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Negative Regulation in Correct Tissue-Specific Expression of Mouse Mammary Tumor Virus in Transgenic Mice

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Mouse mammary tumor virus (MMTV) is an endogenous murine retrovirus that is expressed in the epithelial cells of the mammary and salivary glands, lungs, kidneys, and seminal vesicles and in the lymphoid cells of the spleen and thymus. Several studies have shown that the long terminal repeat (LTR) of this virus can direct the expression of reporter genes to the same tissues in transgenic mice. To determine whether multiple regulatory elements within the LTR are involved in this tissue-specific expression, we have established lines of transgenic mice containing transgenes that have deletions in the MMTV LTR. Deletions of all LTR sequences upstream of −364 or of LTR sequences from −165 to −665 both result in the expression of linked reporter genes such as the simian virus 40 early region or the bacterial enzyme chloramphenicol acetyltransferase in novel sites, such as the heart, brain, and skeletal muscle; expression of endogenous MMTV and transgenes containing the full-length LTR is not detected in these organs. Negative regulation appears to involve more than one region, since deletion of sequences between either −201 and −471 or −201 and −344, as well as sequences upstream of −364, results in inappropriate expression in heart, brain, and skeletal muscle. Therefore, a negative regulatory element(s) in the MMTV LTR can suppress transcription from the viral promoter in several different organs. This represents the first example of generalized negative regulatory elements that act in many different tissues in transgenic mice to prevent inappropriate expression of a gene.

Tissue-specific expression of genes is achieved, at least in part, by the presence of specific sequences termed enhancer elements, usually located upstream of transcription initiation sites (39). Some of these elements represent binding sites for ubiquitous *trans*-acting factors which increase the expression of genes containing these binding sites in a number of different cell types, whereas other elements are apparently recognized by factors found only in specific cell types. It is these latter factors that contribute, at least in part, to the tissue-specific expression of genes.

Negative regulation also has been reported to influence gene expression in specific cell types (24, 45). Some, if not all, genes contain both positive enhancer elements and negative regulatory elements (NREs), which may act as binding sites for factors which inhibit gene expression, possibly by interfering with the binding of positively acting factors (1, 2, 38). In addition, a number of retroviral long terminal repeats (LTRs) have been shown to encode NREs which suppress transcription (5, 14, 37).

The endogenous murine retrovirus mouse mammary tumor virus (MMTV) is expressed in several tissues (18). When transgenic mice were made by using reporter genes under the transcriptional control of the MMTV LTR, the transgenes were expressed in the same tissues as was the endogenous virus, namely, the epithelial cells of the mammary and salivary glands, lungs, kidneys, and seminal vesicles and the lymphoid cells of the spleen and thymus (9, 26, 41, 42). The MMTV promoter is under the transcriptional control of an enhancer sequence, termed a glucocorticoid response element (GRE), that mediates positive transcriptional regulation by hormone-bound receptor complexes in a number of different cell types (13, 47). It is unlikely, however, that the tissue-specific expression of MMTV, or of any other glucocorticoid-regulated gene, is solely the result of transcriptional regulation by the receptor-hormone complex, since virtually every cell type contains these receptors (17). Thus, genes which are regulated by glucocorticoid hormones and yet are expressed in specific cell types must contain additional regulatory elements which supersede control by the GRE.

To define regulatory elements in the MMTV LTR in addition to the GRE which might affect tissue-specific expression, we have established transgenic mice containing various deletions in the MMTV LTR that are linked to several reporter genes. We report here that the tissue-specific expression of this virus is achieved in part by negative regulation, in which DNA sequences mapping upstream from the GRE apparently suppress transcription from the MMTV promoter in tissues in which the virus is not normally expressed.

MATERIALS AND METHODS

Pronuclear injections. DNA injections into single-cell zygotes and implantation into pseudopregnant foster mothers were performed as previously described (9). Swiss Webster mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. DNA fragments for microinjection were isolated from plasmid sequences on low-melting-point agarose gels (International Biotechnologies, Inc., New Haven, Conn.) by using ELUTIP-d columns (Schleicher & Schuell, Inc., Keene, N.H.) or BND-cellulose columns (Accurate Chemical and Scientific Corp., Westbury, N.Y.).

CAT assays. Tissue extracts were prepared by homogenization and sonication. The extracts were centrifuged, and the supernatants were used for chloramphenicol acetyltransferase (CAT) assays. Protein concentrations were deter-

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mined by the colorimetric method (Bio-Rad, Richmond, Calif.), and equal amounts of protein from each sample were used in the CAT assays (29). After thin-layer chromatography, both the acetylated and unconverted chloramphenicol spots were cut out and counted, and specific activities were determined (counts per minute of chloramphenicol acetylated per milligram of protein per minute of reaction time).

RNA analysis. RNA was extracted by guanidine thiocyanate extraction and CsCl gradient centrifugation (7). For Northern (RNA) blot analysis, equal amounts of RNA were subjected to electrophoresis on 1% formaldehyde gels (27), transferred to nitrocellulose filters, and hybridized with labeled probe. The mouse rRNAs served as molecular weight markers. The glyceraldehyde-3-phosphate dehydrogenase (GAPD/H) probe used in Northern blot analysis was isolated from the plasmid GAPD/H (15).

For RNase T_1 mapping (30), labeled RNA probe was synthesized from the vector templates by using [α-^{32}P]UTP and T7 or Sp6 RNA polymerase as specified by the supplier (Promega Biotec, Madison, Wis.). An excess of labeled probe (about 6×10^5 cpm per reaction) was hybridized with 100 μg of total RNA. Hybridizations were performed overnight at 37°C for the CAT probe and at 50°C for the LTR-Tag probe in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M NaCl, 80% formamide]. Approximately 420 U of RNase T_1 (Bethesda Research Laboratories, Gaithersburg, Md.) was added to each reaction in a buffer consisting of 0.01 M Tris (pH 7.6), 0.005 M EDTA, and 0.3 M NaCl before incubation for 1 h at 37°C. The protected fragments were analyzed on 5% sequencing gels with end-labeled *Hpa*II-digested pBR322 as a molecular weight standard.

