The Mouse Mammary Tumor Virus Long Terminal Repeat Directs Expression in Epithelial and Lymphoid Cells of Different Tissues in Transgenic Mice

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A series of transgenic mice was developed that contained the simian virus 40 early region genes under the transcriptional control of the mouse mammary tumor virus long terminal repeat, including the promoter and glucocorticoid response elements. These mice all expressed the transgene in the epithelial cells of a number of different organs, such as lungs, kidneys, and prostate, salivary, and mammary glands, and in Leydig and lymphoid cells. Transcription of the chimeric gene was inducible by glucocorticoids, either after transfection into tissue culture cells or in cells cultured from animals carrying the transgene. Many, but not all, tissues which expressed the simian virus 40 sequences, as determined immunologically and by RNA analysis, developed into tumors, although they showed premalignant features. Since the mouse mammary tumor virus long terminal repeat is expressed in a number of different cell types when inherited through the germ line, the lactating mammary gland-specific transcription of endogenous proviruses must require other factors or sequences to achieve this specificity.

Mouse mammary tumor virus (MMTV) is an endogenous retrovirus of mice which is causally associated with mammary carcinomas and whose transcription is regulated by steroid hormones (37, 39). Expression of viral RNA in the mouse appears to occur predominantly in lactating mammary glands (9, 16, 36). In this organ, new genomic copies of the virus result from reverse transcription of MMTV RNA and reintegration of these sequences into novel chromosomal sites (20). The newly inserted proviruses may cause cellular transformation by activating unique cellular oncogenes, called *int* genes (5, 18, 19, 26). It is not well understood what is required for the specific expression of MMTV.

Previously we examined the sequences encoded by the MMTV provirus which are required for the lactating mammary gland-specific expression by constructing transgenic mice containing an MMTV long terminal repeat (LTR) ligated to the herpes simplex virus type 1 thymidine kinase gene (29). We showed that expression of the transgene occurred in lactating mammary glands and ovaries in females and in testes in males; endogenous MMTV sequences were activated only in the mammary glands in these animals. Others have also shown that MMTV-*c-myc* hybrid-containing transgenic mice express the introduced sequences in lactating mammary glands, resulting in mammary adenocarcinomas (33). These results indicate that elements controlling at least some of the tissue specificity of MMTV expression are encoded by the LTR.

To further map the sequences encoded by the LTR responsible for the lactating mammary gland-specific expression and to determine whether the transcription that we detected in testis resulted from the MMTV or *tk* sequences or a combination of the two genes, we developed a series of transgenic mice in which the simian virus 40 (SV40) early region genes (large T and small t) are transcribed from the MMTV promoter within the LTR. We chose to use SV40 as a marker gene because expression could be examined in

different organs by analyzing both RNA and protein, and because the SV40 large T antigen shows characteristic nuclear immunostaining, determination of precisely which cell type in each organ was expressing the transgene was possible (8, 21, 24). In contrast to mice containing MMTV-*tk* and *myc* transgenes, all the LTR-SV40 transgenic mice showed expression in epithelial and lymphoid cells in several different tissues. Transcription of both large T- and small t-antigen RNA was inducible by glucocorticoids in cells in which expression occurred, and many of these tissues gave rise to tumors. These results indicate that the MMTV LTR is transcriptionally active in several different tissues when it is inherited through the germ line.

MATERIALS AND METHODS

Pronuclear injections. DNA injections into fertilized zygotes (from ICR, C57BL/6, and C3H/HeN mothers) and implantation into pseudopregnant ICR foster mothers were carried out as previously described (29). Mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. (ICR and C57BL/6) and the Frederick Cancer Institute, Frederick, Md. (C3H/HeN).

Plasmid purification. Plasmids were purified from transformed *Escherichia coli* HB101 cells by a modification of the alkaline extraction method (1) followed by CsCl-ethidium bromide equilibrium density centrifugation. DNA fragments for microinjection and synthesis of probes were isolated from low-melting-point agarose gels (International Biotechnologies, Inc., New Haven, Conn.) by using ELUTIP-D columns (Schleicher & Schuell, Inc., Keene, N.H.).

