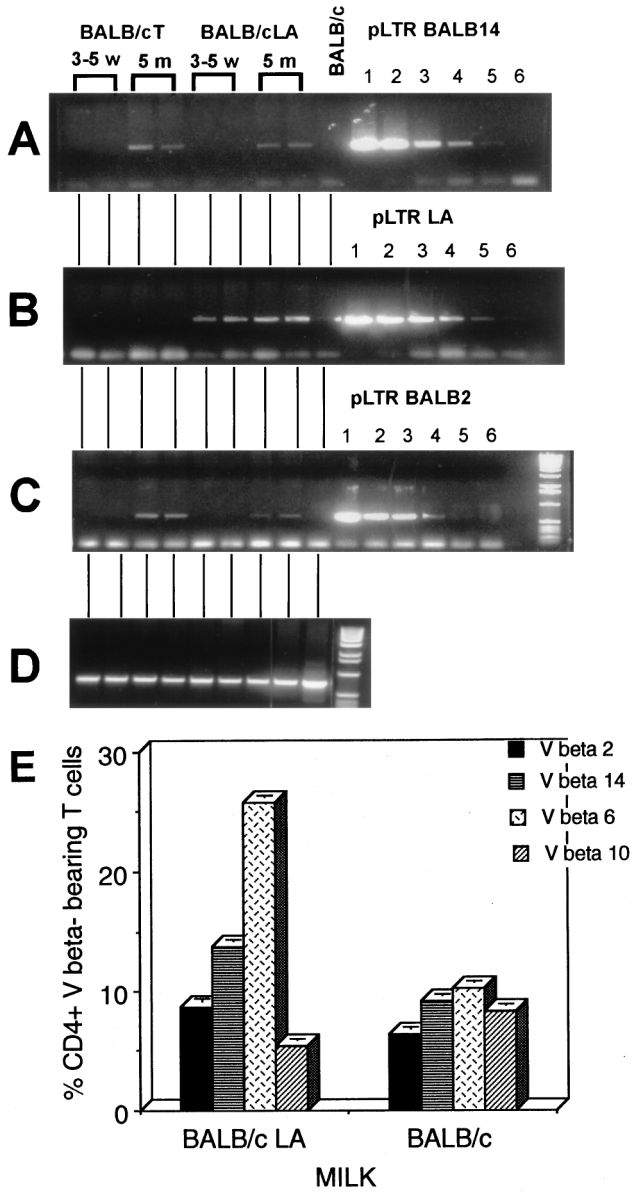


FIG. 6. (A) Detection of the different viruses in the spleens of BALB/cT and BALB/LA mice. PCR was performed with primers specific for BALB14 (A), *Mtv-7*/LA (B), BALB2 (C) or total MMTV (D) (see Materials and Methods) and DNAs isolated from spleens of the indicated mice. Serial dilutions of plasmids carrying LTRs of BALB14 (pLTR BALB14), BALB2 (pLTR BALB2), or LA (pLTR LA) virus were included in this experiment (lane 1, 3×10^7 copies; lane 2, 3×10^6 copies; lane 3, 3×10^5 copies; lane 4, 3×10^4 copies; lane 5, 3×10^3 copies; lane 6, 3×10^2 copies). Abbreviations: 3-5 w, 3- to 5-week-old mice; 5 m, 5-month-old mice. The molecular weight marker is the 1-kb ladder (GIBCO/BRL, Life Technology, Gaithersburg, Md.). (E) Expansion of $V\beta2^+$, $V\beta14^+$, and $V\beta6^+$ $CD4^+$ T cells in adult BALB/c mice injected with BALB/cLA milk. BALB/cLA or BALB/c milk (50 μl) was injected into the footpads of adult BALB/c mice. Four days later, the draining popliteal lymph nodes were isolated and the lymphocytes were analyzed with a FACScan cell sorter (Becton Dickinson). No expansion of $V\beta10^+$ cells, which do not bind to the Sags of the viruses present in these mice, was seen. The data are expressed as the mean plus standard deviation (error bars) ($n = 3$).

and those of the other known Vβ2-deleting MMTVs: MMTV (C4) (46), Mtv-DDO (25), II-TES2 (2), and MMTV(CS) (35) (Fig. 2C). Interestingly, the BALB2 LTR has an 18-bp deletion 19 nt downstream of the Sag stop codon that is identical to that found in MMTV(C3H-K), a virus associated with kidney ade-

nocarcinomas in BALB/cf/Cd mice (50). However, the MMTV (C3H-K) Sag is not functional, since transgenic mice expressing a molecularly cloned MMTV(C3H-K) did not delete $V\beta2^+$ T cells (1). It was suggested that the 18-bp deletion could affect expression of the Sag protein. Since the BALB2 LTR also contains this 18-bp deletion (Fig. 2A) and encodes a functional Sag, other alterations within the viral genome must be responsible for the lack of Sag activity in MMTV(C3H-K).

Comparison of the BALB14 Sag with the other Vβ14-interacting Sags shows that its hypervariable domain is the most diverged from that of MMTV(C3H) (Fig. 2C). It has also been reported that the MMTV(C3H) Sag can stimulate Vβ15-bearing T cells; whether the BALB14 Sag also interacts with this TCR remains to be determined. Such comparisons should help define the amino acid residues involved in Sag/TCR interactions.

