Mouse Mammary Tumor Virus Provirus in T-Cell Lymphomas Lack a Negative Regulatory Element in the Long Terminal Repeat

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The nucleotide sequences of long terminal repeats (LTRs) from several mouse mammary tumor virus (MMTV) proviruses acquired in mouse T-cell lymphomas were determined. All MMTV proviruses cloned from a C57BL/6 lymphoma contained an identical LTR deletion of 491 base pairs (approximately -655 to -165), whereas an MMTV provirus from a BALB/c T-cell lymphoma had a 430-base-pair deletion in the same U₃ region. MMTV proviruses with LTR deletions were acquired in these tumors 10 times more frequently than proviruses with intact LTRs. Because the deletions removed a portion of the glucocorticoid response element or "regulated" enhancer, the transcriptional activity of the deleted MMTV LTRs was assessed in both transient expression and stable transfection experiments. Plasmids were constructed in which the deleted or full-length MMTV LTRs were placed upstream of the chloramphenicol acetyltransferase gene. Results from transfection experiments with these constructs showed that the basal expression of the deleted MMTV LTR in the absence of glucocorticoids was higher than that of the full-length *Mtv-17* or C3H MMTV LTRs under the same conditions. Moreover, the C3H LTR with a similar deletion (-637 to -255) also promoted high basal levels of chloramphenicol acetyltransferase activity. These results, coupled with the observation in lymphomas of high basal levels of transcription from MMTV proviruses with deleted LTRs, suggested that these proviruses lack negative regulatory elements in their LTRs. Loss of the negative regulatory element may contribute to the selective propagation of proviruses with deleted LTRs.

The mouse mammary tumor virus (MMTV) induces a high percentage of breast cancers in murine strains which carry exogenous virus in the milk (15). The mammary tumors which result contain one or more integrated MMTV proviruses (4), and these proviruses activate transcription of nearby cellular loci known as *int* genes (6, 12, 32). Because readthrough transcripts containing MMTV and *int* sequences are rare and because MMTV proviruses may be located either upstream or downstream of the *int* genes, transcriptional activation of these genes is thought to occur through enhancer sequences in the MMTV long terminal repeat (LTR) (6, 12, 32).

T-cell tumors arise in mouse strains which harbor exogenous milk-borne virus (GR and DBA/2) (22, 28) as well as in strains which lack exogenous virus (BALB/c and C57BL/6 [B6]) (8). Some T-cell lymphomas contain additional integrated MMTV proviruses (8, 22, 28) and differ from MMTV-induced mammary cancers in several respects. First, the tumors often fail to produce virions, although MMTV RNA and proteins may be detectable (8). Second, MMTV proviruses cloned from these tumors show extensive restriction enzyme polymorphisms in addition to deletions within the U₃ region of the LTR (8, 9, 20, 22, 29). These deletions remove the 3' end of the long open reading frame within the LTR and, in some cases, the 5' portion of the glucocorticoid response element (GRE) (20, 22, 29).

The GRE is responsible for increased MMTV RNA and protein production from MMTV-infected cells exposed to glucocorticoid hormones (38). The GRE is located within approximately 200 base pairs (bp) of the transcriptional start site and is composed of multiple binding sites for the hormone-activated receptor (38). *cis* linkage of the GRE to other eucaryotic promoters renders them hormone responsive (10, 16, 24). Because the hormone responsiveness

conferred by the GRE is relatively independent of distance from and orientation to the promoter, the GRE has been referred to as a "regulated" enhancer (2). Enhancers also may function in a tissue-specific manner, and it has been reported that the MMTV LTR is expressed in lymphoid cells and in a number of primary epithelial tissues including lungs, salivary and mammary glands, testes, and kidneys (3, 35).

