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A Mouse Mammary Tumor Virus Mammary Gland Enhancer Confers Tissue-Specific but Not Lactation-Dependent Expression in Transgenic Mice

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The long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) is known to contain a number of transcriptional regulatory elements, including glucocorticoid response elements. In this study, we showed that a mammary gland/salivary gland enhancer found in the LTR of this virus directs expression of a heterologous promoter to both virgin and lactating mammary glands in transgenic mice. Using transgenic mice containing hybrid gene constructs with various deletions of the LTR sequences linked to marker genes, we also showed that the dramatic increase in MMTV expression that occurs during lactation is due to the glucocorticoid response elements. Thus, the MMTV LTR encodes two distinct elements, both of which are required for a high level of expression in lactating mammary glands.

Mouse mammary tumor virus (MMTV) is a murine retrovirus that is causally associated with mammary gland adenocarcinomas in mice (10). The virus is expressed at high levels in lactating mammary glands and causes tumors by integrating near and activating the expression of a number of different cellular oncogenes (for a review, see reference 12). Because of the stochastic nature of this integration process, the concomitant increase in viral RNA and production of virus particles that occurs during lactation is probably crucial to the ability of MMTV to infect a sufficient number of cells to cause tumors. In addition, many if not all of the cellular oncogenes activated by MMTV are not normally expressed in mammary glands, and hence sequences encoded within the virus must be directly responsible for their activation. Thus, it is of great interest to understand which DNA sequences govern the regulation of virus expression in the mammary gland.

MMTV has long been used as a model to study steroid hormone regulation of gene expression, since glucocorticoids and other steroid hormones have been shown to stimulate MMTV transcription both in vivo and in tissue culture cells (for a review, see reference 21). The hormone responsiveness characteristic of MMTV is mediated by specific DNA sequences termed glucocorticoid response elements (GREs). In addition to the GREs, a number of transcriptional regulatory elements have been identified in tissue culture experiments, including a nuclear factor I-binding site located near the promoter (7, 11, 19) and additional positive and negative regulatory elements (4–6, 8, 9, 22). Some of these regions have also been shown to function in transgenic mice (1, 15–18).

Both endogenous MMTV RNA and transgenes containing the long terminal repeat (LTR) of this virus linked to marker genes are expressed primarily in mammary glands and at lower levels in a number of other tissues, including the salivary glands, seminal vesicles, spleen, thymus, kidneys, and lungs in mice (1, 3, 18). In an attempt to further define the sequences encoded in the LTR which govern the tissue-specific expression of the virus, we generated ET450 transgenic mice, which contain an MMTV LTR construct with a deletion of all LTR sequences 5′ of bp −365, relative to the start of MMTV transcription, linked to the simian virus 40 (SV40) early region genes (Fig. 1B) (15, 16). RNase T_1 protection analysis was carried out as previously described (2, 16) with RNA isolated from tissues such as lactating mammary gland, salivary gland, and lung. As can be seen in Fig. 2, although mammary and salivary gland expression was still detectable in these animals, the relative level of transgene versus endogenous expression in the mammary gland was drastically reduced compared with the level in mice containing a transgene consisting of the complete LTR linked to SV40 (LTR-Tag [Fig. 1A]). These changes in the relative level of expression detected in different tissues occurred in all three of the ET450 strains analyzed and strongly suggested that although the MMTV promoter was capable of directing mammary gland expression, other regions necessary to promote high levels of expression in this tissue were deleted from this transgene.

The decrease in transgene expression in mammary glands in ET450 mice was concomitant with an increase in the level of transgene expression in lungs relative to that seen in LTR-Tag mice. In previous studies (15, 16), we showed that a negative regulatory element(s) mapped between bp −665 and −201. However, the changes seen in mammary gland expression suggested that a positive acting element, possibly a mammary gland enhancer, was deleted as well. This was confirmed by experiments using a transgene, p1BCAT, containing an internal deletion in the LTR from bp −165 to −665 upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene (15). Although these mice had deletions in the negative regulatory element, the addition of sequences from bp −665 to the 5′ end of the LTR restored mammary and salivary gland expression to levels comparable to those seen in transgenic mice with a complete LTR.

To identify sequences which might be responsible for mammary gland expression, we subcloned fragments between bp −664 and −1182 into a plasmid containing the SV40 late promoter driving a CAT reporter gene and used the constructs in transient-transfection assays. The MMTV LTR sequences were cloned both upstream of the SV40 sequences, as diagrammed in Fig. 1, and downstream of the

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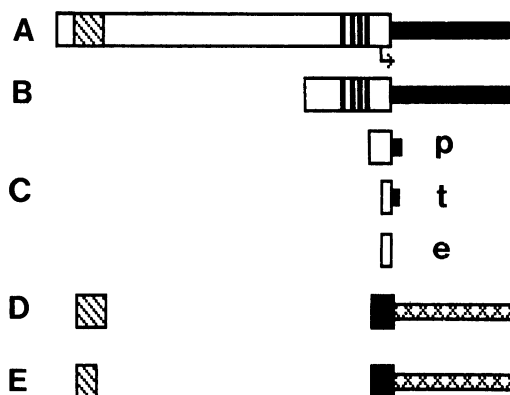