DNA analysis. Genomic DNA was extracted from tails and tissues by proteinase K-sodium dodecyl sulfate digestion followed by phenol-chloroform extraction and ethanol precipitation. Restriction endonuclease digestions were carried out under the conditions recommended by the supplier (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Approximately 15 µg of DNA (unless indicated differently in the figure legend) was electrophoresed on 0.8% agarose gels,

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with *Hind*III-digested lambda DNA as a molecular weight marker, and transferred to nitrocellulose (32). The filters were hybridized for 24 to 48 h at 42°C in hybridization buffer (50% formamide, 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7], 5× Denhardt solution [1× + 0.02% polyvinylpyrrolidone–0.02% Ficoll–0.02% bovine serum albumin], 50 µg of denatured salmon sperm DNA per ml) containing ³²P-labeled probes. Probes were labeled by nick translation (27) or by oligonucleotide-primed synthesis (6). The filters were washed at 50°C in 0.1% SSC–0.1% sodium dodecyl sulfate and exposed to Kodak XAR film with Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The MMTV LTR probe was derived from plasmid p302 (described below), and the SV40 early region probe was isolated from a 3.0-kilobase fragment of pMK16-2 (provided by K. Subramanian).

Histology. Tissue samples were fixed in 10% Formalin and embedded in paraffin by standard procedures, and 6-µm sections were stained with hematoxylin and eosin. The Leydig cell carcinoma was also examined by transmission electron microscopy (not shown).

Immunofluorescence. Tissue samples for immunofluorescence were immediately frozen in liquid nitrogen. Frozen sections of 4 µm were prepared and fixed in cold acetone for 10 min at –20°C. They were then air dried. Primary rabbit antibody against T antigen was a gift from R. Kucherlapati. The sections were incubated with the primary antibody at a 1:10 dilution for 30 min at room temperature. After being extensively washed with phosphate-buffered saline, they were incubated for an additional 30 min with a fluorescein-labeled goat anti-rabbit immunoglobulin secondary antibody (Jackson ImmunoResearch, Avondale, Pa.). After being washed, the sections were dehydrated, mounted, and examined with a fluorescence microscope (Carl Zeiss, Inc., Thornwood, N.Y.).

RNA analysis. RNA was isolated by guanidine thiocyanate extraction and CsCl gradient fractionation by the method of Chirgwin et al. (4). Equal amounts of total RNA (5 µg) were electrophoresed on 1% formaldehyde gels (12), transferred to nitrocellulose filters (34), and hybridized with nick-translated SV40 early-region probe. The mouse rRNAs served as molecular weight markers. Hybridizations and washes were carried out as described above. To ensure that equal amounts of RNA were loaded on the gels, test blots were hybridized to a β-tubulin probe (not shown).

S1 nuclease protection assays. Hybridization probes were end labeled with T4 polynucleotide kinase and [γ-³²P]ATP as specified by the supplier (Boehringer Mannheim). An excess of labeled probe (approximately 5 × 10⁴ cpm per reaction; specific activity, 10⁶ cpm/µg) was hybridized with 10 to 20 µg of total RNA. Hybridizations were carried out overnight at 54°C in 30 µl of hybridization buffer consisting of 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 0.4 M NaCl, and 80% formamide, 1,000 U of S1 nuclease (Boehringer Mannheim) in 0.28 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM ZnSO₄–20 µg of denatured salmon sperm DNA per ml was added, and digestions were carried out at 25°C for 40 min. The protected fragments were analyzed on 5% sequencing gels (15) with end-labeled *Hpa*II-digested pBR322 as markers.