We describe here the LTR alterations from acquired MMTV proviruses in a B6 and a BALB/c T-cell lymphoma. The majority of MMTV proviruses from both tumors had simple deletions in their LTRs which removed a similar U₃ region as detected by both Southern blotting and sequencing analysis. These results and the elevated transcriptional activity of the corresponding MMTV proviruses in the absence of glucocorticoids (9) suggested that acquired proviruses may have a transcription or replication advantage in T cells. To directly test the transcriptional activity of the deleted MMTV LTR, we constructed hybrids with the reporter gene chloramphenicol acetyltransferase (CAT). Transfection experiments showed that the deleted LTR retained glucocorticoid responsiveness. Moreover, basal expression from this LTR was higher than expression from the full-length C3H MMTV LTR, consistent with the loss of a negative regulatory element (30).

MATERIALS AND METHODS

Southern blotting, Northern (RNA) blotting, and hybridization analyses. Lymphoma lines used for Southern analysis and cloning were reported previously (8). Southern blotting and hybridization were performed as described by Dudley and Risser (8). Hybridization probes were obtained by nick translation as described previously (8) and had specific activities of 1×10^8 to 3×10^8 cpm/ μ g. RNA extractions and Northern blotting were performed as stated previously (7).

Molecular cloning. The Charon 4A clones, AA Cl 1 and AA Cl 2, were derived from the B6 lymphoma P3C (9)

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containing acquired MMTV proviruses. The EMBL3 clones, AA Cl 8 and AA Cl 13, were derived from the B6 lymphoma P4C containing acquired MMTV proviruses (7). The B6 lymphomas P4C and P3C appeared to be clonal derivatives of the EL4 tumor (J. Dudley, unpublished data). The λ S31 clone was derived by partial *Mbo*I digestion of S49 BALB/c lymphoma DNA and ligation to EMBL3 DNA as described previously (7). The λ S31 clone had a 12.8-kilobase-pair insert containing the 3' end of an MMTV provirus and adjacent cellular DNA sequences.

MMTV LTR constructs with the CAT gene were derived from two different vectors. The pUC-CAT vector (provided by N. Hopkins and E. Golemis, Massachusetts Institute of Technology) was derived by inserting a 2.4-kilobase CAT fragment from pSV0-CAT (13) into pUC13 (36). The p1B-CAT and p6B-CAT plasmids were constructed by inserting the 3' MMTV LTRs from AA Cl 1 and *Mtv-17*, respectively, into the vector pUC-CAT. The CAT vector used to study transient expression shortly after transfection (pMT2) was obtained from D. Peterson, Texas A&M University. This vector lacks pBR322 sequences which inhibit eucaryotic gene expression (33). The pLC1 plasmid (kindly provided by D. Peterson) contains the C3H exogenous MMTV LTR upstream of CAT in pMT2 (30). All constructions were used to transform *Escherichia coli* HB101 to ampicillin resistance according to standard methods (26).

Dideoxynucleotide sequencing. The method of Sanger was used for DNA sequencing analyses of plasmid subclones as described previously (7). Two 17-mer primers from nucleotides 501 to 517 and from 1082 to 1098 of the *Mtv-8* LTR (17) were used for sequencing the LTR deletions. Primers were synthesized by the Oligonucleotide Synthesis Facility at the University of Texas at Austin.

CAT assays. CAT assays were performed as described by Lopata et al. (23). Cells were disrupted by sonication or by three cycles of freezing and thawing and then were assayed for CAT activity as described in the figure legends.

