Expression Pattern of Mouse Mammary Tumor Virus in Transgenic Mice Carrying Exogenous Proviruses of Different Origins

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To study the tissue specificity of mouse mammary tumor virus (MMTV) gene expression, we developed two series of transgenic mice, containing the MMTV proviral DNA of mammary (GR) and kidney (C3H-K) origin. The expression pattern in the MMTV(GR) transgenic mice is very similar to that observed in infected animals, e.g., a strong preference for viral expression in the lactating mammary glands and lower levels of expression in salivary glands, lymphoid tissues, and male reproductive organs. One line of transgenic mice carrying the C3H-K provirus has a similar expression pattern, indicating that MMTV(C3H-K), despite a striking alteration in the U3 region of its long terminal repeat, can be expressed in the same tissues as the wild-type MMTV.

Mouse mammary tumor virus (MMTV) is a slowly transforming retrovirus that induces neoplastic disease after a long latency period. It can be transmitted either exogenously as viral particles (mainly in the milk) or endogenously through the germ line as stably integrated proviruses, the so-called endogenous MMTV. Moreover, it is the only retrovirus whose expression is significantly stimulated by steroid hormones (for reviews, see references 14 and 34).

Although MMTV is highly tissue specific, being involved in the transformation of mammary gland cells, it has also been associated with murine thymic lymphomas (13, 23, 26) and with kidney adenocarcinomas (11, 44). Cloned MMTV proviruses isolated from lymphomas were found to have alterations in the U3 region of their long terminal repeats (LTR) (2, 19, 23, 27), which were thought to be related to the disease and the tissue-specific expression of MMTV genes in lymphocytes (40, 45). A variant of MMTV, called MMTV(C3H-K) because it originally appeared in kidney tumors of mice infected with MMTV(C3H), was isolated and cloned from an epithelial cell line established from a kidney adenocarcinoma of BALB/cf/Cd mice (15, 44); this variant was also found to have alterations in the U3 region of the LTR, and it was postulated that this change gives the virus a new target cell specificity (44).

The expression of MMTV in various organs of mice has been studied in a number of ways, e.g., by radioimmunoassay and immunofluorescence and by analyses at the RNA level (see, for example, references 16, 21, 35, and 46). From the results it was concluded that in susceptible animals, MMTV RNA and proteins are present mainly in mammary glands and mammary tumors. Salivary glands and lymphoid tissues (spleen, thymus, lymph nodes) usually show a 100- to 1,000-fold-lower level of expression. A low level of expression is also often found in male reproductive organs, e.g., seminal vesicles and/or the prostate and testes. Most authors agree that the heart, lungs, kidneys, liver, ovaries, placenta, uterus, and embryos are negative, as is usually the brain (for reviews, see references 14, 17, 30, and 34).

Transgenic mice represent a system of choice for studying

the contribution of transcriptional elements in the viral LTR or other regions of the genome, e.g., functioning as specific enhancers or coding for transcriptional activators, to the tissue tropism of MMTV. In several studies, the LTR of MMTV, mainly of C3H origin, has been used to drive the expression of transforming genes in transgenic mice (5, 10, 20, 22, 24, 28, 29, 36, 38, 41, 43). A clear preference for transgene expression in the mammary glands was observed in all cases, often related to neoplastic transformation of this organ, indicating that determinants for mammary epithelial cell-specific transcription were located in the wild-type LTR. However, other tissues, such as salivary glands, seminal vesicles, prostate, and lymphoid organs, were often found to express the transgene at lower levels. Other experiments in which the MMTV LTR was tested as a promoter in front of a reporter gene in transgenic mice have been described and showed similar results (9, 33). In all these analyses transgene expression was negative in the liver, which is known to be rich in glucocorticoid receptors; the presence of this receptor therefore does not seem to be sufficient to allow transcription of the transgene to occur. Plasmids containing specific deletions in the MMTV LTR used as a promoter revealed different elements situated in the LTR that affected gene expression in specific cell types (32, 37). These experiments suggested that sequences outside of the hormone-responsive element in the LTR were involved in the tissue-specific transcriptional pattern of MMTV.

