Expression of Endogenous *Mtv* Provirus Transcripts in BALB/c Splenic Lymphocytes (43194)

ROBERT J. PAULEY* AND DIANA M. LOPEZ†,[1]

Michigan Cancer Foundation, Detroit, Michigan 48201 and Department of Microbiology and Immunology,† University of Miami School of Medicine, Miami, Florida 33101*

Abstract. **The presence of antigen(s) related to the exogenous milk-transmitted murine mammary tumor virus on the surface of BALB/c splenic lymphocytes has been documented previously. Since the BALB/c strain lacks murine mammary tumor virus, the presence of murine mammary tumor virus-related antigen(s) on lymphocytes has been ascribed to expression of germinally transmitted *Mtv* transcripts and proviruses were characterized to evaluate this hypothesis. Transcripts from genomic size *Mtv* provirus(es) accumulated in the spleen in an age-dependent manner. Two novel *Mtv* transcripts of 7.8 and 6.4 kb were observed in the spleen. These observations indicate that the transcriptional and translational expression of an endogenous *Mtv* occurs in normal cells of the lymphoreticular lineage.** [P.S.E.B.M. 1991, Vol 196]

The murine mammary tumor virus (MMTV) is an exogenous, milk-transmitted type B retrovirus that promotes mammary neoplasia by insertional mutagenesis of cellular protooncogenes (1). The incidence of mammary neoplasia in inbred mice lacking MMTV is influenced by a variety of factors, including endogenous, germinally transmitted proviral DNA sequences, designated *Mtv*, which are closely related to the proviral form of MMTV in gene organization and sequence (1). Mouse strains devoid of MMTV, i.e., BALB/c, have a low incidence of spontaneous mammary tumors appearing only after 18–30 months of age (2). The possible role of *Mtv* in such mice has not been clearly elucidated (3, 4).

Several studies (5–7) have reported expression of MMTV-related antigen(s) in murine spleen cells in the absence of MMTV infection. Splenocyte preparations enriched for mIg^+ cells, but not Thy-1^+-enriched populations or thymocytes, had the MMTV-related antigen(s) (6). Previous characterization of the BALB/c proviruses by restriction mapping (8–12) has revealed genomic size, *Mtv-8* and *Mtv-9*, and subgenomic, *Mtv-6*, proviruses. We hypothesized that expression of the MMTV-related antigen(s) on BALB/c splenocytes is due to expression of *Mtv* provirus (6).

To test this hypothesis, we have characterized *Mtv* RNA transcripts in BALB/c tissues. The results provide evidence for a positive correlation between *Mtv* transcript accumulation and presence of the MMTV-related antigen in spleen cells.

Materials and Methods

Animals and Tumors. BALB/cCrgl mice were bred by brother-sister mating. The D1-DMBA-3 chemically induced mouse mammary tumor originated from the BALB/c D1 hyperplastic outgrowth line treated with 7,12-dimethylbenz(*a*)anthracene (13) and was passaged *in vivo* by serial subcutaneous transplantation in syngeneic mice. The exogenous MMTV-induced mammary tumors were of spontaneous origin in a BALB/cfC3H colony, which is housed separately from the BALB/c mice. The cloned C3H mouse mammary tumor cell line 34I-101 has been described (14). The spontaneous C3Hf/Se mammary tumor originated from the MMTV-negative C3Hf/Se subline (15).

Flow Cytometric Analyses. Spleen cell suspensions were obtained as described (5). Single-cell suspensions of thymus and tumor cells were obtained by dissociation of the tissue and passage through a stainless steel mesh. Cell preparations were stained with a two-

[1] To whom requests for reprints should be addressed at Department of Microbiology and Immunology R-138, University of Miami School of Medicine, P. O. Box 016960, Miami, FL 33101.

Received May 25, 1989. [P.S.E.B.M. 1991, Vol 196]
Accepted October 22, 1990.

0037-9727/91/1963-0316$3.00/0

step protocol (5) using rabbit antisera raised against a mixture of intact and disrupted purified MMTV. Preimmunization sera were used as background control. Ten thousand cells per sample were analyzed using a fluorescence-activated cell sorter (FACS III; Becton Dickinson FACS Systems, Sunnyvale CA). Analysis of fluorescent cells was based on scatter gating.