Stable transfections and transient assays. A modification of the calcium phosphate precipitation technique was used to obtain stable transfectants (14). The day before transfection, 1.2×10^6 mink lung cells (CCL-64) were plated into 60-mm-diameter dishes in Dulbecco modified Eagle medium containing 10% fetal bovine serum (complete DMEM). At 3 h after replacing the medium, the DNA-calcium phosphate precipitate was added to each plate in a total volume of 1 ml. Each precipitate contained 10 μ g of test DNA–1 μ g of pSV2gpt (31)–0.5 ml of 0.25 M CaCl_2 –0.5 ml of $2\times$ HeBS (0.27 M NaCl, 10 mM KCl, 1.4 mM NaPO_4 [pH 7.1], 0.22% glucose, and 0.04 M HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid] [pH 7.1]). After 15 min at 37°C, 4 ml of complete DMEM was added and the cells were incubated for 7 h before glycerol shock (25% glycerol in DMEM for 1.5 min). After 24 h, the medium was replaced with complete DMEM containing 250 μ g of xanthine per ml and 25 μ g of mycophenolic acid per ml. After growth at 37°C, individual colonies were obtained with cloning cylinders. The number of integrated copies was estimated by Southern blotting of chromosomal DNA from each clone compared with dilutions of purified pUC-CAT DNA digested with *Pst*I or *Bgl*II.

Transient expression assays were performed on cells transfected by electroporation as described by Knutson and Yee (18).

RESULTS

Acquired MMTV proviruses from murine T-cell lymphomas have LTR deletions. Several acquired MMTV proviruses were cloned from the B6 T-cell tumor EL4 (7, 9). Restriction mapping and Southern hybridization analyses indicated that the LTRs from acquired MMTV proviruses were altered. This finding was confirmed by nucleotide sequencing analysis (Fig. 1). The 3' LTR from the MMTV provirus in AA Cl 1 (Cl 1 3' LTR) had a 491-bp deletion in the U_3 region compared with that in the C3H MMTV LTR. The deletion, located between a 4-bp repeat (AGGA) in the C3H LTR, removed the distal glucocorticoid receptor-binding site (1). Identical deletions of approximately 500 bp also were found in additional independent proviruses cloned from EL4 (the 5' and 3' LTRs of the AA Cl 2 provirus, the 3' LTR from the MMTV provirus in AA Cl 8, and the LTR-only provirus in AA Cl 13) (Fig. 1; data not shown). Since only a single U_3 region is used during reverse transcription, these data suggest that most acquired MMTV proviruses in T-cell tumors originated from a single deleted provirus which was a source for multiple rounds of transcription, reverse transcription, and reintegration.

Multiple nucleotide differences also were apparent between LTR clones from the EL4 tumor and the C3H LTR. Many of these differences are located between the deletion and the TATA box around position 1160 (Fig. 1) and may affect the proximal glucocorticoid receptor-binding site (1) or the NF1-binding site (1, 5, 19). The EL4 clones 2 and 13 have an A at position 1120 (Fig. 1) which reportedly reduces NF1 binding (19).

Several MMTV proviruses and their cellular flanking DNAs were cloned from the BALB/c lymphoma S49 (8). A subclone containing the MMTV LTR was sequenced and had a 430-bp deletion compared with the C3H MMTV LTR (Fig. 1). In the C3H LTR, the borders of the deletion were flanked by the 4-bp repeat AAAA, whereas the S49 LTR had only a single copy of this sequence. The 3' border of the S49 deletion was very similar to that found in the B6-derived MMTV proviruses.

The majority of acquired MMTV proviruses from the B6 and BALB/c tumors have similar LTR alterations. The sequencing analysis of several cloned MMTV proviruses from B6 and BALB/c tumors suggested that most or all of the MMTV proviruses acquired in these tumors had similar LTR deletions. To determine whether all acquired MMTV proviruses from each tumor contained this deletion, a region spanning the deleted segment was subcloned from a full-length LTR (Fig. 1). A deletion-specific probe and a full-length LTR probe were labeled by nick translation and hybridized to Southern blots of normal and tumor-derived chromosomal DNA (Fig. 2A and B). Hybridization of deletion-specific probe to DNA from the B6 tumor EL4 identified *Eco*RI fragments typical of the three endogenous B6 proviruses in addition to two additional *Eco*RI fragments (Fig. 2A, compare lanes 1 and 2). Hybridization of deletion-specific probe with S49 chromosomal DNA detected the three endogenous MMTV proviruses as well as five or six additional fragments (Fig. 2A, lane 4). Assuming a single internal *Eco*RI site in each provirus, the deletion-specific probe detected only one and three MMTV proviruses in the B6 and BALB/c tumors, respectively, which were not present in germ line DNA. Since 10 to 20 times this number of proviruses was detectable with the full-length LTR probe, the majority of acquired MMTV proviruses had similar LTR deletions (compare Fig. 2A and B). The full-length LTR

