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Direct Detection of Exogenous Mouse Mammary Tumor Virus Sequences in Lymphoid Cells of BALB/cfC3H Female Mice

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The presence of exogenous mouse mammary tumor virus (MMTV) (C3H) DNA sequences in lymphoid tissue (spleen, bone marrow, and thymus) and nonlymphoid tissue (liver and kidney) of BALB/cfC3H female mice was directly assessed by DNA hybridization methods. Lymphoid tissues were found positive for integrated MMTV(C3H) sequences in females as young as 4 weeks. In most samples, the level of splenic MMTV(C3H) infection was low (2 to 5%). Infection remained throughout the life of the animal. The percentage of spleen samples found positive for exogenous viral infection was significantly higher in females bearing mammary tumors, whether virgin or multiparous. Liver and kidney DNAs were negative for exogenous MMTV sequences, suggesting tissue type selectivity in MMTV infection.

Murine mammary cancer results from a complex interaction between hormonal, genetic, and viral components (1, 15, 21, 24). Murine mammary tumor virus (MMTV) is the primary causative agent, inducing a high incidence of mammary tumors in females of susceptible strains. MMTV is produced in the mammary gland of infected females and transferred to suckling newborn pups. The females then develop mammary tumors with a latency period and incidence characteristic of their strain.

It has been suggested that MMTV is harbored by another cell type or types before infecting mammary epithelium (1, 24), and many different strategies have been utilized for viral detection. Among them are electron microscopy of B particles (3, 11, 35), bioassays establishing transfer of the viral agent from selected tissues of infected mice into uninfected mice (22, 23, 30), and the detection of viral antigens on surfaces of nonmammary cells (12, 13, 17, 27, 37). MMTV has consistently been localized to leukocytes. However, interpretation of these experiments has been complicated by the possible expression of endogenous MMTV sequences, yielding false-positive results for exogenous viral presence. For example, MMTV-specific RNA sequences are found in lymphoid and nonlymphoid cells of BALB/c mice lacking exogenous virus, when treated with viral, chemical, or hormonal agents (2, 16, 28, 39). Moreover, spleen cells of BALB/c and C57BL mice (also lacking exogenous virus) express MMTV-related surface antigens, presumably due to expression of endogenous virus (5, 12, 17, 37).

Direct detection of exogenous MMTV viral sequences in lymphoid cells requires a method which differentiates them from endogenous viral DNA sequences. Previous studies by Cohen et al. (6, 7), using BALB/cfC3H mice, have documented restriction enzyme site heterogeneity with respect to the 5′ *gag-pol* *Pst*I site between the two full endogenous units of BALB/c and the MMTV(C3H) exogenous virus. Fragments diagnostic for exogenous infection can be detected in DNA from infected tissue digested with *Pst*I, size fractionated, Southern blotted, and hybridized with radiolabeled MMTV(C3H).

We have screened *Pst*I-digested DNA from various tissues in BALB/cfC3H females of selected ages and physiological states for exogenous MMTV(C3H) sequences by using a 4.0-kilobase (kb) MMTV(C3H) *gag-pol* fragment as a hybridization probe. (The 4.0-kb subclone was a generous gift from R. Cardiff.) This probe simplifies the restriction pattern produced when whole virus is used as a probe, yet allows differentiation of exogenous from endogenous sequences.

The MMTV sequence complement pertinent to our study with respect to *Pst*I restriction sites in BALB/cfC3H mice is mapped in Fig. 1A. This includes endogenous units II and III and exogenous MMTV(C3H) sequences (6, 7). Each of these three MMTV sequences yields unique *gag-pol* fragments after *Pst*I digestion; endogenous units II and III produce fragments of 5.0 and 5.4 kb, respectively, and exogenous MMTV(C3H) produces a 4.0-kb fragment. The *Pst*I 4.0-kb MMTV(C3H) *gag-pol* fragment can be used as a selective probe to monitor presence of exogenous MMTV sequences (Fig. 1B). BALB/c and BALB/cfC3H liver DNA and mammary tumor DNA were examined. As shown, both liver and tumor samples display the 5.0- and 5.4-kb endogenous fragments, whereas the 4.0-kb genomic band, diagnostic of exogenous infection, is detected only in the mammary tumor sample.