We were interested in comparing the cell- and organ-specific transcription of the MMTV(GR) and MMTV(C3H-K) proviruses. MMTV(GR), produced by the endogenous *Mtv-2* locus, is responsible for the high incidence of mammary tumors in the GR mouse strain (for reviews, see references 14 and 34). The viral DNA was cloned and shown upon transfection into mouse fibroblasts to be correctly transcribed; its expression was stimulated by glucocorticoid hormones. Only small numbers of viral particles were made in these transfected cells (6). In animals infected with MMTV(C3H-K) no mammary tumors were observed (3, 15, 31, 44), and we speculated that the rearrangement in the U3 region of this provirus might render its transcription inefficient in the mammary gland. In this paper, we report the

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analysis of two different series of transgenic mice carrying the complete MMTV(GR) and MMTV(C3H-K) proviruses.

Generation of MMTV(GR) and MMTV(C3H-K) transgenic mice. For the MMTV(GR) transgenic mice, the 8.6-kb *Eco*RI insert from a λ recombinant phage containing the complete information of GR-MMTV in a permuted arrangement (Fig. 1A, clone H) (6) was ligated with T4 DNA ligase to reconstruct a complete copy of proviral DNA. The multimerized MMTV(GR) DNA was injected into (C57BL/6 $\times$ SJL) F_3 fertilized eggs as described previously (18). Six transgenic founder mice were obtained (four males and two females), harboring 5 to 20 tandemly integrated copies of the transgene. In Southern blot analyses we verified that most of these copies were present in a head-to-tail configuration, thus reconstituting normal proviral DNA. The founders were crossed once with BALB/c mice and then maintained as brother-sister matings to generate six transgenic lines named MMTV(GR) A, B, C, E, F, and G.

The 6.1- and 4.7-kb *Eco*RI inserts of plasmids pMKS7 and pMKS10, containing the 5' and 3' portions of integrated proviral DNA and some flanking cellular sequences cloned from a kidney tumor cell line (44), were recombined and grown in the EMBL3 vector (Fig. 1A). The 10.8-kb insert of a phage containing the entire MMTV(C3H-K) proviral DNA was gel purified and used for oocyte microinjections. Four transgenic founder mice (two males and two females) were obtained and crossed to BALB/c mice as for the MMTV(GR) transgenic mice. Four transgenic lines, named MMTV(C3H-K) V, W, X, and Z, were maintained by brother-sister matings. The mice were analyzed by Southern blotting or by polymerase chain reaction on peripheral blood, with primers that amplify specifically the substitution found in the U3 region of the LTR of MMTV(C3H-K) proviral DNA (31). Lines X and W were estimated to harbor about 3 copies of the transgene, whereas line V contained about 5 copies and line Z contained about 10 copies. Subsequent sequencing of the envelope gene contained in plasmid pMKS10, used in the construction of the MMTV(C3H-K) transgenic mice, revealed a point mutation giving rise to a stop codon in the middle of the gene (3); as a consequence, no functional *env* proteins and no infectious viral particles could be produced in these transgenic animals.

Expression of the transgene. Total cytoplasmic RNA was prepared as described previously (44) from several organs from transgenic males and females of different ages. The transgene expression in the MMTV(GR) lines was analyzed by S1 nuclease protection experiments (Table 1; data not shown), using as probe the LTR-containing 1.3-kb *Pst*I-*Pvu*II fragment of MMTV(GR), labeled at the *Pvu*II site as described previously (7, 8). We observed, as in infected animals, a clear preference for viral transcription in the lactating mammary glands. Lower levels of expression were also consistently seen in the salivary glands, male reproductive organs, and lymphoid tissues such as the spleen. In that particular tissue, MMTV transcription, detected at relatively high levels (Table 1), probably originated from both transgene and endogenous proviral loci, since expression of endogenous MMTV in the spleens of different mouse strains has been consistently reported (16, 35, 39).