FIG. 1. Comparison of the U₃ sequence of the C3H MMTV LTR and deleted MMTV LTRs from T-cell lymphomas. Numbering of the bases begins with nucleotide 1 of the C3H MMTV LTR, as described by Majors and Varmus (25). Nucleotides identical to the C3H sequence are represented by dashes, whereas altered bases are indicated by letters. Asterisks indicate deletions of bases. The sequence of the EL4 LTR was obtained from a cDNA clone derived from the B6 lymphoma EL4, as described by Kwon and Weissman (20). The sequence between the two hatch marks includes the MMTV sequence described as a negative regulatory element by Morley et al. (30). The solid bar indicates the region included in the deletion-specific probe; this probe contains a *Hae*III to *Pst*I fragment cloned from the endogenous *Mtv-9* LTR. The hatched bar indicates the GRE as determined by linker-scanning mutations and DNase I protection experiments (1, 34). The TATA sequence is boxed. The start of transcription is shown by the arrow.

FIG. 2. Southern blots of normal and lymphoma-derived DNA. DNA samples were digested with *EcoRI*, subjected to electrophoresis on 0.8% agarose gels, and transferred to nitrocellulose. Duplicate blots were prepared from the same digestions and then hybridized to a nick-translated deletion-specific probe (panel A) or a full-length C3H LTR probe (panel B). Lanes: 1, B6 embryo DNA; 2, EL4 (B6) lymphoma DNA; 3, BALB/c embryo DNA; 4, S49 (BALB/c) lymphoma DNA; 5, RL δ 1 (BALB/c) lymphoma DNA; 6, A/J liver DNA; 7, RADA1 (A/J) lymphoma DNA; and 8, L1210 (DBA/2) lymphoma DNA.

probe identified approximately six additional *EcoRI* fragments not attributable to endogenous MMTV in the BALB/c lymphoma RL δ 1 (Fig. 2B, compare lanes 3 and 5). The deletion-specific probe detected about half this number of non-germ line fragments (Fig. 2A, lane 5). LTR alterations were not detected in A/J and DBA/2 T-cell lymphomas with the deletion-specific probe (Fig. 2A and B, lanes 7 and 8).

Transcription of deleted MMTV proviruses in lymphomas. To determine whether MMTV proviruses with LTR deletions were selectively transcribed relative to the endogenous MMTV proviruses in T-cell lymphomas, we hybridized Northern blots containing EL4 and S49 RNA to deletion-specific (Fig. 3A) or full-length (Fig. 3B) LTR probes. Only RNA species shorter by 500 to 600 bp than full-length MMTV transcripts were detected in EL4 and S49 cells (Fig. 3B, lanes 2 and 3), as expected if deleted LTRs are expressed preferentially. This conclusion is confirmed by results with the deletion-specific probe (Fig. 3A) which failed to detect any hybridizable RNA in EL4 cells (lane 2). However, this probe did detect MMTV RNA in S49 cells (Fig. 3A, lane 3). This result probably reflects the 40-bp homology remaining between the deleted S49 LTR and the deletion-specific probe (Fig. 1). These results indicated that MMTV proviruses with deleted LTRs were transcribed selectively compared with the full-length endogenous proviruses in these lymphomas.