In mammary tumor DNA, the exogenous-specific 4.0-kb band is more intense than the single-copy endogenous sequences of 5.0 or 5.4 kb (Fig. 1B), due to multiple integrations per cell (4, 10, 26, 29). Anticipating that in nontumor tissue the level of infectivity and number of integrations might be lower and thus the fragments more difficult to detect, we first established the limits of detection by Southern blot hybridization (36).

A dilution series was tested in which BALB/cfC3H liver DNA and diminishing amounts of mammary tumor DNA were mixed to determine the lower limit of detection of the 4.0-kb exogenous-specific band (Fig. 2). The tumor DNA sample chosen carries one exogenous MMTV copy per cell as determined by blot hybridization (data not shown). The 4.0-kb band is weakly detected at a liver/tumor dilution ratio of 500:1 (wt/wt), establishing the capability of detecting exogenous MMTV infection in 0.5% of the cells in a given tissue. Our method is approximately 10-fold more sensitive than one previously used (7).

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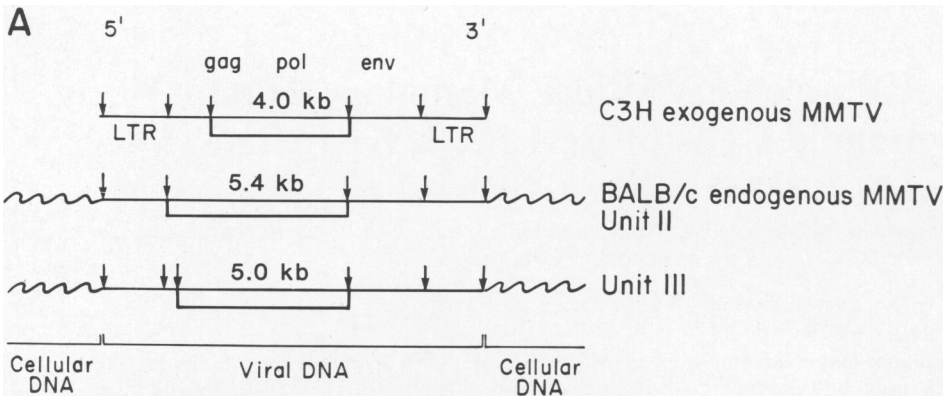

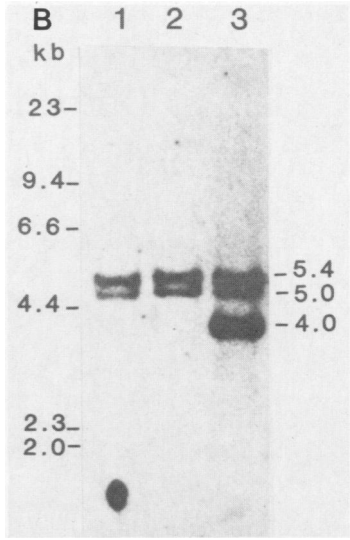

FIG. 1. The 4.0-kb MMTV(C3H) *gag-pol* fragment is used as an exogenous MMTV-specific DNA hybridization probe. (A) Restriction maps of MMTV-related sequences in BALB/cfC3H mice that are pertinent to our study. Arrows, *Pst*I sites. Wavy lines, cellular DNA sequences flanking integrated proviral sequences. Uninfected sequences carry endogenous sequences only. The BALB/cfC3H strain was originally constructed by foster nursing BALB/c pups on C3H lactating females and is maintained as a strain by nursing pups on their natural mothers. The spontaneous mammary tumor incidence in BALB/cfC3H breeding females reflects that of C3H females, which is 90 to 95% (14, 20). The spontaneous mammary tumor incidence in BALB/c(CRGL) breeding females is 0 to 5% (18, 19). (B) A total of 20 μg of cellular DNA was digested with *Pst*I, blotted onto Zeta-probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.), and hybridized with MMTV(C3H) *gag-pol*. Lanes: 1, BALB/c spleen; 2, BALB/cfC3H liver; 3, BALB/cfC3H mammary tumor. The higher-molecular-weight bands of 5.0 and 5.4 kb appear as a single band due to the length of autoradiogram exposure.