Nucleic Acid Analyses. Total cellular RNA isolation and binding to nitrocellulose filters were as described (11, 15). Purification and fractionation of poly(A) RNA were by oligo(dT)-cellulose chromatography and by formaldehyde-agarose gel electrophoresis of formamide-formaldehyde-denatured RNA with transfer to nitrocellulose (11, 15). Molecular hybridization was with radiolabeled MMTV cDNA, prepared from MMTV 70S RNA (3), or with nick-translated cloned (16) or subcloned (15) MMTV DNA. Standard conditions for hybridization, filter washing under stringent conditions, and autoradiography were used (11, 15).

Results

***Mtv* Expression in the BALB/c Spleen.** Initial experiments compared the kinetics of hybridization between total cellular RNA and a representative MMTV cDNA probe to the kinetics of hybridization with MMTV 70S RNA (3). A significant amount of *Mtv* RNA was present in adult virgin female BALB/c spleens (Table I). The amount of *Mtv* RNA in the spleen of young animals was very low but increased with age to levels 15-fold higher than in similar age BALB/c liver or thymus RNA, as well as multiparous retired breeder mammary gland and chemical carcinogen-induced BALB/c mammary tumor RNA, which were all at or below the limit of detection (0.0001%). High levels of RNA were present in a BALB/cfC3H mammary tumor that contained acquired MMTV provirus DNA as a result of milk-transmission of the C3H MMTV.

The presence of MMTV-related antigen(s) was analyzed as a function of animal age using flow cytometric determinations. The percentage of cells with antigen(s) increased with age from 11% at 2 weeks to 26% at 98 weeks in virgin female BALB/c splenocytes, but could not be detected in thymus cells even at 52 weeks of age (Table I). In agreement with the RNA analysis, carcinogen-induced BALB/c mammary tumor cells did not express MMTV-related antigen(s), whereas the majority of MMTV-induced BALB/cfC3H mammary tumor cells reacted positively with the anti-MMTV antibodies.

To identify the nature of the *Mtv* RNA transcripts observed in the BALB/c spleen cells, RNA dot blot analyses were initially performed using total RNA hybridized with the C3H MMTV *gag-pol* probe, a 4-kb *Ps*I fragment (Fig. 1A). *Mtv* RNA was detected in the splenic RNA extracts from 52-week-old (Fig. 1, Lane 1) and 106-week-old (Fig. 1, Lanes 3 and 5) BALB/c mice. Appreciably lower levels of *Mtv* RNA were detected in total liver RNA from the latter mice (Fig. 1, Lanes 4 and 6). *Mtv* RNA was present in total spleen RNA at about 1% of the level observed in BALB/cfC3H mammary tumor RNA (Fig. 1, Lane 2). Additional studies examined *Mtv* RNA in poly(A) RNA from 106-week-old BALB/c spleens and livers by hybridization with the cloned C3H MMTV 1.8-kb *Ps*I *env* fragment alone (Fig. 1B, Lanes 5–8) or in combi-

Table I. *Mtv* RNA and Antigen Expression in BALB/c Tissue as a Function of Age

Tissue[a]	Age (weeks)	% Mtv-RNA[b]	Maximum % hybridization[c]	% MMTV antigen-positive cells[d]
Spleen	2	0.0001	30 (6×10^4)	11 ± 1
	26	0.0003	86 (6×10^4)	17 ± 2
	52	0.0006	76 (6×10^4)	20 ± 2
	78	0.0005	82 (4×10^4)	21 ± 4
	106	0.0015	92 (2×10^4)	26 ± 6[e]
Thymus	2	ND		2 ± 1
	26	0.0001	14 (1×10^5)	2 ± 1
	52	ND		1 ± 1
Liver (same as spleen)	2–106	≤0.0001	8 (1×10^5)	ND
Mammary gland	52	0.0001	26 (6×10^4)	ND
D1 DMBA-3 mammary tumor		0.0001	24 (1×10^4)	0
BALB/cfC3H mammary tumor		0.27	100 (1×10^2)	72 ± 8