RESULTS

Generation of transgenic mice. The MMTV LTR-reporter gene hybrids used to generate the transgenic mouse strains for this study are shown in Fig. 1A. LTag contains a full-length C3H MMTV LTR from the *Ava*I site at −1175 (28 bp from the 5' end of the LTR) to the *Hpa*I site at +101 linked to the simian virus 40 (SV40) early-region genes (8, 9), while LC1-CAT contains the same LTR upstream of CAT (35). Both of these transgenes previously have been shown to direct glucocorticoid-inducible expression of reporter genes from the MMTV LTR (8, 9, 35). Four deletions within the MMTV LTR were also studied. ET450 contains 450 bp (−364 to +101) of the LTR from LTag linked to the SV40 early region, and transgenes pA471 and pA344 are identical to LC1-CAT, except that they have deletions from −201 to −471 and −201 to −344, respectively (C.-L. L. Hsu, C. Fabritius, and J. P. Dudley, submitted for publication). The p1BCAT transgene contains a natural deletion variant of the MMTV LTR (−165 to −655) obtained from a C57BL/6 T-cell lymphoma (19); outside the deletion in the p1B LTR, the p1B sequences are >95% homologous to the C3H LTR. The p1B LTR deletion variant (cloned from the *Pst*I site at −1185 to the *Bst*EII site at +105) inserted upstream of the CAT gene was shown to have elevated basal levels of LTR-directed transcription in transient-transfection assays (19). All injected DNAs lacked plasmid sequences (see Materials and Methods).

Transgenic mice were identified by Southern blot analysis (results not shown). We have studied the expression of LTag transgenes in more than 20 independently derived transgenic strains (8, 9; Y. Choi, Ph.D. thesis, University of Illinois, Chicago, 1988), ET450 in 3 different strains, and pA471, pA344, and p1BCAT in 2 different strains of mice each. These mice had 1 to 10 intact copies of the transgene that were stably inherited through the germ line in a Mandelian fashion, as determined by restriction enzyme digestion and Southern blot analyses of the various pedigrees derived from each founder animal (results not shown).

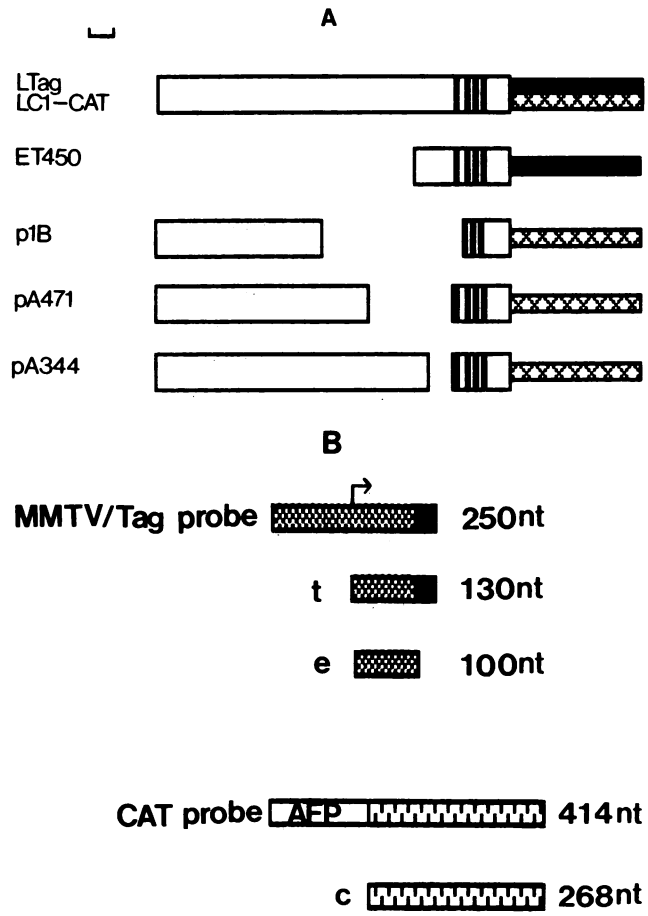

FIG. 1. Diagram of the LTRs of the LTag, ET450, p1B, pA471, and pA344 transgenes (panel A) and the probes used for RNase T_1 protection studies (panel B). (A) Transgene LTag was described by Choi et al. (9). Transgene ET450 was derived from LTag by deletion of the sequences upstream of the *Sau*3A site at −364, relative to the start of MMTV transcription. The LTR in transgene p1BCAT was cloned from a naturally occurring MMTV deletion mutant; the internal deletion is from −165 to −655. pA471 and pA344 were constructed as described previously (Hsu et al., submitted) and contain deletions from −201 to −471 and from −201 to −344, respectively. Symbols: ■, SV40 sequences; ▩, CAT sequences; ▮, GREs in the LTR. (B) RNA probes were generated from a Gemini vector (Promega Biotec, Inc.) containing the MMTV/SV40 junction fragment, as previously described (8). The CAT probe was derived from the same vector containing the α-fetoprotein transcription start site linked to the CAT gene (subcloned from AFP7CAT [36]; A. Siebold, Ph.D. thesis, University of Illinois, Chicago, 1990). The CAT-specific RNA isolated from the tissues of p1BCAT mice protects a 268-nt fragment of probes generated from this plasmid. Abbreviations: t, transgene; e, endogenous; c, CAT RNA-protected fragment.