To analyze MMTV(C3H-K) transgenic mice, we used an RNase protection assay that could distinguish between transcripts derived from the transgene and from endogenous MMTV proviruses (Fig. 1B). A 1.27-kb fragment from plasmid pMKL7 (44), containing the 3' LTR of an integrated MMTV(C3H-K) provirus, from the *Pst*I site at the 5' end of the LTR to a *Bam*HI site located 3 bp outside of the LTR in

FIG. 1. Generation of MMTV transgenic mice and probes used in this study. (A) The 8.6-kb insert of clone H (6) representing the complete information of MMTV(GR) is shown with the sites for *Eco*RI (E) and *Bam*HI (B). (B) The construction of MMTV(C3H-K), from the inserts of plasmids pMKS7 and pMKS10 (44), is depicted below. The black box in the U3 region of the LTR represents the MMTV(C3H-K)-specific sequences (KSS). Adjacent cellular sequences are indicated by wavy lines, and the *Sal*I site from the EMBL3 vector (S) used to cut out the insert before microinjection is also shown, as is the approximate location of the coding regions for the viral genes (*gag*, *pol*, *env*, and *orf*). (C) Probe for RNase protection experiments. The relevant sites in the MMTV(C3H-K) LTR used in the construction are indicated by their positions relative to the 5' end of viral RNAs. Dotted lines represent pSP65 plasmid sequences, and the black box (KSS) represents the MMTV(C3H-K) specific sequences. Below are shown the riboprobe and the fragments protected by the 5' and 3' ends of viral RNAs. Only the 280-nt fragment protected by the 3' ends of viral RNAs is specific for MMTV(C3H-K) expression (see text). (C) Probes used for the in situ hybridization experiments. Probe M is a subclone from pMKS7, and probe K is a subclone from pMKS10 (panel A). The sites used for the constructions are indicated by their positions relative to the 5' end of MMTV(C3H-K) RNAs. Thin lines and *Eco*RI and *Hind*III sites are part of the pGEM-1 vector. Below are indicated the sense and antisense runoff transcripts generated by SP6 or T7 RNA polymerases that were used as probes in the experiments.

TABLE 1. Transgene expression in MMTV(C3H-K) and MMTV(GR) mice

Transgenic line	Sex ^a	Relative level of transgene expression ^b								
		UT	TE	SV	MG	SG	LI	KI	SP	BR
MMTV(C3H-K)										
V	F(l)	ND			—	—	—	—	+	—
	M		+	—	ND	ND	—	—	+	—
W	F(l)	+			+++	+	—	—	++	++
	F(v)	+			+	+	—	—	++	++
			+	+	ND	+	—	—	++	++
X	F(l)	+			+	+	—	+	++	+
	F(v)	—			+	+	—	+	++	+
	M		ND	—	ND	+	—	+	++	+
Z	F(l)	—			—	—	—	—	+	—
	M		+	+	ND	—	—	—	+	—
MMTV(GR)										
A	F(l)	—			+++	+	—	—	++	—
	M		+	++	ND	++	—	—	++	—
B	F(l)	+			+++	+	—	—	++	+
C	F(l)	—			+++	+	—	—	++	+
E	F(l)	+			++	+	—	—	+	—
F	F(l)	+			+++	+	—	—	++	ND
G	F(l)	—			+++	+	—	—	++	+

^a F(l), lactating females; F(v), virgin females; M, males.

^b Measured by RNase protection or S1 analysis with SC7 total RNA as an internal standard. Symbols: —, not detectable; +, low; ++, intermediate; +++, high. Abbreviations: ND, not done; UT, uterus; TE, testes; SV, seminal vesicles; MG, mammary glands; SG, salivary glands; LI, liver; KI, kidney; SP, spleen; BR, brain.

adjacent cellular sequences, was cloned in the antisense orientation in the vector pSP65 (Fig. 1B). The plasmid was linearized with *Dra*I, which cuts 403 bp from the 3' end of the LTR, to produce a runoff antisense transcript of 437 nucleotides (nt), labeled with [α -³²P]UTP, and used in an RNase protection assay essentially as described by Melton et al. (25), with 0.5 to 20 μ g of cytoplasmic RNA. Owing to the sequence substitution in the U3 region of the MMTV(C3H-K) LTR (KSS in Fig. 1) (44), the 280-nt fragment protected by the 3' end of viral RNAs is specific for transgene expression. Transcription from endogenous MMTV proviruses gave protected fragments of variable size (but with a maximum of 200 nt), as a result on one hand of the sequence divergence between the probe and the endogenous MMTV and on the other hand of the segregation of the endogenous loci in individual mice. Readthrough transcription of MMTV(C3H-K) RNAs past the poly(A) addition signal gave a longer protected fragment of 403 nt, indicated by r in Fig. 2. The riboprobe used also yielded a 133-nt protected fragment corresponding to the 5' end of viral RNAs; in this case, however, it was not possible to tell whether it resulted from expression of the transgene or of endogenous proviruses.

