Endogenous Mouse Mammary Tumor Virus Is Expressed in Several Organs in Addition to the Lactating Mammary Gland

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We studied the transcription of endogenous mouse mammary tumor virus (MMTV) in transgenic and normal strains of mice of different genetic backgrounds. Although the lactating mammary gland in all strains showed the highest level of MMTV expression, detectable levels of viral RNA were also found in the lungs, kidneys, salivary glands, seminal vesicles and/or prostate gland, testes, and lymphoid tissue in mice which contain different endogenous proviruses. Transcription in transgenic mice containing the MMTV long terminal repeat linked to the simian virus 40 early region also occurred in these same organs. These results indicate that the MMTV long terminal repeat functions in several organs in addition to the lactating mammary gland.

Mouse mammary tumor virus (MMTV) is an endogenous murine retrovirus which is causally associated with mammary carcinomas (1, 15, 17). Transcription of MMTV has been reported to occur primarily in the lactating mammary gland (14, 27), although demethylation of endogenous proviruses, an event associated with gene activation, has been found in several organs in addition to this tissue (6, 7, 9). Most inbred strains of mice contain several proviruses, which can be distinguished genetically and by restriction fragment length polymorphisms (4, 10). Not all of the endogenous proviruses are transcriptionally active, but most mouse strains contain at least one expressed copy of MMTV (14, 27).

Recently, we bred transgenic mice containing the MMTV long terminal repeat (LTR) linked to both the herpes simplex virus type 1 thymidine kinase gene (LTR-tk) (20) and the simian virus 40 (SV40) early region (LTR-Tag) (2). In the case of the LTR-tk construct, transcription of the transgene was detected at high levels in both the lactating mammary gland and testes, while the LTR-Tag sequences were expressed in these two organs and in the lungs, kidneys, salivary glands, seminal vesicles, and lymphoid tissue of several independently derived mice. Work by others with the MMTV LTR linked to different marker genes has also resulted in transgenic mice expressing the transgene in many of these same organs (24, 25). We now report that transcription of endogenous MMTV also occurs in these organs and that the expression of MMTV LTR-containing transgenes reflects that of the provirus.

We investigated the transcription of endogenous MMTV proviruses by both S1 nuclease protection studies and Northern (RNA) blot analysis. We first compared transcription of the LTR-Tag transgene with that of the endogenous provirus(es) in the same tissue by S1 nuclease protection (28). Two ³²P-labeled 5'-end probes were prepared (Fig. 1a); probe MTV is protected by RNA molecules which contain MMTV LTR sequences 3' to those present in LTR-Tag and is specific for endogenous transcription, while probe Tag is labeled in the SV40 portion of LTR-Tag and is specific for RNA coded for by the transgene. RNA (10 to 20 µg) isolated from various organs from male and female LTR-Tag transgenic mice was hybridized to both of these probes, digested with S1 nuclease, and analyzed on 5% denaturing polyacryl-

amide gels. Fragments of 136 nucleotides (nt) from probe MTV and of 132 nt from probe Tag were protected by RNA from the spleen, lymph nodes, lungs, and seminal vesicles (Fig. 1b). We also observed a faint band in brain tissue, but in subsequent analyses, this tissue was negative (data not shown). Transgene and MTV-specific RNAs were also detected in the kidneys, salivary glands, and thymus of these mice (data not shown). Thus, all organs which expressed the transgene also transcribed endogenous MMTV RNA.

We next examined whether endogenous MMTV transcription occurred only in transgenic mice, possibly as a result of SV40 large-T-antigen expression, which has been shown to regulate the expression of several genes (22, 23). It was also possible that the presence of MMTV RNA in different organs reflected the genetic background of these mice or was due to position effects on the LTR-Tag transgene and/or the endogenous proviruses. RNA was analyzed from inbred strains C57BL/6 and C3Hf/HeN, which contain different endogenous proviruses (see below), and outbred ICR mice of both sexes. S1 nuclease protection analysis revealed that the subset of organs which produced RNA capable of protecting the 136-nt fragment from the endogenous probe MTV (Fig. 2) was identical to that which expressed the transgene. Analysis of RNA isolated from the tissues of two other strains of mice, C57B1/Ks and BALB/c, produced the same results (data not shown). Thus, it appears that the expression of endogenous MMTV is not affected by the SV40 early region, position effects, or genetic background.