Isolation of stable transfectants containing MMTV LTR-CAT constructs. The LTR deletion in the acquired MMTV proviruses from B6 and BALB/c T-cell lymphomas spans the carboxyl terminus of the long open reading frame as well as the most 5' glucocorticoid receptor-binding sites in the GRE (2) (Fig. 1). To detect alterations in GRE (enhancer) func-

FIG. 3. MMTV RNA in S49 and EL4 cells. Cytoplasmic RNA was extracted from GR mouse mammary tumor cells, EL4 (B6) lymphoma cells, and S49 (BALB/c) lymphoma cells and separated on 1% formaldehyde gels. Then, 10 μ g of poly(A) RNA was loaded in each lane and hybridized to either an LTR deletion-specific probe (panel A) or a full-length LTR probe (panel B). Lanes: 1, GR RNA; 2, EL4 RNA; 3, S49 RNA.

FIG. 4. Construction of MMTV LTR-CAT plasmids. A subclone of the AA CI 1 3' LTR (EL4-deleted LTR) was digested with *Hind*III and *Xba*I and was ligated to pUC-CAT digested with the same enzymes. Recombinant DNA from this ligation was cut with *Bst*EII and *Xba*I, the ends were filled with Klenow, and ligated. For p6B-CAT construction, a proviral subclone of AA CI 6 (containing the *Mtv-17* LTR) was digested with *Sal*I and *Bst*EII and ligated to *Sal*I- and *Xba*I-cleaved pUC-CAT. After ligation, the cohesive ends were filled with Klenow and the ligation was repeated.

tion, we inserted the truncated MMTV LTR from an EL4 provirus upstream of the CAT gene in vector pUC-CAT (p1B-CAT; Fig. 4). CAT plasmids containing the full-length endogenous *Mtv-17* LTR also were constructed. Plasmids with either the deleted or the full-length MMTV LTRs were cotransfected separately with pSV2gpt into mink lung cells. After mycophenolic acid selection, individual clones were grown in the presence or absence of dexamethasone before harvesting cells for CAT assays (Fig. 5). As predicted, the *Mtv-17* LTR-driven CAT was responsive to glucocorticoids (from 10- to 33-fold induction in different clones) (Table 1). The induced and basal (uninduced) levels of CAT expression did not correlate with the numbers of integrated copies as determined by Southern blotting (Table 1).

The basal CAT activity of cells containing deleted LTR constructs (p1B-CAT) appeared to be elevated compared with the basal CAT expression of cells containing the *Mtv-17* LTR (Fig. 5, Table 1). This difference was not related to the number of integrated copies (compare uninduced expression from clones 6B3 and 1B3) (Table 1). CAT activity in clones

containing the deleted MMTV LTR was increased from 24- to 99-fold in the presence of dexamethasone despite the loss of the distal glucocorticoid receptor-binding site (1) (Table 1). Cooperation between partially defective LTRs in tandemly integrated plasmids may yield higher hormone induction than that observed in transient expression assays (see below).

Transient expression from MMTV LTR-CAT constructs. Because the basal level of CAT expression from the MMTV LTR may be affected by the plasmid integration sites in stably transfected cells (11), we also used transient assays. The deleted LTR from the B6 lymphoma EL4 was inserted into the vector pMT2 (33) (p1BMT) for comparison with a similar construct containing the exogenous C3H MMTV LTR (pLC1) (30). Purified DNA from each construct was introduced into mink lung cells by electroporation. The day after electroporation, half of each sample was exposed to dexamethasone for 24 h and then cells were collected and tested for CAT activity. As predicted, the C3H LTR construct responded well to glucocorticoid induction (24-fold)