Expression of the constructs lacking the 5' end of the MMTV LTR. Transgenic mice containing LTag sequences have been shown to express the SV40 early-region RNA and protein in the epithelial cells of mammary and salivary glands, lungs, kidneys, and prostate, the Leydig cells of the

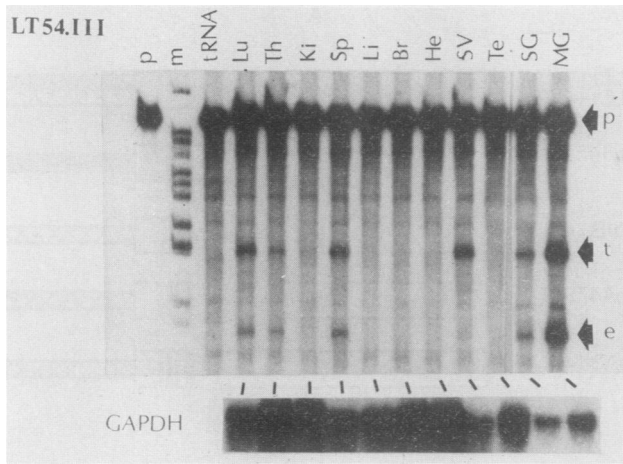

FIG. 2. RNase T_1 protection assay with RNA isolated from several tissues of a representative LTag transgenic mouse. Total RNA (50 μg) from the lungs (Lu), thymus (Th), kidneys (Ki), spleen (Sp), liver (Li), brain (Br), heart (He), seminal vesicle (SV), testes (Te), salivary gland (SG), and mammary gland (MG) of mouse LT54.III was used with the MMTV-TAG probe (Fig. 1B). The bottom of the figure, marked GAPDH, shows a Northern blot analysis of 10 μg of the same RNA after hybridization with the GAPD/H probe. Lane p, probe; lane m, molecular weight marker. Abbreviations: p, probe; t, transgene; e, endogenous.

testes, and the lymphoid cells of the spleen and thymus (9, 26, 41, 42). To determine whether deletions of the 5′ end of the LTR would affect the tissue distribution of expression, RNA was isolated from various organs of ET450 transgenic mice and used in an RNase T_1 protection assay. A uniformly labeled RNA hybridization probe that spanned the junction of the LTR-SV40 early region (Fig. 1B) was hybridized to tissue RNA, digested with RNase T_1, and subjected to electrophoresis on denaturing polyacrylamide gels. Endogenous MMTV RNA protects only the MMTV sequences in the probe (100 nucleotides [nt]) (e in Fig. 1B) whereas the transgene transcript protects both MMTV and SV40 sequences (130 nt) (t in Fig. 1B). When RNA from mice carrying the LTag transgene (containing the full-length LTR) was used (Fig. 2, strain LT54.III), the pattern of transgene expression mimicked that of the endogenous virus; both protected bands were detected in the lungs, thymus, kidneys, spleen, seminal vesicles, salivary gland, and mammary gland. Longer exposures of this autoradiogram (not shown) also revealed MMTV LTR-directed transcription in the testis, but no expression of either the transgene or the endogenous virus was seen in the heart, brain (Fig. 2) or skeletal muscle (not shown). This difference in expression was not due to the quality of the RNA obtained, since Northern blots with the same RNA preparations revealed intact GAPD/H mRNA in each tissue. In addition, a transgenic mouse containing the MMTV LTR linked to the bacterial enzyme CAT gene exhibited the same pattern of expression (Table 1). More than 20 independently derived transgenic strains containing the entire MMTV LTR gave identical patterns of tissue-specific expression (8, 9; Choi, Ph.D. thesis).

Similar RNase T_1 protection analysis was performed with RNA isolated from the organs of the ET450 mouse strains, lacking the MMTV LTR sequences upstream of −364. Transgene and endogenous MMTV expression were again detectable in the lungs, thymus, kidneys, spleen, seminal vesicles, testes, salivary gland, and mammary gland of one

TABLE 1. CAT activity in lactating LC1-CAT transgenic mouse

Tissue	CAT activity[a]
Mammary gland	1,912
Salivary gland	101
Thymus	38
Lung	416
Spleen	251
Kidney	0
Brain	0
Heart	0
Skeletal muscle	340[b]
Liver	0

[a] CAT activity is expressed in counts per minute per milligram of protein.
[b] Skeletal muscle was contaminated with lactating mammary gland.

such strain, ET2.2 (Fig. 3A). In addition, however, transgene expression was detected in heart, brain, and skeletal muscle of these animals, despite the absence of detectable endogenous MMTV expression. This result was not specific for the ET2.2 strain, since RNA from two other independently obtained ET450 transgenic strains gave similar results (Fig. 3B and C). Thus, deletion of the MMTV LTR sequences between −1200 and −364 resulted in expression of the transgene in heart, brain, and skeletal muscle but not in liver. These results suggest the presence of an NRE upstream of −364 in the MMTV LTR.