As a positive control in these experiments, RNA from a serially transplanted kidney tumor (44), harboring eight copies of MMTV(C3H-K), was used. This tumor, SC7, expresses high levels of MMTV(C3H-K) RNA and was used as internal standard for quantitation in all experiments. Representative results from such analyses are shown in Fig. 2 for the MMTV(C3H-K) W line, and the results of all expression studies are summarized in Table 1.

In the MMTV(C3H-K) W transgenic mice, the highest levels of transgene-specific transcripts were observed in the mammary glands of lactating females. Relatively high levels of expression (about 20 to 50 times lower than in lactating mammary glands) were also observed in the spleen and brain of all mice of that line tested. After longer exposure of the

autoradiographs, expression was detected in the salivary glands, testes, uterus, and seminal vesicles. None of the mice of that line expressed the transgene to a significant level in the kidneys or the liver. Nontransgenic control littermates (female W28 compared with female W30 in Fig. 2) had low levels of endogenous viral transcripts in the spleen, mammary glands, salivary glands, and seminal vesicles. Mixed expression (transgene and endogenous) was often seen, e.g., in seminal vesicles or salivary glands (male W61 in Fig. 2). Low-level expression of the transgene was observed in the mammary glands of virgin females, confirming our previous observations that MMTV(C3H-K) RNA synthesis is hormonally stimulated (44). The expression pattern in the W line, apart from the relatively high levels of transcription in the brain, is very similar to the one observed in MMTV(GR) transgenic mice and in MMTV-infected animals (14, 17, 30, 34), indicating that the MMTV(C3H-K) proviral DNA can be expressed in the same tissues as the wild-type mammary MMTV.

Transgenic mice from the MMTV(C3H-K) X line expressed relatively high levels of the transgene in the spleen and low levels in the mammary glands, salivary glands, brain, uterus, and kidneys (Table 1). Transgene-specific transcripts were not detected in the liver or the seminal vesicles. High levels of MMTV endogenous expression were seen in lactating mammary glands, and lower levels were detected in seminal vesicles and salivary glands. Mice of the MMTV(C3H-K) V line expressed low levels of the transgene in the testes and the spleen, whereas in the Z line, low-level transcription of MMTV(C3H-K) RNA was observed in the testes, spleen, and seminal vesicles (Table 1).

To check whether rearrangements of the transgene or recombinations with endogenous MMTV proviruses occurred in MMTV(C3H-K) transgenic mice, we performed Southern blots on expressing tissues (data not shown). No rearrangements or additional proviruses were detected in these samples compared with nonexpressing tissues. No

FIG. 2. RNase protection analysis of MMTV(C3H-K) W transgenic mice. Total RNA from various organs was assayed by using the riboprobe depicted in Fig. 1B. Male W61 and female W55 mice are 4-month-old virgin littermates. W30 and W28 are 9-month-old lactating female littermates after their sixth pregnancies (W28 is a nontransgenic control). In each assay, 20 μ g of RNA was used, except for the positive control SC7 (0.5 μ g) and mammary glands (10 μ g for W55 and 5 μ g for W30). The positions of the transgene-specific protected fragment (280 nt) and the other protected fragment (133 nt, see text) are indicated. The arrowhead denoted r corresponds to readthrough transcription of MMTV(C3H-K) RNA (see the text). The films were exposed for 12 to 15 h with intensifying screens at -70°C , and low levels of expression were detected on longer exposures. Abbreviations: p, 437-nt riboprobe; m, *Sau3AI* pUC13 marker; SC7, positive control RNA (see text); yeast, yeast RNA; SV, seminal vesicles; TE, testes; SG, salivary glands; LI, liver; KI, kidneys; SP, spleen; BR, brain; UT, uterus; MG, mammary glands.