FIG. 5. CAT assays on cells stably transfected with MMTV LTR-CAT plasmids. Purified DNA from the plasmids in Fig. 4 was transfected into mink cells as described in Materials and Methods. Cells were grown in the presence or absence of 10^{-5} M dexamethasone (DEX) for 24 h before cell extract preparation. Each lane contained lysate from 1.7×10^6 cells (150 μ g of protein). CAT assays were performed at 37°C for 2 h in a total volume of 200 μ l. Each pair of lanes depicts extracts from different cell clones transfected with pUC-CAT (promoterless CAT vector), the *Mtv-17* LTR construct p6B-CAT, or the deleted MMTV LTR construct p1B-CAT. The right-hand lane shows the activity of 0.5 U of purified CAT (Sigma). Acetylated (AcCM) and unacetylated (CM) forms of chloramphenicol are indicated.

and had very low CAT activity in the absence of the hormone (Fig. 6, Table 2) (30). In contrast, the deleted MMTV LTR from the EL4 tumor (p1BMT) (–655 to –165) showed high basal levels of expression in the absence of glucocorticoids and low-level glucocorticoid induction (about twofold). The basal level of CAT activity from the deleted EL4 LTR appeared to be approximately sevenfold greater than that from pLC1 (Fig. 6, Table 2).

The MMTV LTRs from EL4-derived proviruses also have a number of point mutations (Fig. 1) which may affect expression from the LTR in the absence of hormones. To determine whether the deletion was responsible for elevation of basal expression, a similar deletion extending from –637 to –255 in the C3H MMTV LTR was constructed from the pLC1 plasmid (pS255). This plasmid then was introduced into mink lung cells by electroporation. Extracts from these

FIG. 6. Transient expression assays with MMTV LTR-CAT constructs. A subclone of AA Cl 1 containing the 3' MMTV LTR was digested with *Hind*III and *Bst*EII before filling the cohesive ends with Klenow. The pMT2 vector (MT) also was cleaved at the unique *Hind*III site upstream of the CAT gene before filling the ends with Klenow. After blunt-end ligation and transformation, DNA samples from individual recombinants were screened for those containing the MMTV LTR in the correct orientation to the CAT gene (plasmid p1BMT) (1B). The pLC1 plasmid (LC) has the full-length C3H MMTV LTR (30). Construction of pS106 (S106) (deletion from –637 to –106) involved double digestions at the unique *Stu*I and *Sac*I sites of pLC1, polymerization with Klenow to fill the ends, and religation. The pS255 plasmid (S255) (deletion from –637 to –255) was constructed by digestion of pLC1 at the unique *Stu*I site, digestion with BAL 31 nuclease, and cleavage with *Eco*RI. The resulting fragment was used to replace the equivalent region in pLC1. The size of the deletion was confirmed by sequencing. Forty μ g of DNA from each construct was electroporated into 5×10^6 mink lung cells (voltage 1,500, capacitance 75 μ F). Cells were divided into two 100-mm plates and incubated at 37°C. After 1 day, the medium was removed and replaced with complete DMEM with or without 10^{-6} M dexamethasone for 24 h as indicated. One hundred micrograms of cell extract was analyzed in CAT assays at 43°C. Acetylated (AcCM) and unacetylated (CM) forms of chloramphenicol are indicated.

cells showed high basal CAT activity (4.5-fold compared with that of pLC1) as well as glucocorticoid-induced CAT expression (Fig. 6, Table 2). Similar deletions extending from the *Stu*I site at –637 to various points, including –426, also showed high basal expression (data not shown). However, a plasmid containing a deletion from –637 to –106 (pS106) expressed CAT at a basal level comparable to that of pLC1 (Fig. 6). As expected, loss of GRE sequences in addition to those deleted in the EL4 LTR (p1BMT) effectively abolished the response to dexamethasone.