We next examined tissues other than mammary gland for exogenous MMTV sequences. Lymphoid (spleen, bone marrow, and thymus) and nonlymphoid (liver and kidney) tissues from BALB/cfC3H female donors of various ages and physiological states were assayed to establish which tissues are susceptible to MMTV(C3H), whether infection occurs transiently or permanently, and whether it correlates with changes in mammary gland development. DNA was analyzed from tissues of young (3 to 6 weeks), adult (2 months and older), virgin, parous, mammary tumor-bearing, and non-tumor-bearing females.

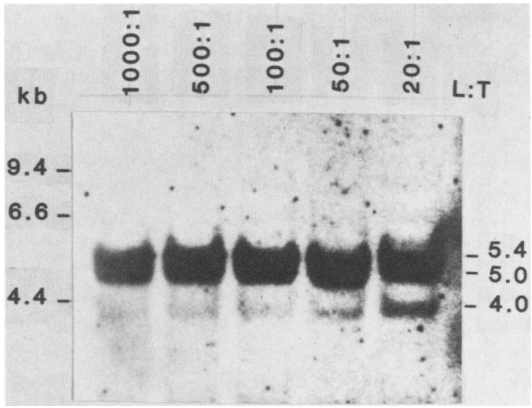

FIG. 2. Dilution series. Diminishing amounts of tumor DNA were mixed with an excess of liver DNA to establish liver/tumor dilution ratios of 1,000:1, 500:1, 100:1, 50:1, and 20:1. A total of 20 μg of DNA was used per lane and processed as described in the legend to Fig. 1B.

Exogenous virus was evident in lymphoid tissues of all ages examined; the youngest mice showing splenic infection of exogenous MMTV(C3H) were 4 weeks old (Table 1). Splenic infection may occur earlier, but at a level too low for this detection method; Ritter and Nandi (32, 33) did report transfer of viral infectivity with blood cells of 2-week-old BALB/cfC3H females. The earliest age at which exogenous MMTV infection of mammary epithelium has been reported is 6 weeks, in experiments detecting B particle production in BALB/cfC3H mammary cells in vitro (P. Nakayama, M.S. thesis, University of California, Berkeley, 1968). Thus, splenic infection appears to precede mammary gland infection.

*Bam*HI is another restriction enzyme useful in determining the presence of exogenous MMTV(C3H) DNA sequences in tissues of BALB/cfC3H mice, yielding a band diagnostic for exogenous infection at 0.7×10^6 or approximately 1 kb (6). Our results with *Pst*I were confirmed with *Bam*HI; a 1.2-kb exogenous-specific band is evident in BALB/cfC3H spleen and tumor DNA but absent in liver DNA.

MMTV(C3H) infection was evident in the spleens of adult females regardless of their physiological state (e.g., virgin, parous, or mammary tumor bearing). Similar results were obtained with bone marrow and thymus DNA. In contrast, liver and kidney DNAs from all donors tested were negative for exogenous sequences, showing selectivity of MMTV

TABLE 1. Incidence of infection with exogenous MMTV (C3H) in mouse tissue

Tissue	Age	Virgin	Parous	% Positive (no. tested)	
				Without mammary tumor	With mammary tumor
Spleen	3 wk	+		0 (7)	X[a]
	4–6 wk	+		44 (18)	X
	Adult[b]	+		29 (7)	100 (5)
	Adult		+	50 (6)	74 (19)
Bone marrow	Adult	+		25 (8)	75 (4)
	Adult		+	25 (4)	100 (2)
Thymus	Adult	+		44 (9)	50 (6)
	Adult		+	25 (4)	100 (2)

[a] X, Mouse is too young for mammary cancer to be possible.
[b] Adult, Females 2 months and older.