BALB/cT mice that foster nursed on (BALB/cT × AKR/J)F_1 mothers showed deletion of $V\beta6^+$ and $V\beta8.1^+$ T cells in addition to $V\beta2^+$ and $V\beta14^+$ T cells (38; this paper). Deletion of these last two subsets is normally associated with *Mtv-7* or similar viruses. *Mtv-7*, which is present in the genomes of AKR/J but not BALB/c mice, is expressed only in cells of the immune system and encodes one of the most powerful Sags thus far identified; it causes profound stimulation of Sag-cognate T cells both in vivo and in vitro (30, 40). Because the reciprocal hybrids [(AKR/J × BALB/cT)F_1] did not transmit *Mtv-7*-like viruses, it appeared that the recombinant viruses were generated in the (BALB/cT × AKR/J)F_1 mice upon productive infection of their lymphoid cells with one of the exogenous MMTVs present in BALB/cT mice following by copackaging with *Mtv-7* and consequent recombination. Sequence analysis of the LA virus LTR, as well as the other recombinant viruses produced in the (BALB/cT × AKR/J)F_1 lactating mammary gland, demonstrated that these viruses indeed resulted from recombination between the *Mtv-7* endogenous and the BALB14 exogenous viruses.

In contrast to its endogenous counterpart, *Mtv-7*, LA virus was highly expressed in the mammary gland. We reported recently that many of the endogenous MMTVs could be divided into two groups according to whether they were or were not expressed in the mammary gland (39a, 42). *Mtv-7* belongs to the second group; although it is silent in mammary gland cells, expression of this provirus could be easily detected in lymphoid cells (Fig. 3A and B; 42). The different MMTV LTRs are highly homologous to each other outside the Sag hypervariable region. However, all MMTVs that are expressed in the mammary gland [i.e., MMTV(C3H), MMTV(SW), *Mtv-1*, *Mtv-6*, and *Mtv-17* (42)] have a T at position 520, whereas those expressed in lymphoid tissue (*Mtv-7*, *Mtv-9*) have a G (Fig. 4; 41). Both the BALB14 and the BALB2 viruses have a T at nt 520 and are expressed in the mammary gland. LA virus, as well as all of the other recombinant viruses expressed in (BALB/cT × AKR/J)F_1 mammary glands, acquired this T residue through recombination with BALB14 virus (Fig. 4). Therefore, only new recombinant *Mtv-7*-like MMTVs with a T residue at position 520 were expressed in the mammary gland of (BALB/cT × AKR/J)F_1.

This T residue falls in a consensus binding site for the STAT transcription factor family (24, 45). Previous work on STAT factors has shown that STAT5 plays a role in the prolactin-mediated stimulation of milk protein genes such as β-casein (49) and β-lactoglobulin (5). We have evidence that alteration of this T to G at position 520 prevents binding of one member of this family, STAT5b (39a). STAT5 proteins are thought to mediate transcriptional response to cytokines such as prolactin but not to determine tissue-specific expression in the mammary gland. Indeed, none of the STAT transcription factors have been shown to be responsible for tissue specificity. However, the STAT proteins have been shown to interact with other transcription factors (18, 44), and such combinatorial action of multiple factors may contribute to this specificity. Further studies are in progress to delineate the role of the STAT5b protein in the mammary gland-specific transcription of MMTV (39a).

Surprisingly, even though the BALB2 and BALB14 viruses are both highly expressed in BALB/cT mice and both contain the same STAT consensus sequence, only BALB14 participated in the generation of LA virus and the other recombinant MMTVs found in (BALB/cT × AKR/J)F_1 females. These data suggest that the BALB14 and *Mtv-7* RNAs are copackaged more efficiently or that there is selection for BALB14 sequences upstream of the *sag* hypervariable domain that contribute to the recombinant virus's infectivity. These could include either other regions of BALB14 that contribute to expression of the virus or viral structural proteins. We have found that in BALB/cT mice, BALB14 is present in more tumors than is BALB2, indicating that indeed other features of the former are important for its infectivity or tumorigenicity (not shown).

Although *Mtv-7*-like recombinant viruses represented a minute fraction of the total viruses transmitted by (BALB/cT × AKR/J)F_1 females to foster-nursing BALB/cT pups, the LA virus became highly expressed in both lymphoid and mammary gland cells within three generations of transmission in BALB/cLA mice. Moreover, it was the predominant virus found in mammary tumors. What accounts for its high infectivity? One possibility is that LA virus acquired from *Mtv-7* a powerful Sag with the strong T-cell stimulatory activity that allowed it to amplify very effectively in lymphoid cells. In support of this, injection of BALB/cLA milk into BALB/c footpads resulted in very strong stimulation of Vβ6-bearing T cells. In addition, LA virus was more abundant than either BALB2 or BALB14 in the lymphoid cells of BALB/cLA mice. Viruses without Sags do not amplify in the cells of the immune system and therefore never get to the mammary gland (17a). Conversely, recombinant viruses with strong Sags may be selected because they can more highly amplify in the immune system and, as a result, more viruses are ultimately transmitted to the mammary gland.

MMTV induces mammary tumors by insertional activation of cellular oncogenes. Because integration is a stochastic event, the higher the viral load, the more rapidly mice develop mammary tumors. Because of their high virus titer in both lymphoid and mammary gland cells, MMTVs with strong Sags such as LA virus should be highly tumorigenic. Indeed, we found that BALB/cLA mice had a higher incidence of mammary gland tumors than BALB/cT mice (Table 2). Interestingly, another *Mtv-7*-bearing exogenous virus, MMTV(SW), which induces a vigorous Sag-dependent T-cell response in vivo after injection into naive BALB/c mice, is inefficient at inducing mammary gland tumors (19). This implies that viral genes in addition to Sag play a role in the infectivity or tumorigenicity of MMTV.

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