Protein analysis. Approximately 2 mg of protein from sonic extracts of tissue was immunoprecipitated with pAB412, an anti-SV40 large T monoclonal antibody (a gift of A. J. Levine), as previously described (2). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13% polyacrylamide), the proteins were electrophoretically

FIG. 1. Diagram of pLTR-Tag. Abbreviations: B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; G, *Bgl*II; L, *Bgl*II; S, *Sac*I; P, *Pst*I. Symbols: MMTV LTR with the glucocorticoid regulatory element in the hatched area; SV40 small t-antigen coding sequences; large T-antigen coding sequences.

blotted to nitrocellulose filters. The filters were incubated in blocking buffer (50 mM Tris hydrochloride [pH 7.5], 0.2 M NaCl, 1% bovine serum albumin) for 4 h at 4°C and then incubated with pAB412 in blocking buffer overnight. After being extensively washed with TBSN (50 mM Tris hydrochloride [pH 7.5], 0.2 M NaCl, 0.1% Nonidet P-40), the blots were incubated for 45 min at room temperature with biotinylated goat anti-mouse immunoglobulin secondary antibody (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), washed again, incubated with streptavidin-horseradish peroxidase, and developed as specified by the supplier (Bethesda Research Laboratories).

RESULTS

Construction of pLTR-Tag transgenic mice. We constructed a plasmid containing the SV40 early region under the control of the MMTV LTR (Fig. 1). The MMTV LTR sequences were isolated from plasmid p302 (provided by J. Majors), which contains the LTR sequences from an *Ava*I site at –1200 (28 base pairs [bp] from the 5' end of the LTR) to a *Hpa*II site at +101, relative to the start of MMTV transcription, cloned from C3H strain viral DNA (14). Both the *Ava*I and *Hpa*II sites have been converted to *Bam*HI sites. This fragment was ligated into a plasmid containing a *Bgl*II linker at the *Stu*I site of the SV40 early region at position 5190 on the SV40 map and +34, relative to the start of early gene transcription (provided by K. Subramanian). The resulting plasmid, pLTR-Tag, was transfected into both mouse L cells and HeLa cells and was shown to support dexamethasone-inducible transcription of the SV40 early region initiating within the LTR (see Fig. 3B).

A 4.5-kb *Bgl*I-to-*Bam*HI fragment of plasmid pLTR-Tag was used for microinjections (Fig. 1); all of the SV40 early control region elements and plasmid sequences (the *Bgl*II site within the SV40 origin is 40 bp from the LTR junction), which have been shown to affect expression in transgenic mice (3, 7, 35), are absent from this fragment. Approximately 500 copies of this insert were microinjected into each nucleus of one-cell fertilized zygotes from ICR Swiss Webster, C57BL/6, or C3H/HeN strains. The embryos were implanted, and DNA isolated from tails of the resultant offspring was analyzed by dot blot or Southern blot analysis, as previously described (29); in some cases, Caesarean sections were performed and placental DNA was analyzed as well.

Six independent transgenic mice, designated LTag1 through LTag6, were obtained. Two of these (LTag4 and LTag6) were delivered by Caesarean section and found to be dead in utero. Examination of Southern blots of *Pst*I-digested DNA, which cuts once within the injected sequences, from each of these mice revealed that they carried different copy numbers and configurations of the input DNA

FIG. 2. Southern blot analysis of normal and tumor tissue from pLTag transgenic mice. (A and B) DNAs were restricted with *Pst*I; blots were hybridized with T-antigen probe. (A) Analysis of DNA from LTag1 and LTag2. Numbers above the lanes represent the amount of DNA (in micrograms) loaded on the gel. Abbreviations: C, normal C3H/HeN DNA; Lu, lung; Mg, mammary gland; K, kidney; Te, testis; Tu, seminal vesicle tumor. Solid arrows, bands found in LTag2 DNA; open arrows, bands found in LTag1 DNA. (B) Analysis of DNA from LTag4. LTag1 seminal vesicle tumor DNA (1, Tu), normal C3H/HeN DNA (C), and LTag4 placental DNA (4, P) (15 μ g of each) were loaded on the gel. Hatched arrow, bands found in LTag4 DNA. (C) DNA was digested with *Cla*I and *Bcl*I, which generates a 3.3-kb fragment, and the blot was hybridized with T-antigen probe. Lanes: 1, DNA from pLTag1; 3.1, DNA from an LTag3 offspring; 3, DNA from LTag3.