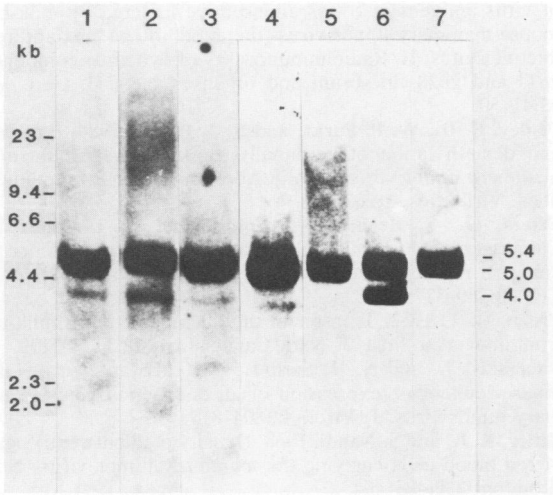

FIG. 3. Splenic infection of BALB/cfC3H females. A total of 20 μg of BALB/cfC3H DNA was used for each lane and processed as described in the legend to Fig. 1B. Lanes: 1, 20:1 liver/tumor dilution; 2, 4-week virgin spleen; 3, adult, virgin spleen from non-mammary tumor bearer; 4–6, adult, multiparous spleen from mammary-tumor bearer; 7, liver.

infection. Non-tumor-bearing primiparous or multiparous females resembled virgins in the incidence of lymphoid infection. However, in mammary tumor-bearing females, the incidence of splenic, bone marrow and thymic infection was markedly increased (Table 1): 50% or more were positive for infection in tumor-bearing females, versus 50% or fewer in non-tumor-bearing females.

The relative intensity of the 4.0-kb band varies when compared with the single-copy endogenous bands, reflecting variation in the number of cells infected for a given tissue or in the number of viral integrations per cell. The range in splenic infection levels is shown in Fig. 3. Splenic DNA samples from mammary tumor-bearing females may show a more intense 4.0-kb band when compared with spleen DNA samples from non-mammary tumor bearers (lanes 6 versus 2 or 3).

MMTV integrates randomly or at a large number of preferred sites in chromosomal DNA (15). Because of the cell heterogeneity in lymphoid tissues, it is not possible to demonstrate whether MMTV(C3H) sequences are integrated in spleens with restriction enzymes, as is possible with mammary tumors (4, 10, 26, 29). Unintegrated linear and circular forms of MMTV DNA have been found in both murine (8) and in vitro infected heterologous cells (34). Molecular weight sizes consistently range around 9 kb. To test whether the 4.0-kb *Pst*I fragment found in lymphoid tissues represented integrated exogenous MMTV, undigested spleen DNA samples were fractionated in a 0.8% agarose gel, Southern blotted, and hybridized with radiolabeled MMTV. Only high-molecular-weight DNA (>23 kb) was specific for MMTV DNA sequences. There were no smaller MMTV-specific DNA sequences, including those in the 9.0-kb range. These samples were positive for MMTV(C3H) sequences when assayed with *Pst*I. In addition, the DNA preparation procedures used select for high-molecular-weight DNA. Thus, we conclude that the exogenous MMTV(C3H) sequences detected in lymphoid cells by *Pst*I are integrated in the cellular DNA.

The exact mechanism(s) of MMTV-induced transformation of mammary epithelium is currently an area of intense investigation (26, 29). Although the evidence is not as definitive as with mammary cell neoplasia, it is possible that MMTV is also oncogenic for lymphoid cells (8). Numerous T cell lymphomas have amplified MMTV sequences (8, 9, 20), and express MMTV-related antigens on their surfaces (25, 31, 38). However, it has not been possible in these cases to directly implicate MMTV in the etiology of these tumors.

The data presented here demonstrate that exogenous MMTV can infect lymphoid cells and integrate into the DNA. It follows, then, to question the role that lymphoid cells may play in the infection of mammary epithelium by MMTV. The putative mode of MMTV transfer from lymphoid cells to mammary cells is not known. Since MMTV B particles are not produced in lymphoid tissues (11), viral transfer in particle form appears unlikely. Alternate forms such as unencapsulated RNA or DNA may be transferred by cell-to-cell contact. Given the sensitivity of our exogenous-specific hybridization assay, it may be possible to determine the lymphoid cell type or types directly involved with MMTV-mediated transformation.

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