Constructs containing an internal MMTV LTR deletion. To further delineate the LTR sequences responsible for LTR-directed transcription in certain tissues, we used a naturally occurring LTR deletion (−165 to −655) linked to CAT for construction of transgenic mice. Two strains of transgenic mice obtained with this deleted LTR-CAT transgene (p1BCAT) were analyzed for the tissue-specific distribution of CAT expression. CAT enzymatic activity in the founders and offspring of both the p1BCAT 1 and p1BCAT 5 strains was detected in mammary and salivary glands, lungs, kidneys, thymus, spleen, and seminal vesicles (Table 2), a pattern similar to that seen for the expression of endogenous MMTV and for transgenic mice containing the entire LTR (Fig. 2 and Table 1) (18). However, unlike endogenous MMTV expression or expression in LTag or LC1-CAT mice, CAT activity from the deleted LTR transgene was detectable in heart, skeletal muscle, and brain of both transgenic strains. This pattern of expression was identical to that seen for the ET450 transgenic mice (Fig. 3) and localized at least one NRE between −364 and −655 in the MMTV LTR. In general, CAT activity in all tissues was higher in lactating females than in virgin females and males (see next section). In addition, we also occasionally detected very low levels of CAT activity in the livers of lactating females, but did not detect expression in the livers of males or virgin females.

To more finely map the sequences within the MMTV LTR conferring negative regulation, we also made transgenic mice containing two deletions between −201 and −471. Transgene pA471 contains a deletion encompassing the entire region between −201 and −471, while pA344 contains a deletion between −201 and −344. When CAT activity was examined in extracts prepared from the tissues of mice containing either of these transgenes, a pattern of expression similar to that seen in the p1BCAT transgenic strain was seen; that is, the high levels of expression were seen in mammary and salivary glands, lungs, kidneys, thymus, spleen, and seminal vesicles and was detectable in heart, skeletal muscle, and brain of both the pA471 and pA344 transgenic strains, as is shown for one strain of each in Table

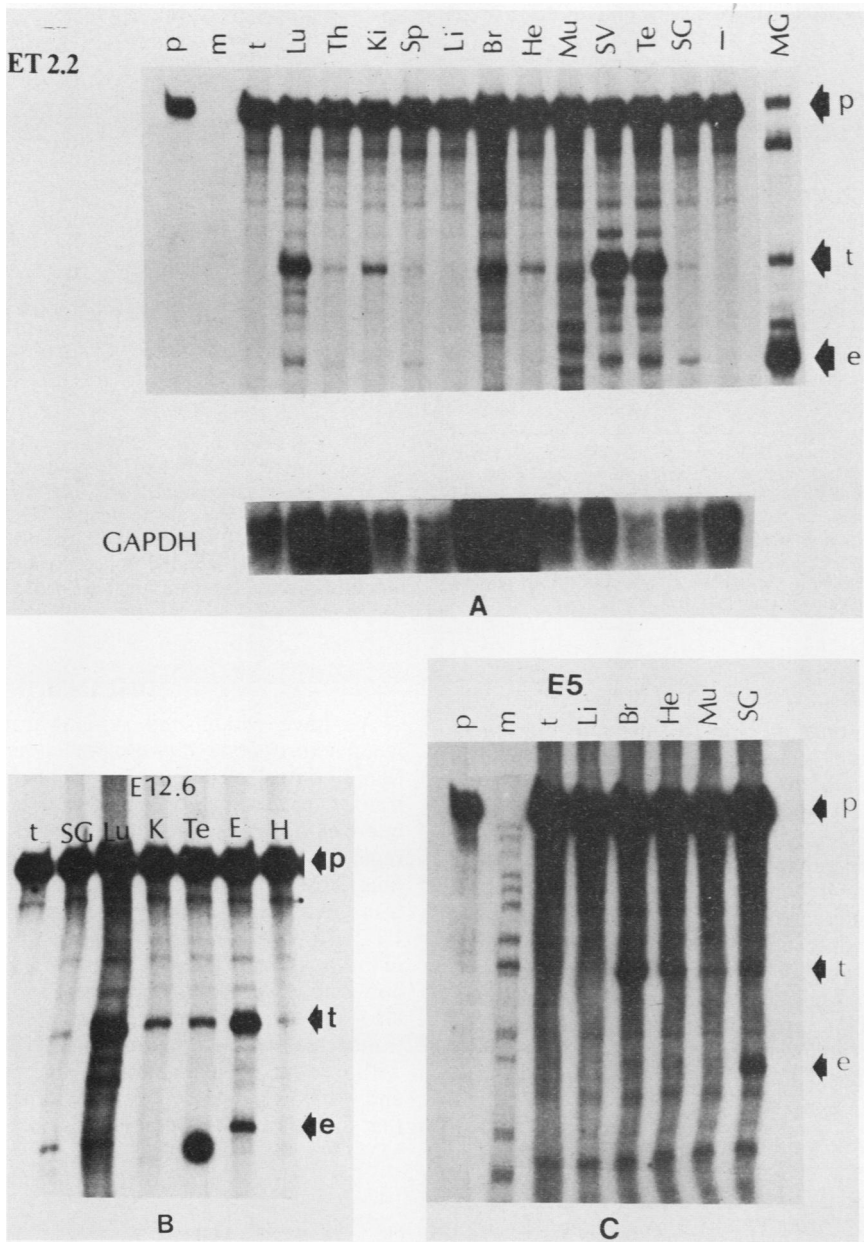

FIG. 3. RNase T_1 protection assay with RNA isolated from three different ET450 transgenic mouse strains. (A) ET2.2 tissues; (B) E12.6 tissues; (C) E5 tissues. For abbreviations; see the legend to Fig. 2.

3. Similar results were obtained with the other strains. This result implies that sequences between −344 and −201, in addition to sequences upstream of −364, play a role in the negative regulation of MMTV expression in heart, brain, and skeletal muscle.