correlation was evident between the copy number of the transgenes and the levels of transgene-specific expression. Differences of MMTV(C3H-K) expression in the independently derived transgenic lines were observed (Table 1), as was reported for MMTV/*myc* and MMTV/*neu* transgenic mice (29, 38). The basis of this observation might reside in the structure of the integrated genes or their chromosomal location. The levels of expression of the transgene in the kidneys were low or undetectable, suggesting that no dominant positive kidney-specific transcriptional elements are present in the MMTV(C3H-K) genome. The observation that levels of transgene expression are relatively high in the brains of mice of the MMTV(C3H-K) W line and lower in those of mice of the X line is interesting, although this did not seem to affect the behavior of the mice. The LTR of MMTV(C3H-K), when compared with the one of MMTV(C3H), has a deletion of 18 bp between -186 and -204 [relative to the MMTV(C3H) RNA start site] and a replacement of 113 bp between -220 and -333 by 91 bp of unknown origin (44); in the other regions, the two LTRs diverge by only 3.5% in their nucleotide sequence (3). We can speculate that the substitution found in the MMTV(C3H-K) LTR removed a negative regulatory element and thus allowed the provirus to be expressed in the brain of the transgenic mice. On the other hand, the results with line W indicate that sequences missing in the MMTV(C3H-K) LTR are not required for high-level expression in the mammary gland.

In situ hybridization experiments. To localize and characterize more precisely which cells expressed the MMTV(C3H-K) provirus in the transgenic mice, we performed a series of in situ hybridizations. Two probes, depicted in Fig. 1C, were used. The first, probe M, detected

transcripts originating from the transgene as well as those initiated at endogenous MMTV loci, whereas the second, probe K, revealed only transgene-specific transcripts. The appropriate MMTV(C3H-K) proviral fragments were subcloned in the pGEM-1 vector. Sense and antisense riboprobes were labeled with [α - ^{35}S]UTP (18), reduced when necessary to an average size of 100 nt by limited alkaline hydrolysis (12), and hybridized to cryostat sections from different organs of the MMTV(C3H-K) transgenic mice essentially as described previously (18). Hybridizations with the control sense probes revealed only background staining (data not shown). Representative results obtained with the antisense probes are shown in Fig. 3.

As a positive control, we used SC7, the serially transplanted kidney tumor that was used in the RNase protection experiments described above. As shown in Fig. 3A, the subcutaneously inoculated tumor mass expressed high levels of MMTV(C3H-K) RNA, but viral transcription was totally absent in the neighboring tissues of the nude mouse, e.g., in the epidermis, dermis, and hair follicles.

Figure 3B shows a section of the lactating mammary glands of a 7-month-old female of the C3H/OIJ strain sacrificed 10 days after bearing its fifth litter; this section was hybridized to the M probe. C3H mice are infected with an exogenous, milk-borne mammary tumor virus, MMTV(C3H), which is responsible for the high incidence of mammary tumors observed in this mouse strain (4). High levels of MMTV(C3H) RNA were observed in epithelial alveolar cells of the mammary glands, but the pattern of expression was patchy, some alveoli being highly positive and some others completely negative (Fig. 3B), as was already noted by observing immunofluorescence in the RIII mouse strain (21). A similar pattern of MMTV(C3H-K) expression was ob-

FIG. 3. In situ hybridization experiments. Frozen sections of different tissues of control mice or MMTV(C3H-K) transgenic mice were hybridized to antisense probe M or K (see Fig. 1C) and counterstained with hematoxylin and eosin. (A) Kidney tumor SC7, harboring eight copies of MMTV(C3H-K) per cell, subcutaneously inoculated in the nude mouse and hybridized to probe K. Note the heavily labeled tumor mass and surrounding negative skin. Magnification, $\times 80$. (B) Mammary glands of a 7-month-old lactating C3H female hybridized to probe M. Expression is restricted to epithelial alveolar cells, but some alveoli remain completely negative, as do the intralobular conjunctive tissue and blood vessels. Magnification, $\times 50$. (C) Lactating mammary glands of a 7-month-old MMTV(C3H-K) W16 female hybridized to probe M. Note the similar patchy expression to the one observed in panel B. Magnification, $\times 40$. (D) Adjacent section to the one shown in panel C but hybridized to probe K. Note the perfect superposition of silver grains compared with panel C. Magnification, $\times 40$. (E) Detail of salivary glands of a 7-month-old MMTV(C3H-K) W25 male, hybridized to probe K. Expression is limited to rare epithelial acinar cells. Magnification, $\times 310$. (F) Spleen of an 8-month-old MMTV(C3H-K) X13 female, hybridized to probe K. Highly expressing cells are found associated with the red pulp. Magnification, $\times 200$. (G) Brain of a 7-month-old MMTV(C3H-K) W25 male, hybridized to probe K. Expression is restricted to a subset of neurons in the third and fifth layers of the temporal lobe of the cerebral cortex. Note the cerebellum in the top right angle. Magnification, $\times 32$. (H) Detail of a region of panel G. Magnification, $\times 200$.