[a] Tissues were from the specified age female virgin BALB/c mice, BALB/c retired breeder mammary glands, and mammary tumors.
[b] The level of *Mtv* RNA, quantitated by RNA excess hybridization, is expressed as a percentage of total cellular RNA.
[c] The maximum percentage of hybridization of the MMTV cDNA probe is specified, with the equivalent Rot at which the maximum percentage of hybridization was observed is specified in parentheses. In most cases, equivalent Rot values greater than 5×10^4 could not be obtained. About 3% mismatching between 106-week-old mouse spleen RNA and the cDNA probe was determined.
[d] Determined by counting 10,000 cells/sample in a FACS III.
[e] The mice used as spleen donors for the fluorescence studies were 98 weeks old.

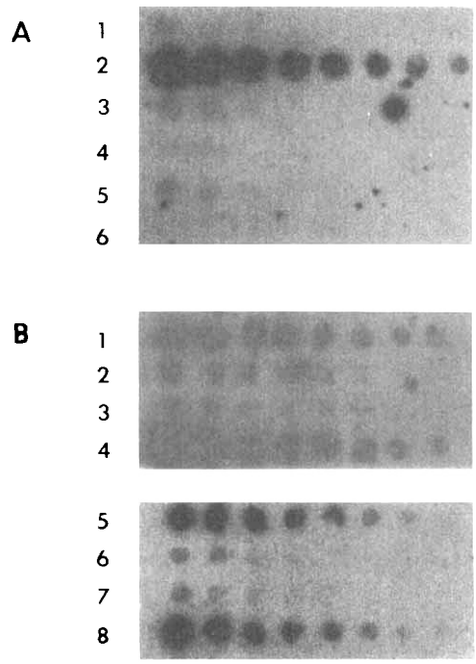

Figure 1. Identification of *Mtv* RNA transcripts in BALB/c tissue. (A) Total RNA, 3 μg and serial 1/1 dilutions (left to right), were denatured, spotted onto nitrocellulose, hybridized with the radiolabeled cloned C3H MMTV *gag-pol* probe, and autoradiographed for 5 days. The RNA were from pooled 1-year-old virgin BALB/c spleens (Lane 1), a spontaneous BALB/cfC3H mammary tumor (Lane 2), a pool of 2-year-old virgin BALB/c spleens (Lane 3), a 2-year-old virgin BALB/c liver (Lane 4), another pool of 2-year-old virgin BALB/c spleens (Lane 5), and another 2-year-old virgin BALB/c liver (Lane 6). (B) Poly(A) RNA, 3 μg (Lanes 1–3 and 5–7) or 0.18 μg (Lanes 4 and 8) and serial 1/1 dilutions (left to right), were denatured, spotted onto nitrocellulose, hybridized, then autoradiographed for 17 days. Hybridization probes were a mixture of the C3H MMTV *gag-pol*, and *env* probes in Lanes 1–4 or the *env* probe alone, Lanes 5–8. Lanes 1 and 5 contained pooled 2-year-old virgin BALB/c spleen poly(A) RNA, Lanes 2 and 6 contained multiparous 7-day lactating BALB/c mammary gland poly(A) RNA, Lanes 3 and 7 contained pooled 2-year-old virgin BALB/c liver poly(A) RNA, and Lanes 4 and 8 contained C3H mammary tumor cell line 34I-101 poly(A) RNA.

nation with the *gag-pol* probe (Fig. 1B, Lanes 1–4). BALB/c spleen poly(A) RNA hybridized intensely with the *env* region probe (Fig. 1, Lane 5) and somewhat more intensely with the combined probes (Fig. 1, Lane 1). BALB/c multiparous lactating mammary gland (Fig. 1, Lanes 2 and 6) and liver (Fig. 1, Lanes 3 and 7) poly(A) RNA had approximately one-tenth the levels of *Mtv* transcripts observed in the spleen. In Figure 1, Lanes 4 and 8 represent the levels of MMTV poly(A) RNA from the cloned MMTV producing C3H mammary tumor cell line 34I-101 used as a positive control.