We also performed RNase T_1 protection assays with RNA isolated from the tissues of p1BCAT mice (Fig. 4). Total mammary-gland RNA was shown to protect a 268-nt fragment when hybridized to a CAT-specific riboprobe (Fig. 1B and 4A). We did not determine the transcription initiation site for the CAT-specific RNA isolated from these tissues, but RNA extracted from mink lung cells transfected with the p1BCAT construct initiated from the MMTV U3/R junction (19). Longer exposures of the autoradiogram in Fig. 4 showed a faint 268-nt fragment protected by total thymus RNA (results not shown). Moreover, when we used poly(A)$^+$ RNA from the salivary gland, spleen, and brain of p1BCAT transgenic mice in an RNase protection assay, we also could detect a 268-nt CAT-specific fragment (Fig. 4B). The CAT assays were more sensitive than the RNase protection experiments with the p1BCAT transgenic animals because of the instability of the CAT mRNA (24; our unpublished results), and CAT activity was consistent with the CAT RNA levels observed in tissues (i.e., mammary gland CAT-specific RNA and CAT activity levels are >50-fold higher than in any another tissue). Thus, CAT expression was regulated most probably at the RNA level.

Hormone regulation of the deleted LTR constructs. To demonstrate the hormone regulation of CAT expression in p1BCAT transgenic mice, we treated virgin females with 100 μg of dexamethasone in corn oil for 7 days and then prepared tissue extracts for CAT assays. Virgin females of the same

TABLE 2. CAT activity in two strains of p1BCAT transgenic mice

Tissue	CAT activity[a] in:			
	Strain 1		Strain 5	
	Male	Female[b]	Male	Female[b]
Mammary gland	39	>64,000[c]	33	>47,000[c]
Salivary gland	114	1,470	152	638
Thymus	92	211	72	372
Lung	26	138	205	203
Spleen	7	53	8	189
Brain	8	36	6	43
Kidney	19	28	24	11
Heart	3	26	4	50
Skeletal muscle	3	1,443[d]	8	14,672[d]
Liver	0	1	0	ND[e]

[a] CAT activity is expressed in counts per minute per milligram of protein per minute of reaction time. Each number represents the average of two or three independent assays on different mice, except for skeletal muscle and liver assays, which were performed on one mouse each.

[b] The female tissues all were obtained from lactating mice (8 days after parturition).

[c] These assays were performed in the nonlinear portion of the assay (i.e., virtually all the chloramphenicol was acetylated).

[d] Skeletal muscle from lactating females was contaminated with mammary tissue.

[e] ND, Not determined.

age were injected with corn oil alone for the same period as a control. All expressing tissues, including lungs and thymus (Fig. 5), showed dexamethasone-inducible CAT activity. The level of hormone responsiveness was similar to that obtained with p1BCAT constructs in stable transfections of mink lung cells (19) and confirmed that the CAT activity was under the transcriptional control of the MMTV LTR. In addition, the relative increase in activity in hormone-treated animals was similar to that seen for the lactating female with respect to the male (Table 2) or virgin female (not shown); this implies that most, if not all, of the increase in transgene and endogenous MMTV expression detected in the tissues of lactating mice is due to an increase in the circulating levels of glucocorticoid hormones.

TABLE 3. CAT activity in pA471 and pA344 transgenic mice

Tissue	CAT activity[a] in:			
	pA471		pA344	
	Male	Female[b]	Male	Female[b]
Mammary gland	ND[c]	164	ND	2,457
Salivary gland	1	4	170	840
Thymus	5	5	444	2,686[d]
Lung	88	27	68	517
Spleen	1	7	72	1,312[d]
Brain	8	1	381	946
Kidney	1	11	5	12
Heart	12	1	50	22
Skeletal muscle	3	0.2	15	298

[a] CAT activity is expressed in counts per minute per milligram of protein per minute of reaction time. Each number represents the average of two or three independent assays on different mice.

[b] The female tissues were all obtained from lactating mice (8 days after partuition).

[c] ND, Not determined.

[d] The relatively high level of CAT activity in the lymphoid tissues of these animals appears to be unique to this strain; another pA344 transgenic strain showed expression in thymus and spleen at about 1/100 of the levels detected in lactating mammary gland, as was seen for all other transgenic strains (E. Mok and S. R. Ross, unpublished results).

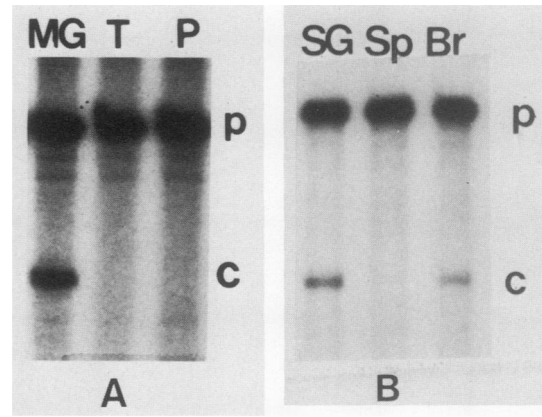

FIG. 4. RNase T_1 protection assay with RNA isolated from the tissues of p1BCAT 1 transgenic mouse tissues. Total RNA (50 μg from the mammary gland [MG] and thymus [T]) (A) or poly(A)$^+$ RNA (20 μg from the salivary gland [SG] and brain [Br], 10 μg from the spleen [Sp]) (B) was used with the AFP-CAT probe (Fig. 1B). A faint band can be detected in lane Sp upon longer exposure of the autoradiogram. Abbreviations: p, probe; c, CAT RNA-protected fragment.

DISCUSSION

We have established several transgenic mouse strains which express one of two reporter genes under the transcriptional control of deleted forms of the MMTV LTR. Three transgenic strains with constructs lacking MMTV LTR sequences upstream of −364 (ET450) expressed SV40 early-region RNA in mammary and salivary glands, testis, thymus, spleen, kidneys, lungs, and seminal vesicles, as did transgenic strains with transgenes containing the full-length LTR. However, ET450 transgene expression was detectable in brain, skeletal muscle, and heart, whereas full-length LTR constructs were not (compare Fig. 2 and 3). CAT activity also was observed in brain, skeletal muscle, and heart from transgenic strains containing MMTV LTR-CAT constructs with internal LTR deletions (−201 to −344, −201 to −471, and −655 to −165). Together, these results strongly argue that there is at least one NRE between −655 and −201 in the MMTV LTR.