served in the lactating mammary glands of the MMTV(C3H-K) W line, an example of which is shown in Fig. 3C and D. The patchy transcription pattern in lactating mammary glands was observed also in the other MMTV transgenic lines studied, even in those expressing only endogenous MMTV (data not shown).

In the salivary glands, expression of the transgene was restricted to rare epithelial acinar cells, as shown for a MMTV(C3H-K) W transgenic mouse (Fig. 3E). The spleen was the only organ consistently positive for transgene expression in all transgenic mice analyzed (Table 1). An example of in situ hybridization is shown in Fig. 3F, where silver grains were observed over some cells of the red pulp. An example of the unusually high level of transgene expression in the brain of MMTV(C3H-K) W mice is shown in Fig. 3G and H. Interestingly, viral transcription was limited to certain neurons in the third and fifth layers of the temporal lobe of the cerebral cortex. In the MMTV(C3H-K) X transgenic line, where the expression in the brain is lower (Table 1), the localization of silver grains was similar (data not shown).

Expression of the transgene was observed in the spleen of all transgenic mice analyzed. MMTV RNA and proteins have been found in the spleen of different mouse strains (infected and noninfected) (14, 16, 30, 35, 39, 46). Our in situ hybridization experiments did not allow us to define the type of cells involved, but, as suggested by others (39, 42), it is likely that subsets of T or B lymphocytes are the cells expressing MMTV in the transgenic mice. Moreover, our recent experiments showing clonal deletion of V β 14-bearing T cells in MMTV(GR) transgenic animals indicate that the transgene must be expressed at least in a subset of B cells (1). Purification of different subpopulations of hematopoietic cells from the spleen, lymph nodes, or blood will clarify this point.

Abnormalities among transgenic mice. So far we have not observed any disease in the MMTV(C3H-K) transgenic mice. In one case only, an 11-month-old female of the V line developed mammary tumors after five pregnancies. An RNase protection experiment as described above revealed large amounts of MMTV RNA in the tumors, resulting exclusively from transcription of endogenous proviruses (data not shown). At autopsy, none of the transgenic animals of the four lines of MMTV(C3H-K) showed macroscopic abnormalities.

Transgenic females of the MMTV(GR) A line were used as foster mothers for BALB/c newborns. Neither the transgenic females nor the foster-nursed mice developed mammary tumors after forced breeding. None of the mice of the other MMTV(GR) lines had mammary tumors after forced breeding. At autopsy a small percentage (about 5%) of the

transgenic males of the MMTV(GR) A line showed enlargement of the seminal vesicles and/or prostate, but no progression to solid tumors was observed. Interestingly, this phenomenon was also reported in MMTV-*int-2* transgenic mice (28). There was no strict correlation between the levels of MMTV expression (analyzed by S1 mapping) and the abnormalities observed.

The absence of mammary tumors in the MMTV(GR) mice could be attributed to a smaller amount of virus produced by the transgenic females than by infected strains such as the C3H/OUJ mice. Indeed, viral envelope glycoprotein was detected by enzyme-linked immunosorbent assay in the milk of MMTV(GR) transgenic females after the second pregnancy, but, at most, at 10-fold lower levels than in corresponding C3H/OUJ females (data not shown). Finally, we could not exclude the appearance of point mutations in the transgene during the generation and the maintenance of transgenic mice, giving rise to nonfunctional viral proteins.

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