DISCUSSION

We have identified several deletions found within the LTRs of MMTV proviruses acquired in murine T-cell lymphomas. The 491-bp deletion which is present in several different MMTV proviruses in the EL4 tumor appeared to be identical to that identified in an MMTV cDNA clone from this lymphoma (20; Fig. 1). The identification of proviruses with the same deletions in the 3' and 5' LTRs may indicate that the original alteration occurred in the 3' U_3 region during transcription and was transferred to the 5' LTR and subsequent proviruses by reverse transcription. Acquired MMTV proviruses from the BALB/c lymphoma S49 also contained a 430-bp deletion with a 3' endpoint similar to that found in the B6 lymphoma. The similarity between the B6

TABLE 1. Summary of CAT assays from stable transfectants

Construct	Dexamethasone addition	% Chloramphenicol acetylated	Dexamethasone induction (fold)	DNA copies ^a
6B1 ^b	–	0.2		
	+	6.5	33	>100
6B3 ^b	–	0.2		
	+	3.4	17	25
6B7 ^b	–	0.1		
	+	1.0	10	>100
1B2 ^c	–	0.9		
	+	88.9	99	>100
1B3 ^c	–	3.2		
	+	89.5	28	10
1B7 ^c	–	0.2		
	+	8.8	44	ND ^d
1B9 ^c	–	0.3		
	+	7.1	24	NT ^e

^a Estimated copy number from *Bgl*II and *Pst*I digests.

^b 6B1, 6B3, and 6B7 are independent mycophenolic acid-resistant clones obtained by cotransfection of p6B-CAT (*Mtv-17* LTR) and pSV2gpt.

^c 1B2, 1B3, 1B7, and 1B9 are independent mycophenolic acid-resistant clones obtained by cotransfection of p1B-CAT (EL4-truncated LTR) and pSV2gpt.

^d None detected.

^e Not tested.

TABLE 2. CAT assays from transient transfection experiments

Plasmid	Deletion limits	Dexamethasone addition	% Chloramphenicol acetylated	Dexamethasone induction (fold)	Increase in basal level (fold)
pLC1 ^a		—	2.2	23.8	1.0
		+	51.7		
p1BMT ^b	-655 to -165	—	15.5	2.4	7.2
		+	37.2		
pS106 ^c	-637 to -106	—	1.9	1.8	0.9
		+	3.4		
pS255 ^d	-637 to -255	—	9.8	7.4	4.5
		+	72.1		

^a pLC1 contains the full-length C3H LTR.

^b p1BMT contains the truncated LTR from the B6 EL4 tumor (CI 1 3' LTR).

^c pS106 was derived by internal deletion of the pLC1 LTR from *StuI* (-637) to *SacI* (-106).

^d pS255 was obtained by BAL 31 deletion of the pLC1 LTR starting at the *StuI* (-637) site.

and BALB/c deletions suggested that MMTV proviruses with deleted LTRs were propagated selectively in T-cell lymphomas. This conclusion was supported by results showing that proviruses with deleted LTRs were acquired at least 10 times more frequently than proviruses with full-length LTRs in these tumors (Fig. 2). Proviruses with LTR deletions also were detected in the BALB/c lymphoma RL δ 1 although in lower abundance than in EL4 or S49. Moreover, the proviruses with deleted LTRs were expressed more efficiently than the endogenous MMTV proviruses in the EL4 and S49 lymphomas (Fig. 3).

Because the LTR deletions remove the 5' portion of the GRE and because this element is thought to be a regulated enhancer, the transcriptional activity of the deleted LTRs was determined in transient expression assays and in stable transfectants. Glucocorticoid induction from the B6 deleted LTR was evident in stable transfectants despite the loss of the distal glucocorticoid receptor-binding site (-180 to -170) which is known to dramatically affect hormone inducibility (1). Although the level of induction appeared to be higher in clones transfected with deleted EL4 LTR constructs than that observed with intact *Mtv-17* LTR constructs, these results probably reflect lower-level expression from a defective *Mtv-17* promoter (19). To determine the basal level of expression and the relative level of induction, transient expression experiments also were performed. In these experiments, the EL4 LTR (p1BMT) (deletion of -655 to -165) and the C3H LTR provided a similar level of reporter gene expression in the presence of hormone. However, due to the high basal expression of the deleted LTR, induction of CAT activity by glucocorticoids was low. In contrast, Buetti et al. (1) found that linker-scanning mutations in the distal hormone-binding site affected the relative hormone inducibility as well as the final level of reporter gene expression achieved. Thus, hormone inducibility and level of gene expression may be affected by the spacing of regulatory elements in the MMTV LTR.