The *Mtv* transcripts detected in the spleen of BALB/c mice were further characterized by Northern blot analyses (Fig. 2). Figure 2 represents the results after autoradiography for 6, 20, and 70 hr, respectively. BALB/c spleen poly(A) RNA (Fig. 2, A and B, Lane 5) clearly demonstrated the existence of *Mtv* RNA species of 7.8 and 6.4 kb. BALB/c retired breeder mammary gland contained primarily a 1.7-kb *Mtv* RNA transcript

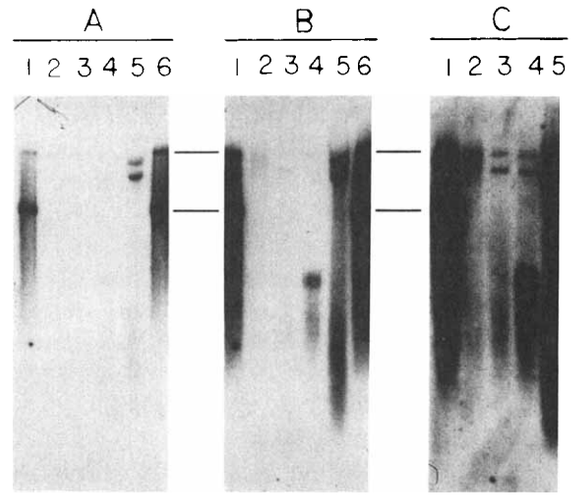

Figure 2. Molecular sizing of *Mtv* transcripts. Poly(A) RNA were fractionated in a 1.2% agarose gel with 2.2 *M* formaldehyde and transferred to nitrocellulose for hybridization. Following hybridization with the radiolabeled C3H MMTV long terminal repeat, *gag-pol* and *env* probes, autoradiography was for 6, 20, and 70 hr (A, B, and C, respectively). Lanes 1 and 6 contained 0.27 and 0.53 μg, respectively, of the cloned C3H mammary tumor cell line 34I-101 poly(A) RNA, Lane 2 has 0.5 μg of C3Hf/Se spontaneous mammary tumor RNA, Lane 3 has 10 μg of 2-year-old female virgin BALB/c liver RNA, Lane 4 has 10 μg of multiparous 7-day lactating BALB/c mammary gland RNA, and Lane 5 has 10 μg of 2-year-old female virgin BALB/c spleen RNA. The arrows on the left margin indicate the migration, top to bottom, of the 4.7-kb, 3.1-kb, 1.9-kb and 1.6-kb rRNA molecular weight standards. The upper and lower horizontal lines indicate the migration of the 9 kb and 3.8 kb, respectively, C3H MMTV RNA molecular weight standards.

detectable after 20 hr of autoradiography (Fig. 2B, Lane 4). In addition, low amounts of a 8.9-kb genomic size transcript and a 7.4-kb transcript were detected in mammary gland RNA with longer exposures (Fig. 2C, Lane 4), a result consistent with a report of *Mtv gag-, pol-,* and *env*-related RNA in BALB/c mammary glands (17). The aged virgin BALB/c liver poly(A) RNA contained a very low amount of 8.9- and 7.4-kb *Mtv* transcripts that were observed only after prolonged exposure (Fig. 2C, Lane 3). None of the BALB/c tissues contained a detectable amount of the spliced 3.8-kb *Mtv env* transcript, that is the most abundant MMTV RNA in the control C3H mammary tumor cell line (Fig. 2, Lanes 1 and 6). Both MMTV-infected C3H tumor cells (Fig. 2, Lanes 2 and 6) and MMTV-negative C3Hf/Se mammary tumor (Fig. 2, Lane 2) RNA had 8.9-kb genomic size transcripts. These observations demonstrate that BALB/c spleen contained *Mtv* structural gene transcripts.

Discussion

These studies have examined the hypothesis that the BALB/c splenocyte MMTV-related antigen is encoded by RNA transcripts from a BALB/c genomic size *Mtv* provirus(es). The possibility that the *env* gene is the source of the cross-reactive antigen(s) is suggested by evidence that the MMTV-related antigen was reac-

tive with antibodies specific to MMTV virion protein (6) and that at least some BALB/c splenic lymphocytes had an antigen that cross-reacted with a MMTV envelope glycoprotein monoclonal antibody (7).