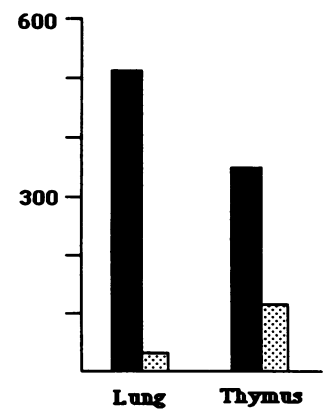

FIG. 5. Histograms of CAT activity in p1BCAT 1 mouse tissues. The *y* axis is CAT specific activity (counts per minute of acetylated chloramphenicol per milligram of protein per minute of reaction time). Extracts were made from dexamethasone-injected (■) and control (▨) virgin females.

NREs have been implicated in the transcriptional regulation of a number of eucaryotic genes, including MMTV (19, 35). Langer and Ostrowski identified a negative regulatory region located between −166 and −138 upstream of the MMTV transcription start site (25), which does not appear to be involved in the increased expression of the MMTV LTR in brain, heart, and skeletal muscle, since it is intact in all the transgenes described here. Morley et al. (35) noted that the deletion of MMTV LTR sequences between −455 and −364 elevated basal-level CAT expression from LTR-CAT constructs in transient assays in mouse L cells (35). Like transcriptional enhancers, the action of these NRE sequences was relatively orientation independent with respect to the MMTV promoter. Our results are consistent with the existence of an NRE in this region; however, we also found that deletion of sequences from −201 to −344 resulted in more promiscuous expression than was detected in transgenic mice containing the complete LTR.

We have also identified an NRE between −201 and −344 in a tissue culture cell assay; both naturally occurring (−665 to −165) and laboratory-constructed (−637 to −255) MMTV LTR deletions promoted high basal levels of CAT activity in transient transfections of mink lung cells (19; Hsu et al., submitted), and MMTV proviruses harboring such deletions appear to be preferentially transcribed in T-cell tumors (12, 19). Since all of the sequences deleted from the pA344 transgene are contained in the ET450 transgene, the region(s) conferring negative regulation must either (i) contain more than one element which is necessary to suppress expression in the inappropriate tissues, (ii) include a single element which spans the two deletions, or (iii) be subject to spacing effects which alter the function of a single element. We favor the first explanation since different gel retardation patterns are obtained by using labeled DNA fragments from each region (Hsu et al., submitted).

The majority of MMTV proviruses acquired in T-cell lymphomas have similar LTR deletions which encompass the upstream NRE(s) (11, 19, 23, 31, 32), and these deletions confer increased levels of LTR-directed transcription in the absence of glucocorticoid hormones (19). It has been suggested that these deletions might result in an MMTV LTR that allows preferential viral expression in T cells (3, 44). We show in this study that an LTR isolated from a T-lymphoma-acquired MMTV is not T-cell specific, since p1BCAT mice still show the same pattern of expression in tissues as the endogenous virus or transgenic mice containing the full-length LTR. Moreover, the T-cell-derived LTR is still hormone responsive in the animal. This implies that other elements in the MMTV provirus or additional events involving virus replication in T lymphomas affect the acquisition of additional copies of MMTV in these cells.

Using MMTV LTR-human growth hormone (HGH) constructs in transgenic mice, Stewart et al. reported that deletion of LTR sequences upstream of −741 profoundly lowered HGH expression in all tissues, including spleen, kidney, salivary gland, testis, and lung tissue (41). Curiously, transgenic mice containing full-length LTR constructs had high HGH expression in brain, a tissue in which endogenous MMTV transcription is not detectable (18) (Fig. 2 and 3); mammary gland expression of HGH was not monitored. HGH expression was also regulated by glucocorticoids and was expressed at the highest levels in salivary glands of both male and female mice. This apparent discrepancy with our results may be due to the influence of an enhancer found in intron 1 of the HGH gene (34, 40) on the MMTV promoter; the HGH gene has been shown to alter the pattern of expression of other tissue-specific promoters in transgenic mice (43). We have monitored LTR-regulated expression with two different reporter genes whose tissue-specific transcription is very similar to that of the endogenous MMTV RNA and have not seen such reporter gene effects.

Cell-type-specific expression due to the influence of both positive and negative regulators of transcription has been observed from a number of promoters (10, 16, 28, 33, 45). Many enhancer elements have been reported to be cell type specific, and much evidence suggests that the mechanism of enhancer action depends upon the binding of protein factors to these elements. Therefore, tissue-specific expression may rely on the presence of these factors in certain cell types. Alternatively, it could result from the absence of certain proteins which inhibit gene transcription in certain cells. Consistent with this hypothesis, immunoglobulin gene expression can be suppressed in B cells by fusing them with nonlymphoid cells (20) or T cells (48). Moreover, expression of a transfected IgH gene can be activated in nonlymphoid cells by treatment with protein synthesis inhibitors (21), and certain deletions within the immunoglobulin heavy chain (IgH) enhancer can activate expression of a reporter gene in CV1 monkey epithelial cells (22) and mouse 3T3 cells (46). Binding of negative regulatory proteins in nonlymphoid cells may obstruct the action of positively acting proteins present in all cells. In other cases, such as the α-fetoprotein gene, cell-type-specific proteins may bind to enhancer and promoter elements and to a negative regulatory region which is distinct from the enhancer (4, 36). All of our constructs, which contain deletions from −165 to −655 and the other deleting sequences upstream of −354, elevate transcription in brain, heart, and skeletal muscle. Thus, in contrast to the α-fetoprotein gene, for which the element(s) suppressing adult gene expression functions only in the liver (4), factors which bind to the upstream NRE in the MMTV LTR must be present in a large number of tissues, an observation supported by both transfection and gel retardation experiments (19, 35; Hsu et al., submitted).