The most obvious difference between the transcriptional activity of the deleted B6 LTR and the intact C3H LTR was the level of CAT expression in the absence of hormone induction (Fig. 6, Table 2). Deletion of a similar region from the C3H LTR also produced high basal levels of CAT activity from the MMTV LTR, suggesting that the deleted LTRs lack negative regulatory elements. This higher level of gene expression likely is due to higher transcriptional activity from the MMTV promoter, since no cryptic plasmid initiation sites have been detected in the pLC1 or pMT2

vectors (30). Moreover, RNase protection experiments with p1B-CAT (deleted LTR) stable transfectants indicated that CAT RNA synthesis initiates at the LTR U₃-R junction (data not shown).

Morley et al. (30) have identified a negative regulatory element in the MMTV LTR between positions -455 and -364 relative to the site of transcription initiation. All of the lymphoma-derived proviruses with deleted LTRs lack these sequences (20, 22, 27, 29; Fig. 1), supporting a biological role for the negative regulatory element. Thus, despite a number of studies which failed to detect a negative regulatory element in the MMTV LTR (1, 24, 38), our findings support those of Morley et al. (30) and suggest some other interesting features. For example, deletion of 60 bp between -165 and -106 (compare the constructs p1BMT, pS255, and pS106 in Fig. 6) resulted in loss of both high basal CAT activity and glucocorticoid-regulated expression. Several explanations are possible for the low basal level of the -106 construct, including (i) the deletion of a positive regulatory sequence between -165 and -106 or (ii) the loss of GRE sequences which maintain basal expression. Morley et al. (30) reported that a deletion lacking all MMTV LTR sequences upstream of -106 had high basal expression. Although this discrepancy may be attributed to technical differences in the experimental design (our constructs contained 5' LTR sequences) or to the cell types used (mink versus mouse cells), an LTR sequence between -204 and -156 which contributes to basal MMTV transcription was suggested by the results of Lee et al. (21). Internal deletions between the *SacI* site (approximately -100) and -156 showed basal transcription levels similar to those of a full-length MMTV LTR, yet deletions between *SacI* and -204 elevated reporter gene expression about sixfold in the absence of glucocorticoids (21).

In summary, several BALB/c and B6 T-cell lymphomas have acquired MMTV proviruses with LTR deletions between -165 and -665 which include negative regulatory elements that map between -455 and -364 in the U₃ region (30). MMTV LTR-CAT constructs containing this deletion displayed low levels of glucocorticoid responsiveness but, more strikingly, elevated CAT expression in the absence of hormone. In addition, independently isolated proviruses from the same tumor contained identical deletions which were more extensive than those defined for the negative regulatory element (30). Thus, there may be a selective advantage for LTR deletions which confer elevated transcriptional activity in the absence of hormone or functional

hormone receptors. In support of this, transcription of acquired MMTV proviruses in EL4 and most lymphomas is not stimulated by the addition of glucocorticoids (9; J. Dudley, unpublished data). MMTV proviruses with deleted LTRs have been described only in T cells and in kidney tumors (9, 20, 22, 27, 29, 37) and have not been isolated from mammary tumors. Perhaps MMTV transcription is regulated by a different mechanism (factors) in mammary glands and there is no selection for proviruses with deleted LTRs.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA34780 from the National Institutes of Health and by the Morrow Kelley Professorship in Microbiology.

We thank David Peterson for providing the pLC1 and pMT2 plasmids, Nancy Hopkins and E. Golemis for the pUC-CAT plasmid, and Henry Bose and Bill Folk for helpful suggestions on the manuscript.

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