The evidence presented here (Fig. 1 and 2) demonstrates *Mtv* provirus transcript accumulation from the *env* gene region and likely the *gag-pol* region in spleens of BALB/c mice. Therefore, these transcripts are encoded by a genomic size *Mtv*. Other investigators (18) have reported the presence of two large, approximately 9 kb and 7.8 kb, MMTV RNA in GR mammary tumor cells. Although *Mtv* expression in normal tissues is generally considered to be highly mammary tissue specific (3, 19), there is evidence for *Mtv* RNA in normal mouse spleen (15, 20). Sarkar and Gunzburg (20) have reported that BALB/c spleen *Mtv* transcripts were not initiated within the *Mtv* long terminal repeat and concluded that the subgenomic *Mtv-6* was the template for BALB/c spleen *Mtv* RNA. King *et al.* (21) have recently shown the lipopolysaccharide-inducible expression of *Mtv* RNA in both normal B lymphocytes and an inducible B cell lymphoma, CH12. These authors concluded that the *Mtv-9* transcripts accumulated in these cells, an interpretation consistent with the data presented here for genomic size *Mtv* provirus transcript accumulation. In other unpublished studies, we have found data suggesting that the *Mtv-9* 5′ and 3′ long terminal repeats in BALB/c spleen DNA are hypomethylated. This finding is compatible with the observations of King *et al.* (21).

The reasons why *Mtv* transcripts can be detected in the spleen, but not in the thymus, are unclear. In our unpublished work, we have found that hypomethylation of *Mtv* proviral DNA is essentially identical in the thymus and the spleen. The most likely reason for the lack of thymic *Mtv* RNA is the absence of the appropriate cell type in thymic tissue. Indeed, in previous reports (5, 6) we have shown that the majority of the splenic cells expressing MMTV antigens are B lymphocytes. Therefore, B cells, but not T cells, likely contain factors necessary for *Mtv* expression.

The data presented herein demonstrate that in BALB/c mice, an important example of an exogenous MMTV-negative strain, there is expression of MMTV-related RNA and protein in the spleen, which increases with age. In C3Hf strains, which are another example of MMTV-negative mice in which mammary tumors are of late occurrence, *Mtv* provirus 3.8-kb *env* transcripts accumulate (15). These transcripts are encoded by the *Mtv-1* provirus (22). As shown in this study, no 3.8-kb *env* transcripts are detectable in the BALB/c spleens. In recent experiments using Western blots, a 68-kDa band that reacts with anti-MMTV antisera has been detected in the spleens of BALB/c mice. No 52-kDa band could be seen in the same type analyses. Preliminary evidence indicates that the 68-kDa molecule represents an unprocessed precursor of the *env* gene products (Yang *et al.*, manuscript in preparation). The data presented in this manuscript support the results obtained with the Western blots, inasmuch as the RNA extracted from the spleens of BALB/c mice hybridizes with a probe containing the *env* gene region. The MMTV-related macromolecules, therefore, probably represent the selective expression of one or more *Mtv* proviruses in splenic lymphoid cells of the B cell lineage.

This research was supported by Grants CA 28999 (R. J. P.) and CA 25583 (D. M. L.) from the National Cancer Institute, NIH.

We gratefully appreciate the excellent technical help of Mrs. Lynn Herbert and Mr. Mantley Dorsey, Jr. We thank Ms. Pat Washington for preparing the manuscript.