FIG. 1. Diagram of the LTR-Tag, ET450, RNase T_1 protection probes, and 179 and 102 transgenes. (A) LTR-Tag transgene, containing the *Pst*I-to*Hpa*II fragment from the C3H LTR upstream of the SV40 coding sequences (1, 2, 16). (B) ET450 transgene, containing the *Sau*3A (bp −365)-to-*Hpa*II fragment upstream of the SV40 coding sequences (16). (C) RNase T_1 protection probes. A fragment spanning the junction between the MMTV LTR and Tag was used to create a probe, as previously described (2, 16). (D) 180 transgene, containing bp −1166 to −987 of the MMTV LTR upstream of the SV40 promoter in plasmid pCAT (Promega Biotec, Inc.). (E) 102 transgene, containing bp −1166 to −1065 of the MMTV LTR in the pCAT plasmid. Abbreviations: p, probe; t, transgene; e, endogenous.

CAT reporter gene (not shown). The transfections were done in normal mammary gland (NMuMG) cells and Hepa (mouse hepatoma) cells. The results in Table 1 show that both a 180-bp fragment (bp −987 to −1166) (Fig. 1C) and a 102-bp fragment (bp −1065 to −1166) (Fig. 1D) conferred enhanced levels of expression relative to the parental construct lacking any enhancer (pCAT) in NMuMG but not Hepa cells. This enhancement was both position and orientation independent, as can be seen for the 102-bp fragment; similar results were obtained with the 180-bp construct (data not shown). As a control, a plasmid containing the SV40 enhancer was also transfected into these cells; this plasmid showed no cell type specificity. Thus, sequences from bp −1166 to −1065 appeared to function as a mammary gland

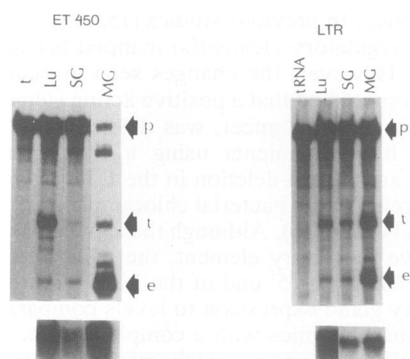

FIG. 2. Transgene and endogenous MTV expression in ET450 and LTR-Tag mice. RNase T_1 protection analysis was carried out as previously described (2, 16) with the probe diagrammed in Fig. 1. Abbreviations: t, tRNA control; Lu, lung; SG, salivary gland; MG, mammary gland; p, probe; t, transgene; e, endogenous. As a control, 20 μg of total RNA was subjected to Northern blot analysis with an actin probe (bottom panel).

TABLE 1. CAT activity in tissue culture cells transiently transfected with MG enhancer constructs

Fragment[a]	Position[b]	Sp act[c] in indicated cells	
		NMuMG	Hepa
MMTV 180-bp fragment	Upstream	612	8.2
MMTV 102-bp fragment	Upstream	635	4.6
MMTV 102-bp fragment	Downstream	707	8.2
No enhancer		189	5
SV40 enhancer		440	249

[a] The 180-bp fragment is from bp −1166 to −987 and the 102-bp fragment is from bp −1166 to −1065, relative to the start of transcription.

[b] Upstream means 5′ of the SV40 promoter sequences and downstream means 3′ of the SV40 polyadenylation signal, in the same transcriptional orientation as MMTV. Similar results were obtained with the fragments in the opposite transcriptional orientation.

[c] Tissue culture cells were transfected by calcium phosphate precipitation of DNA and assayed for CAT activity as previously described (16). Similar results were obtained in three independent experiments; shown are the representative results from one experiment. Specific activity is given as counts of acetylated chloramphenicol per minute/milligrams of protein assayed/minutes of reaction time.

enhancer in tissue culture cells. Our 102-bp region includes approximately 10 to 15 bp of overlap with enhancers identified recently by others by transient transfection of MMTV LTR sequences linked to marker genes into different cultured mammary gland cell lines (6, 22).

To determine whether these sequences were also functional in mice, the 180-bp construct was used to generate transgenic mice. CAT assays using tissues from transgenic mice from two independently derived 180 strains, 14 and 17, showed that expression of the transgene was directed primarily to mammary and salivary gland tissue (Table 2); other tissues such as spleen, thymus, lung, kidney, and liver tissue showed only low levels of expression. CAT activity was also seen in the brain of these animals (not shown) and was most likely due to the SV40 promoter, which has been shown to function in this tissue (14). Therefore, the 180-bp fragment acts as a strong enhancer of transcription primarily in mammary gland and salivary gland tissue in transgenic mice. Moreover, this enhancer functions in the absence of the MMTV promoter and GREs to confer tissue-specific expression to the heterologous SV40 promoter.