Although the endogenous MMTV sequences in inbred mice have been well characterized, it was possible that the transcription we detected in organs other than the mammary gland originated from previously unidentified truncated MMTV sequences, since the probe we used protected only a small portion of the LTR. For example, it has been reported that the MMTV-homologous RNA found in BALB/c spleen comes from *Mtv-6*, a deleted provirus which contains primarily LTR sequences (21). To determine whether bona fide MMTV RNA was transcribed in each of the different organs, we isolated poly(A)⁺ RNA, fractionated it on formaldehyde-agarose gels, and subjected it to Northern blot analysis with a probe containing most of the MMTV genome, pMTV2, a gift from J. Majors (18). RNA isolated from ICR lungs, salivary glands, and spleen contained the three MMTV transcripts of 8,900, 3,800, and 1,700 nt, as did RNA from the control GR mammary carcinoma cell line (Fig. 3a). The

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FIG. 1. Expression of endogenous MMTV in LTR-Tag transgenic mice. (a) Diagram of the pLTR-Tag plasmid (2) and the probes used for S1 nuclease mapping of LTR-Tag (Tag) and endogenous MMTV (MTV) expression. Probe Tag is a 5'-end 230-nt *SacI*-*HindIII* fragment 32 P labeled at the *HindIII* site (asterisk) in SV40. A total of 132 nt spanning that LTR-Tag junction are protected by RNAs initiating within the MMTV LTR. Probe MTV is a 5'-end 236-nt *SacI*-*PvuII* fragment 32 P labeled at the *PvuII* site (asterisk) in MMTV. A fragment of 136 nt is protected by MMTV-specific RNAs. Abbreviations: S, *SacI*; H, *HindIII*; P, *PvuII*. The small stippled box represents small-t-antigen-coding sequences, and the large stippled box represents large-T-antigen-coding sequences. The large open boxes represent the MMTV LTR. The arrows indicate the transcription initiation sites. (b) S1 nuclease mapping of Tag and endogenous MMTV expression in transgenic mice. The Tag probe (230 nt) was hybridized to 15 μ g of total RNA from transgenic animals; a protected fragment of 132 nt was seen. The MTV probe (236 nt) was hybridized to 10 μ g of total RNA from transgenic animals (2); the protected fragment was 136 nt. Abbreviations: B, brain; Lu, lung; SV, seminal vesicle; LN, lymph nodes; t, tRNA; M, *HaeIII*-cut pBR322 markers; P, probe.

level of expression of endogenous MMTV in these three organs was about 500-fold lower than that in lactating mammary gland; 10 to 20 μ g of poly(A)⁺ RNA from the three organs was loaded on the gel, while 2 μ g of GR total RNA was analyzed (GR cells contain approximately the same amount of MMTV RNA as does ICR lactating mammary gland) (14; D. Henrard, unpublished data). These three transcripts were not unique to ICR mice; Fig. 3b also shows the results when 20 μ g of poly(A)⁺ RNA isolated from the salivary glands of virgin BALB/c, C57BL/6, and C3Hf/HeN mice was analyzed. All of the organs in which we detected

endogenous MMTV transcripts by S1 nuclease protection assays contained these three transcripts (data not shown).

The strains of mice that we used for these experiments contain different endogenous proviruses (Fig. 2) (10). Transcription in C57BL/6 mice most likely is from *Mtv-9* (19), since *Mtv-8* is integrated into a silent repetitive element and is hypermethylated in mammary glands and other organs (5) and since *Mtv-17* has a mutation in its nuclear factor 1-binding site (a cloned *Mtv-17* LTR is not expressed after transfection; D. Peterson, personal communication). BALB/c mice express two endogenous proviruses, *Mtv-9* and *Mtv-6*, which is truncated and contains primarily LTR sequences (3). Finally, *Mtv-1* is known to be transcriptionally active in the lactating mammary gland of C3H/He mice (26), which do not contain *Mtv-9*. Therefore, since at least two different endogenous proviruses, *Mtv-1* and *Mtv-9*, which have different integration sites (chromosomes 7 and 12, respectively), are expressed in the various organs, their activation seems not to be the result of position effects. In addition, the genetic background of the mice appears not to