(Fig. 2A and B; Table 1) (copy numbers were estimated by quantitative dot blot analysis [not shown]). In addition, DNA digested with *Cla*I and *Bcl*I and hybridized to both an SV40 early region (Fig. 2C) and an MMTV LTR probe (not shown) revealed a band of 3.3 kb (see Fig. 1 for restriction map), as shown for LTag1, LTag3, and LTag3.1 (an LTag3 offspring), indicating that at least one functional copy of the injected DNA was present in each mouse (the increased mobility of the band in lane 3.1 is due to a lower amount of DNA on the gel).

One animal, LTag2, showed an amplification of the transgene in at least two tissue samples. Southern analysis of DNA isolated from a kidney and a lung of LTag2, both of which had carcinomas (see below), showed about a 5-fold and a 20-fold amplification of the transgene, respectively, relative to nontumor tissue (Fig. 2A). However, since this animal did not produce any offspring, it was not clear whether this was a true amplification of chromosomal DNA

or whether this founder mouse was a somatic mosaic and merely had an increase in the number of transgenic tumor cells in these two tissues.

Expression of the transgene. At puberty, all four of the live transgenic mice (LTag1, LTag2, LTag3, and LTag5) were mated with animals of the same strain. Only LTag3 and LTag5, both females, produced offspring; two others, LTag1 and LTag2, never mated. SV40 tumor antigen expression analysis was examined in the tissues from all six transgenic mice and the offspring of LTag3 and LTag5. RNA isolated from a number of tissues from all the transgenic mice was analyzed by Northern blot (RNA blot) to determine whether the transcripts for both the large and small SV40 tumor antigens were being made (Fig. 3A) and by S1 nuclease protection experiments (Fig. 3B and C) to determine whether transcription initiated within the MMTV LTR. In addition, Western blot (immunoblot) analysis on protein extracts (Fig. 4A) and immunofluorescence studies with

TABLE 1. Expression of pLTR-Tag sequences and tumor formation in LTag mice

Mouse	Sex	Copy no. ^a	Strain	Age ^b	Tissue ^c											
					Br	He	Sp	Sk	Te	Pr	S.G.	M.G.	Int.	Kid	Lu	Li
LTag1	M	4	ICR	3.5 months	-/-	-/-	+/-	-/-	+Le	+A	+Dy	NA	-/-	+A	+A	-/-
LTag2	F	2	ICR	3.5 months	-/-	-/-	+/-	-/-	NA	NA	+Dy	NT/Dy	-/-	+A	+A	-/-
LTag3	F	1.5	C3H/HeN	5 months	-/-	-/-	+Ly	-/-	NA	NA	+Dy	+/-	+/- ^e	+Dy	+/-	-/-
LTag4	ND ^d	>50	C3H/HeN	<1 day	-/-	NT	NT	-/-	NT	NT	NT	NA	+/- ^e	NT	+A	NT
LTag5	F	2	ICR	5 months	-/-	-/-	+Ly	-/-	NA	NA	+/-	+Dy	+/- ^e	+/-	+Dy	-/-
LTag6	F	6	C57BL/6	<1 day	-/-	-/-	+/-	-/-	NA	NA	NT	NA	NA	+/-	+/-	-/-

^a Number of copies of transgene estimated by quantitative dot blot (not shown).

^b Age at death. LTag1, LTag2, LTag3, and LTag5 were sacrificed. LTag4 and LTag6 were dead in utero.

^c Expression (measured by Northern or S1 analysis of RNA and by immunofluorescence)/tumor formation (+, positive; -, negative).

^d ND, Not determined.

^e Expression was limited to lymphoma cells in Peyer's patches. Abbreviations: Br, brain; He, heart; Sp, spleen; Sk, skeletal muscle; Te, testis; Pr, prostate; S.G., salivary gland; M.G., mammary gland; Int., intestine; Kid, kidney; Lu, lung; Li, liver; NA, not applicable; NT, not tested; Le, Leydig cell tumor; A, adenocarcinoma; Ly, lymphoma; Dy, dysplasia.