1. Dickson C. Molecular aspects of mouse mammary tumor virus biology. Int Rev Cytol **108**:119–147, 1987.
2. Nandi S, McGrath CM. Mammary neoplasia in mice. Adv Cancer Res **17**:353–414, 1973.
3. Pauley RJ, Medina D, Socher SH. Murine mammary tumor virus expression during mammary tumorigenesis in BALB/c mice. J Virol **29**:483–493, 1979.
4. Slagle BL, Butel JS. Exogenous and endogenous mouse mammary tumor viruses: Replication and cell transformation. In: Medina D, Kidwell W, Heppner G, Anderson E, Eds. Cellular and Molecular Biology of Mammary Cancer. New York: Plenum Press, pp275–286, 1987.
5. Charyulu V, Sigel MM, Durden DL, Lopez DM. Mouse mammary tumor virus (MMTV) antigen(s) are present in B lymphocytes of BALB/c mice. Int J Cancer **24**:813–818, 1979.
6. Lopez DM, Charyulu V, Paul RD. B cell subsets in spleens of BALB/c mice: Identification and isolation of MMTV-expressing and MMTV-responding subpopulations. J Immunol **134**:603–607, 1985.
7. Tax A, Ewert D, Manson LA. An antigen cross-reactive with gp52 of mammary tumor virus is expressed on a B cell subpopulation of mice. J Immunol **138**:2368–2371, 1983.
8. Kozak C, Peters G, Pauley R, Morris V, Michalides R, Dudley J, Green M, Davisson M, Prakash O, Vaidya A, Hilgers J, Verstraeten A, Hynes N, Diggelmann H, Peterson D, Cohen JC, Dickson C, Sarkar N, Nusse R, Varmus H, Callahan R. A standardized nomenclature for endogenous mouse mammary tumor viruses. J Virol **61**:1651–1654, 1987.
9. Cohen JC, Majors JE, Varmus HE. Organization of mouse mammary tumor virus-specific DNA endogenous to BALB/c mice. J Virol **32**:483–490, 1979.
10. Groner B, Buetti E, Diggelmann H, Hynes NE. Characterization of endogenous and exogenous mouse mammary tumor virus proviral DNA with site-specific molecular clones. J Virol **36**:734–745, 1980.
11. Pauley R, Parks W, Popko B. Expression and demethylation of germinally-transmitted BALB/c mouse mammary tumor virus DNA in Abelson MuLV B-lymphoid cell lines. Virus Res **1**:381–400, 1984.
12. Peters G, Placzek S, Brookes S, Kozak C, Smith R, Dickson C. Characterization, chromosome assignment and segregation analysis of endogenous proviral units of mouse mammary tumor virus. J Virol **59**:535–544, 1986.
13. Medina D, DeOme KB. Response of hyperplastic alveolar nodule outgrowth-line D1 to mammary tumor virus, nodule-inducing

virus and prolonged hormonal stimulation acting singly and in combination. J Natl Cancer Inst **42**:303–310, 1969.
14. Parks WP, Hubbell EF, Goldberg RJ, O'Neil FJ, Scolnick EM. High frequency variation in mammary tumor virus expression in cell culture. Cell **8**:87–93, 1976.
15. Popko BJ, Pauley RJ. Organization of mouse mammary tumor virus sequences in normal and neoplastic C3Hf/HeSed mouse tissues. J Virol **52**:328–335, 1984.
16. Majors, JE, Varmus HE. Nucleotide sequence host-proviral junctions for mouse mammary tumor virus. Nature **289**:253–258, 1981.
17. Knepper JE, Medina D, Butel JS. Differential expression of endogenous mouse mammary tumor virus genes during development of the BALB/c mammary gland. J Virol **51**:518–521, 1986.
18. Groner B, Haynes NE, Diggelmann H. Identification of mouse mammary tumor virus-specific mRNA. J Virol **30**:417–420, 1979.
19. Hu W-S, Fanning TG, Cardiff RD. Mouse mammary tumor virus specific methylation patterns of proviral DNA in normal mouse tissues. J Virol **49**:66–71, 1984.
20. Sarkar A, Gunzburg WH. Spleen specific expression of an MMTV related transcript associated with the *Mtv-6* locus in BALB/c mice. Virology **154**:233–239, 1986.
21. King LB, Lund FE, White DA, Sharma S, Corley RB. Molecular events in B lymphocyte differentiation. Inducible expression of the endogenous mouse mammary tumor proviral gene, *Mtv-9*. J Immunol **144**:3218–3227, 1990.
22. Crouse CA, Pauley RJ. Molecular cloning and sequencing of the *Mtv-1* LTR: Evidence for a LTR sequence alteration. Virus Res **12**:123–138, 1989.