During lactation, the levels of circulating glucocorticoids

TABLE 2. Expression of the 180 construct in tissues from transgenic mice

Tissue	Sp act[a]	
	Strain 14	Strain 17
Lactating mammary gland	25.1	17.2
Virgin mammary gland	23.8	15.1
Salivary gland	9.0	16.6
Spleen	0.8	3.3
Thymus	0.0	2.0
Lung	0.0	2.2
Kidney	0.0	1.4
Liver	0.0	0.0
Heart	0.0	ND

[a] Specific activity is presented as counts of acetylated chloramphenicol per minute/milligrams of protein assayed/minutes of reaction time. Data are averages for three animals, with the exception of lactating mammary gland and thymus data for strain 17, which represent one animal. ND, not determined.

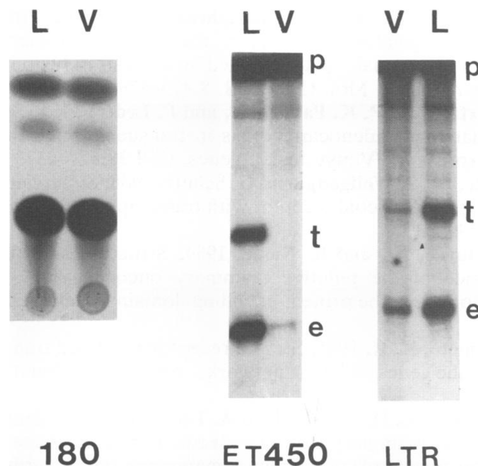

FIG. 3. Expression in lactating and virgin mammary glands of 180, ET450, and LTR-Tag mice. RNase T_1 protection analysis as described in the legend to Fig. 2 was carried out for the ET450 and LTR-Tag transgenic mice (LTR), while CAT assays were carried out for the 180 mice. Abbreviations: L, lactating; V, virgin; p, probe; t, transgene; e, endogenous.

and progesterone are very high, concurrent with a large increase in MMTV transcripts. It was not known whether the increase in MMTV RNA is due exclusively to transcriptional activation of the LTR by binding of hormone-receptor complexes to the GREs or whether there are also indirect effects on MMTV expression. For example, stimulation of MMTV could also be the result of increases in the activity of transcription factors responsive to circulating hormone levels. It was therefore of interest to determine whether lactation would have an effect on the 180 mice which contain a transgene that includes only the mammary gland/salivary gland enhancer (MG/SGE). We used three different types of transgenic mice to study the effect of lactation upon MMTV gene expression: (i) LTR-Tag mice, which contain the whole MMTV LTR linked to the SV40 T-antigen (Tag) reporter gene; (ii) ET450 mice, which are identical to LTR-Tag mice except that the transgene contains a deletion of all 5′ LTR sequences upstream of bp −364, including the MG/SGE; and (iii) 180 mice, which contain only the MG/SGE upstream of the SV40 promoter. A comparison by RNase T_1 protection analysis of the transgene expression levels in lactating and virgin mammary glands shows that in both LTR-Tag and ET450 mice, the transgene level mimics the level of endogenous viral RNA; that is, in both cases the endogenous RNA and transgene levels increase during lactation (Fig. 3). A similar increase was seen in response to lactation in other tissues (not shown). We had also previously shown that p1BCAT transgenic mice, which contain both the GREs and the mammary gland enhancer, express much larger amounts of the transgene in lactating animals (15, 16). In contrast, 180 mice show no change in CAT enzyme expression levels in mammary gland tissue during lactation (Fig. 3 [compare lanes V and L] and Table 2). Therefore, the hormonal stimulation of MMTV expression seen during lactation must act primarily through the GREs, and the MG/SGE and GREs can apparently function as independent regulatory elements in vivo.

Thus, the MMTV LTR encodes a mammary gland enhancer in the region between bp −1166 and −987 that confers tissue-specific expression in transgenic mice. Moreover, this enhancer functions in both lactating and nonlactating mammary glands. This is significant for several reasons. MMTV usually integrates either upstream in the opposite transcriptional orientation from or downstream in the same orientation as the *int* genes that it activates (12, 13, 20). Such integrations would result in the placement of the MG/SGE close to the *int* gene, which may allow this enhancer element to be the effector of *int* gene activation by MMTV. Interestingly, *int* gene expression in tumors is not regulated by glucocorticoid hormones (12); it is possible that the MG/SGE, not the GREs, provides the activating activity. Because the MG/SGE functions in both lactating and nonlactating mammary glands, *int* gene expression in this tissue could be elevated even after parturition, which would increase the probability that the secondary events necessary for full-blown transformation will occur.

For integration near cellular oncogenes to occur, large amounts of MMTV virions must be produced in the mammary gland. Our results indicate that the increase in virus production that occurs during lactation is the result of hormonal stimulation. Thus, the MG/SGE probably causes the turn-on of MMTV expression during mammary gland development and hormonal stimulation during pregnancy and lactation results in a huge amplification of viral gene expression. This hormonally stimulated increase in MMTV not only increases the efficiency of mammary gland transformation but also results in large amounts of viral particles in the milk, which increases the likelihood that the subsequent generation will acquire the virus.

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