FIG. 3. Northern blot analysis and S1 nuclease protection of pLTR-Tag probe by various RNAs. (A) Northern blot analysis of RNA from LTag1 testis (Te) and seminal vesicle (Tu) and NIH 3T3 cells (N). Numbers at right represent sizes of RNA. (B) Diagram of protected probe and S1 protection analysis of RNA isolated from mouse L cells transfected with pLTR-Tag (lanes 1 and 2) and from salivary gland cultured cells isolated from LTag3 (lanes 3 and 4). Symbols: □, MMTV LTR; ■, SV40 sequences; *, labeled end of the molecule. Cells were treated with 0.1 μ M dexamethasone (+) or left untreated (–) for 24 h prior to harvest. (C) RNA isolated from LTag1, LTag2, LTag3, and LTag6 normal and tumor tissue (numbers at top of figure). Abbreviations: M, *Hpa*II pBR322 markers; P, 231-bp probe; t, tRNA; E, epididymis; S, spleen; T, testis; K, kidney; Lu, lung; B, brain; H, heart; Tu, seminal vesicle tumor; Li, liver; LN, lymph node.

rabbit anti-SV40 T-antigen antibody on frozen sections from these tissues were carried out (Fig. 4B).

Northern analysis of RNA from tissues showed two transcripts of approximately 2,600 and 2,300 nucleotides, corresponding to the small t and large T spliced mRNAs, respectively, as seen in Fig. 3A for RNA isolated from the testis and seminal vesicle of LTag1. The small t- and large T-antigen-hybridizing RNAs initiated at the MMTV transcription start site within the LTR in all expressing tissues, since a 130-bp junction fragment labeled at a *Hind*III site within SV40 and extending 109 bases into the MMTV LTR was protected from S1 nuclease digestion (Fig. 3B). Transcription of this RNA was inducible with dexamethasone, either after transfection of the plasmid pLTR-Tag into L cells (Fig. 3B, lanes 1 and 2) or HeLa cells (not shown) or into

fibroblasts cultured from the tissues of the transgenic mice (Fig. 3B, lanes 3 and 4). A 130-bp fragment was also protected by RNA isolated from lung, spleen, kidney, and salivary gland tissue of all the animals and from prostate and testis tissue of LTag1 (Fig. 3C; Table 1). However, no protection of this fragment was seen with RNA isolated from brain, heart, or liver tissue (Fig. 3C) or from skeletal muscle (Table 1 and data not shown).

All of the tissues which expressed the transgene as determined by RNA analysis also expressed SV40 early-region proteins when analyzed by immunofluorescence and Western blots. Fig. 4A shows Western analysis on extracts from LTag1 seminal vesicle and testis tissue with large T monoclonal antibody pAB412 (2), which recognized a protein of about 95 kilodaltons, corresponding to large Tag, in tissues from transgenic but not normal animals.

We used rabbit polyclonal anti-SV40 T-antigen antibody to immunostain sections of tissues from the transgenic animals and found that the nuclei of positive cells were diffusely and strongly immunostained, a pattern which is characteristic for T antigen, as shown for spleen tissue of LTag5 and kidney tissue of LTag2 (Fig. 4B). No staining was seen in any cell type in the kidney other than the ductal epithelial cells (Fig. 4B); this was also the case in the lungs, salivary glands, prostate glands, and mammary glands of all the animals (not shown). In addition to detecting Tag staining in the epithelial cells of expressing tissues, we saw immunostaining of single lymphoid cells in nonexpressing tissues (not shown) and in the spleen and lymph nodes.

In summary, expression of the transgene was detected in the epithelial cells of the salivary glands, lungs, kidneys, and lactating mammary glands and in lymphoid cells in both the spleens and other organs, of all mice examined, (Table 1). In addition, in the one adult male transgenic mouse obtained, expression was seen in the epithelial cells of the prostate and in Leydig cells of the testes.