FIG. 2. S1 nuclease analysis of endogenous MMTV RNA from the tissues of different mouse strains. Total RNA (10 μ g) from each tissue, except for the ICR mammary gland (MG) (1 μ g), was hybridized to the MTV probe; a protected fragment of 136 nt was seen in positive tissues. Abbreviations: C₅, C57BL/6; C, C3Hf/HeN; B, brain; K, kidney; Lu, lung; Li, liver; SG, salivary gland; Sp, spleen; MG, mammary gland. Strain C3Hf/HeN contains proviruses 1, 6, 8, 11, and 14; strain C57BL/6 contains proviruses 8, 9, and 17; and strain BALB/c contains proviruses 6, 8, and 9.

FIG. 3. Northern blot analysis of poly(A)⁺ RNA to detect endogenous MMTV transcripts. Cloned MMTV DNA (pMTV2) (18) was used as a probe. (a) Poly(A)⁺ RNA from lung (Lu, 15 μ g), salivary gland (SG, 10 μ g), spleen (Sp, 20 μ g), and brain (Br, 10 μ g) and total RNA from lactating mammary gland (MG, 2 μ g) from an ICR mouse. (b) Salivary gland poly(A)⁺ RNA (20 μ g) from female BALB/c (B), male C3Hf/HeN (C3), and female C57BL/6 (C5) mice. The sizes corresponding to the three MMTV transcripts are indicated (8.9, 3.8, and 1.7 kilobases); rRNAs (18S and 28S) were used as molecular weight markers.

be involved in expression, since *Mtv-9* is probably the provirus that is activated in all three strains tested that contain it.

MMTV proteins have been detected in several of the organs in which we detected transcription; for example, both the spleen (13) and seminal vesicles (11) have been shown by immunological methods to contain MMTV *gag* and *env* proteins. In our studies, we used virgin and lactating female and male C57BL/6, C57BL/Ks, BALB/c, C3Hf/HeN, and ICR mice. Neither C57BL nor BALB/c mice are known to produce virus (8); thus, it is unlikely that the transcription that we detected was due to exogenous infection of the organs. Although other studies, including our own (20), have failed to detect MMTV transcription in the organs that we now report on, our use of S1 nuclease protection assays and large amounts of poly(A)⁺ RNA for Northern blot analysis probably afforded us much greater sensitivity.

In conclusion, our results indicate that MMTV has a much wider range of specificity than was previously thought. Although expression of this virus occurs in a number of different organs, it is probably limited to only a few cell types. In a previous study, we used immunofluorescence staining of tissue sections to detect SV40 T-antigen expression and showed that expression of the transgene in LTR-Tag mice was limited to the ductal epithelial cells in the lungs, kidneys, salivary glands, mammary glands, and seminal vesicles, to the lymphoid cells of the spleen and thymus, and to Leydig cells in the testes (2). It is most likely that the endogenous virus is transcribed in these cell types as well.

Why is the primary site of MMTV-induced carcinogenesis the mammary gland, if expression occurs in all these organs? The level of expression of the endogenous provirus, which is about 500-fold greater in the lactating mammary gland than in any other organ, may increase the frequency of viral reintegration next to cellular oncogenes or, alternatively, the MMTV LTR may function as an enhancer for the increased

expression of cellular oncogenes in the lactating mammary gland. Recently, it was shown that MMTV proviruses with rearranged LTRs are associated with T-cell leukemias (16) and kidney adenocarcinomas (29); it has been hypothesized in both cases that these rearranged LTRs allow MMTV expression in these organs. Our results indicate that kidney epithelial cells and possibly T cells are capable of expressing detectable levels of endogenous MMTV. Interestingly, the SV40 early genes in LTR-Tag mice (2), the *myc* gene in LTR-*myc* mice (12), and the *c-Ha-ras* gene in LTR-*ras* mice (24) all cause tumors in the same tissues in which expression of the endogenous provirus occurs, despite the fact that the provirus is expressed at much higher levels in the lactating mammary gland. We are presently examining the role of the LTR and other regions of the MMTV genome in determining the relative levels of expression in different organs by breeding transgenic mice containing MMTV LTR deletions and deletions of other regions of the virus.

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