Although many other groups have defined NREs that suppress gene transcription in a number of different cell types in tissue culture, our study represents the first example of an element that functions in the animal. It is now well established that positive regulatory elements are involved in the control of tissue-specific gene expression, but the role of NREs in this process is less clear. Tissue-specific extinguishers, identified by cell fusion and chromosome transfection studies, may be involved in the suppression of liver gene expression outside the liver, but the specific DNA sequences through which tissue-specific extinguishers act have not been defined (6). The NRE(s) defined in our study may represent such sequences, since it appears to act dominantly in certain tissues, such as the heart, brain, and skeletal muscle, to prevent the activation of MMTV transcription by the glucocorticoid hormone receptor and other transcription factors. Because quantitative comparisons of the absolute levels of gene expression among different transgenic strains is not possible, we cannot determine whether the NRE functions in tissues such as mammary and salivary glands, lungs, spleen, thymus, etc. (i.e., whether the levels of transgene expression in these organs are higher in mice containing LTR deletions than in mice containing full-length LTRs). However, in these tissues, positive transcriptional regulatory elements, including the GRE, are dominant over the NRE(s).

Finally, the transgenic mice with the deleted LTRs all express the transgene in the same tissues as do the trans-

genic mice with a full-length LTR. This argues that sequences responsible for expression in mammary and salivary glands, thymus, spleen, lungs, and seminal vesicle are present downstream of −165. Thus, there are at least two types of elements in the MMTV LTR which contribute to cell-type-specific expression, one type which appears to negatively regulate the MMTV promoter and another, presumably positive type, which may be the GRE or the MMTV promoter itself.

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LITERATURE CITED

1. **Adler, S., M. L. Waterman, X. He, and M. G. Rosenfeld.** 1988. Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain. Cell **52**:685–695.
2. **Akerbloom, I. E., E. P. Slater, M. Beato, J. D. Baxter, and P. L. Mellon.** 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science **241**: 350–353.
3. **Ball, J. K., H. Diggelmann, G. A. Dekaban, G. F. Grossi, R. Semmler, P. A. Waight, and R. F. Fletcher.** 1988. Alterations in the U3 region of the long terminal repeat of an infectious thymotropic type B retrovirus. J. Virol. **62**:2985–2993.
4. **Camper, S. A., and S. M. Tilghman.** 1989. Postnatal repression of the α-fetoprotein gene is enhancer independent. Genes Dev. **3**:537–546.
5. **Ch'Ang, L.-Y., W. K. Yang, F. E. Myer, and D.-M. Yang.** 1989. Negative regulatory element associated with potentially functional promoter and enhancer elements in the long terminal repeats of endogenous murine leukemia virus-related proviral sequences. J. Virol. **63**:2746–2757.
6. **Chin, A. C., and R. E. K. Fournier.** 1987. A genetic analysis of extinction: trans-regulation of 16 liver-specific genes in hepatoma-fibroblast hybrid cells. Proc. Natl. Acad. Sci. USA **84**:1614–1618.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
8. **Choi, Y., I. Lee, and S. R. Ross.** 1988. Requirement for the Simian virus 40 small tumor antigen in tumorigenesis in transgenic mice. Mol. Cell. Biol. **8**:3382–3390.
9. **Choi, Y. C., D. H. Henrard, I. Lee, and S. R. Ross.** 1987. The mouse mammary tumor virus long terminal repeat directs expression in epithelial and lymphoid cells of different tissues in transgenic mice. J. Virol. **61**:3013–3019.
10. **Colantuoni, V., A. Pirozzi, C. Blance, and R. Cortese.** 1987. Negative control of liver-specific gene expression: cloned human retinol-binding protein gene is repressed in HeLa cells. EMBO J. **6**:631–636.
11. **Dudley, J., and R. Risser.** 1984. Amplification and novel locations of endogenous mouse mammary tumor virus genomes in mouse T-cell lymphomas. J. Virol. **49**:92–101.
12. **Dudley, J. P., A. Arfsten, C.-L. L. Hus, C. Kozak, and R. Risser.** 1986. Molecular cloning and characterization of mouse mammary tumor proviruses from a T-cell lymphoma. J. Virol. **57**:385–388.
13. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor super-family. Science **240**:889–895.
14. **Flanagan, J. R., A. M. Krieg, E. E. Max, and A. S. Khan.** 1989. Negative control region at the 5′ end of murine leukemia virus long terminal repeats. Mol. Cell. Biol. **9**:739–746.
15. **Fort, P., P. H. L. Marty, M. Piechaczyk, S. E. Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the rat glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13**:1431–1442.
16. **Goodbourn, S., H. Burstein, and T. Maniatis.** 1986. The human β-interferon gene enhancer is under negative control. Cell **45**:601–610.
17. **Gustafsson, J.-A., J. Carstedt-Duke, L. Poellinger, S. Okret, A.-C. Wikstrom, M. Bronnegard, M. Gillner, Y. Dong, K. Fuxe, A. Cintra, A. Harfstrand, and L. Agnati.** 1987. Biochemistry, molecular biology, and physiology of the glucocorticoid receptor. Endocr. Rev. **8**:185–234.
18. **Henrard, D., and S. R. Ross.** 1988. Endogenous mouse mammary tumor virus is expressed in several organs in addition to the lactating mammary gland. J. Virol. **62**:3046–3049.
19. **Hsu, C.-L. L., C. Fabritius, and J. Dudley.** 1988. Mouse mammary tumor virus proviruses in T-cell lymphomas lack a negative regulatory element in the long terminal repeat. J. Virol. **62**:4644–4652.
20. **Imler, J.-L., C. Lemaire, C. Wasylyk, and B. Wasylyk.** 1987. Negative regulation contributes to tissue specificity of the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. **7**:2558–2567.
21. **Ishihara, T., A. Kudo, and T. Watanabe.** 1984. Induction of immunoglobulin gene expression in mouse fibroblasts by cycloheximide treatment. J. Exp. Med. **160**:1937–1942.
22. **Kadesch, T., P. Zervos, and D. Ruezinsky.** 1986. Functional analysis of the murine IgH enhancer: evidence for negative control of cell-type specificity. Nucleic Acids Res. **14**:8209–8221.
23. **Kwon, B. S., and S. M. Weissman.** 1984. Mouse mammary tumor virus-related sequences in mouse lymphocytes are inducible by 12-*O*-tetradecanoylphorbol-13-acetate. J. Virol. **52**:1000–1004.
24. **Laimins, L., M. Holmgren-Konig, and G. Khoury.** 1986. Transcriptional "silencer" element in rat repetitive sequences associated with the rat insulin 1 gene locus. Proc. Natl. Acad. Sci. USA **83**:3151–3155.
25. **Langer, S. L., and M. C. Ostrowski.** 1988. Negative regulation of transcription in vitro by a glucocorticoid response element is mediated by a *trans*-acting factor. Mol. Cell. Biol. **8**:3872–3881.
26. **Leder, A., P. K. Pattengale, A. Kuo, T. A. Stewart, and P. Leder.** 1986. Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development. Cell **45**:485–495.
27. **Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical re-examination. Biochemistry **16**:4743–4751.
28. **Levy, D. E., D. S. Kessler, R. Pine, N. Reich, and J. E. Darnell, Jr.** 1988. Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. Genes Dev. **2**:383–393.
29. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
30. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
31. **Michalides, R., E. Wagenaar, J. Hilkens, J. Hilgers, B. Groner, and N. E. Hynes.** 1982. Acquisition of proviral DNA of mouse mammary tumor virus in thymic leukemia cells from GR mice. J. Virol. **43**:819–829.
32. **Michalides, R., E. Wagenaar, and P. Weijers.** 1985. Rearrangements in the long terminal repeat of extra mouse mammary tumor proviruses in T-cell leukemias of mouse strain GR result in a novel enhancerlike structure. Mol. Cell. Biol. **5**:823–830.
33. **Miyazaki, J., E. Appela, and K. Ozato.** 1986. Negative regulation of the major histocompatibility class I gene in undifferentiated embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA

83:9537–9541.
34. **Moore, D. D., A. R. Marks, D. I. Buckley, G. Kapler, F. Payvar, and H. M. Goodman.** 1985. The first intron of the human growth hormone gene contains a binding site for glucocorticoid receptor. Proc. Natl. Acad. Sci. USA **82**:699–702.
35. **Morley, K. L., M. G. Toohey, and D. O. Peterson.** 1987. Transcriptional repression of a hormone-responsive promoter. Nucleic Acids Res. **15**:6973–6989.
36. **Muglia, L., and L. B. Rothman-Denes.** 1986. Cell type-specific negative regulatory element in the control region of the rat alphafetoprotein gene. Proc. Natl. Acad. Sci. USA **83**:7653–7657.
37. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813–823.
38. **Sakai, D. D., S. Helms, J. Carlstedt-Duke, J.-A. Gustafsson, F. M. Rottman, and K. R. Yamamoto.** 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2**:1144–1154.
39. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eukaryotic gene transcription. Trends Genet. **1**:224–230.
40. **Slater, E. P., O. Rabenau, M. Karin, J. D. Baxter, and M. Beato.** 1985. Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol. Cell. Biol. **5**:2984–2992.
41. **Stewart, T. A., P. G. Hollingshead, and S. L. Pitts.** 1988. Multiple regulatory domains in the mouse mammary tumor virus long terminal repeat revealed by analysis of fusion genes in transgenic mice. Mol. Cell. Biol. **8**:473–479.
42. **Stewart, T. A., P. K. Pattengale, and P. Leder.** 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell **38**:627–637.
43. **Swanson, L. W., D. M. Simmons, J. Arriza, R. Hammer, R. Brinster, M. G. Rosenfeld, and R. M. Evans.** 1985. Novel developmental specificity in the nervous system of transgenic animals expressing growth hormone fusion genes. Nature (London) **317**:363–366.
44. **Theunissen, H. J. M., M. Paardekooper, L. J. Maduro, R. J. A. M. Michalides, and R. Nusse.** 1989. Phorbol ester-inducible T-cell-specific expression of variant mouse mammary tumor virus long terminal repeats. J. Virol. **63**:3466–3471.
45. **Tripputi, P., S. L. Guerin, and D. D. Moore.** 1988. Two mechanisms for the extinction of gene expression in hybrid cells. Science **241**:1205–1207.
46. **Weinberger, J., D. Baltimore, and P. Sharp.** 1986. Distinct factors bind to apparently homologous sequences in the immunoglobulin heavy-chain enhancer. Nature (London) **322**:846–848.
47. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209–252.
48. **Zaller, D. M., H. Yu, and L. A. Eckhardt.** 1988. Genes in the presence of an immunoglobulin enhancer or promoter are negatively regulated by a T-lymphoma cell line. Mol. Cell. Biol. **8**:1932–1939.