Expression of the transgene is heritable. Both LTag3 and LTag5 were mated with normal ICR males in an attempt to create new strains of mice containing the pLTR-Tag plasmid. These mice produced two and three litters, respectively; approximately 50% of each litter was transgenic, indicating a single chromosomal integration (not shown). We isolated RNA from the organs of neonates and on day 17 of gestation from offspring of both these mice and carried out S1 nuclease mapping and immunofluorescence studies (not shown) on sections of their organs. Expression of the transgene was detected in the lung, kidney (Fig. 5), salivary gland, and spleen (not shown) tissue of all LTag3 and LTag5 offspring that were transgenic. All of the transgenic LTag3 and LTag5 offspring expressed the transgene in these four tissues, but not in liver, brain, or heart tissue (Fig. 5); because of the limited amount of tissue, we were not able to carry out similar analysis on prostate and testis tissue. Although both the LTag3 and LTag5 founder mice had lymphomas, none of the offspring had developed these or any tumors prenatally or neonatally (D. Henrard, Y. Choi, I. Lee, and S. R. Ross, manuscript in preparation).

Correlation of transgene expression with tumor induction. All six founder mice developed neoplastic diseases; they included renal cell carcinoma, lung carcinoma, prostate and/or seminal vesicle carcinoma, pre-B-cell lymphoma, Leydig cell tumor of the testis, and ovarian hilus cell tumor (Table 1; Henrard et al., in preparation). The mammary and salivary gland ductal epithelial cells of several of the mice also showed dysplasia; however, no neoplasms were seen in tissue from these two organs (Table 1). Two of the animals,

LTag3 and LTag5, also developed lymphomas; the lung and kidney tissue of these animals showed only a hyperplastic morphology, although T antigen was detected by immunostaining.

DISCUSSION

In this study we showed that a transgene containing the SV40 early region under the control of the MMTV LTR is capable of being expressed in the ductal epithelial cells of lungs, kidneys, and prostate, mammary, and salivary glands, and in Leydig and lymphoid cells. Thus it appears that the LTR encodes sequences that determine epithelial and lymphoid cell expression and that the transcription of endogenous provirus(es) in only lactating mammary gland epithelial cells must require other factors or sequences in addition to the LTR to achieve this higher order of specificity.

The expression detected in the LTR-Tag mice might be the result of integration of the injected DNA at a chromosomal site(s) in all six mice that allows expression in several

FIG. 4. Western blot analysis and SV40 immunostaining. (A) Western blot analysis of protein from LTag3 spleen and LTag1 seminal vesicle tissue, with a monoclonal antibody to large T-antigen. Abbreviations: n, spleen and seminal vesicle extracts from normal C3H/HeN (spleen) and ICR (seminal vesicle) mice; Tag, protein band of about 95 kilodaltons represents the SV40 large T-antigen protein. The lower-molecular-mass bands are the mouse immunoglobulin heavy and light chains, recognized by the secondary antibody (rabbit anti-mouse immunoglobulin). (B) Immunostaining of LTag5 lymph node and LTag2 kidney tissue. Panel 1, Section of diffuse pre-B cell lymphoma in spleen, hematoxylin-eosin stained; magnification, $\times 140$. Panel 2, Immunostaining of spleen section with rabbit anti-SV40 antibody; note staining of nuclei. Magnification, $\times 540$. Panel 3, Section of LTag2 kidney; note infiltrating adenocarcinoma (*), a dilated tubule with atypical, papillary epithelial proliferation (tub), and atrophic ducts (g). Hematoxylin-eosin stained; magnification, $\times 140$. Panel 4, Same kidney displaying staining for SV40 T antigen at the nuclei of tubular epithelial cells. Magnification, $\times 540$.

FIG. 5. S1 nuclease protection of pLTR-Tag RNA from LTag3 and LTag5 offspring. RNA was isolated from the tissues of LTag5.1, LTag5.4, and LTag3.1. Abbreviations are the same as for Fig. 3. RNA from the seminal vesicle tumor (Tu) of LTag1 was included as a positive control.

tissues. However, at least five of the six mice examined in our study expressed the transgene in the same set of tissues: in the epithelial cells of the kidneys, lungs, and salivary glands and in the spleens (we were unable to extensively study expression in the sixth mouse because of tissue degradation). This expression could also result from cryptic regulatory effects by the SV40 early region on the MMTV LTR. A number of groups have successfully used the SV40 sequences as a marker gene for tissue-specific expression, and only a small percentage of these transgenic mice showed aberrant expression (for a review, see reference 23). It seems unlikely, therefore, that the expression that occurs in our transgenic mice results only from integration or other effects.

It is also possible that the MMTV LTR allowed a low level of expression in many cells of different tissues or that only a few cells in the tissue expressed the construct and then went on to form tumors which transcribed the transgene at a higher level than normal tissue. However, we did detect T-antigen expression in the tissues of newborn offspring of these transgenic mice at a time when no obvious histological changes had yet occurred in these animals, and the level of expression in these tissues, either by RNA analysis or by immunofluorescence, did not appear to be lower than that seen in tumors (Fig. 5; Henrard et al., in preparation). In addition, the spectrum of pathologic changes in adult LTag mice, involving many cells in both the kidneys and lungs, including dysplastic change, in situ carcinomas, and infiltrating carcinomas, seemingly representing a chronological sequence of carcinogenesis, and the immunofluorescence results, which showed staining of all the tubular epithelial cells, indicate that expression is occurring simultaneously in many different cells of the same organ (Henrard et al., in preparation).

Some of the tissues in which we saw expression and which gave rise to tumors have been previously implicated as sites of MMTV expression; for example, immunological studies have shown high levels of MMTV proteins in B cells (13) and in the prostate glands, seminiferous tubules, and coagulating glands of viremic mice (10), and both B cells (30) and a Leydig cell carcinoma (36) have been identified which express MMTV RNA. Interestingly, one animal in our study

developed an ovarian hilus cell tumor, which is an androgen-producing tumor in females, equivalent to Leydig cell tumors in males (I. Lee, unpublished results); in our previous study we also detected expression of the *tk* RNA in both these tissues (29). Thus it seems that the MMTV LTR, rather than the *tk* sequences, directs expression in these organs.

It has recently been proposed that rearrangements and/or deletions within the MMTV LTR between -650 and -165 upstream from the MMTV transcription initiation site allow its expression in T cells and kidney epithelia and hence cause lymphomas (17) and adenocarcinomas (38), respectively. The Southern blot analysis shown in Fig. 2 indicated that gross deletions of the MMTV sequences had not occurred in any of the LTag transgenic mice. More detailed restriction mapping of the LTRs present in the transgene in DNA isolated from various tissues of LTag1 through LTag6 indicated that similar deletions had not occurred in these mice (not shown). Therefore, expression in the different tissues does not appear to have resulted from gross rearrangement of the LTR sequences.

The LTRs of several murine leukemia viruses, which can function in many cell types, show enhancement of expression in the cell type in which they cause tumors (28, 31, 40). Recently it has also been shown that transgenic mice carrying a Rous sarcoma virus LTR-containing plasmid expressed the introduced sequences in the expected tissues (22). Our results suggest that for MMTV, the LTR may not be the sole determinant of tissue-specific expression. The level of expression of the MMTV provirus in mammary glands may be enhanced by the presence of the LTR, but there may also be regions of the MMTV genome outside the LTR which affect its transcription in other organs. For example, transcription of MMTV in the spleens of BALB/c mice has been reported to originate from a deleted provirus consisting of sequences primarily from the LTR (30).

The widespread but tissue-specific expression seen with the LTag mice was unexpected in light of our results with an MMTV LTR-HSV-1 *tk* plasmid (29) and those reported for the LTR/*myc* transgenic mice (11, 33). Both of these constructs contained MMTV sequences from outside the LTR; pLTR₂*tk* contains about 0.8 kb of the MMTV *pol* gene, while the LTR/*myc* plasmids contain 1.5 kb of the MMTV envelope coding region, which has been shown to encode putative glucocorticoid regulatory elements (25). We can test whether these other regions of MMTV encode tissue specificity by creating new transgenic mice containing plasmids bearing different regions of the MMTV genome.

ACKNOWLEDGMENTS

We thank Raju Kucherlapati and Katherine Knight for critically reviewing the manuscript and Barry Mellovitz for technical assistance.

This research was supported by Public Health Service grant HD-19444 from the National Institutes of Health and a Basil O'Connor grant from the March of Dimes.

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