Negative Regulatory Element Associated with Potentially Functional Promoter and Enhancer Elements in the Long Terminal Repeats of Endogenous Murine Leukemia Virus-Related Proviral Sequences

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Three series of recombinant DNA clones were constructed, with the bacterial chloramphenicol acetyltransferase (CAT) gene as a quantitative indicator, to examine the activities of promoter and enhancer sequence elements in the 5' long terminal repeat (LTR) of murine leukemia virus (MuLV)-related proviral sequences isolated from the mouse genome. Transient CAT expression was determined in mouse NIH 3T3, human HT1080, and mink CCL64 cultured cells transfected with the LTR-CAT constructs. The 700-base-pair (bp) LTRs of three polytropic MuLV-related proviral clones and the 750-bp LTRs of four modified polytropic proviral clones, in complete structures either with or without the adjacent downstream sequences, all showed very little or negligible activities for CAT expression, while ecotropic MuLV LTRs were highly active. The MuLV-related LTRs were divided into three portions and examined separately. The 3' portion of the MuLV-related LTRs that contains the CCAAC and TATAA boxes was found to be a functional promoter, being about one-half to one-third as active as the corresponding portion of ecotropic MuLV LTRs. A *MboI*-*BglII* fragment, representing the distinct 190- to 200-bp inserted segment in the middle, was found to be a potential enhancer, especially when examined in combination with the simian virus 40 promoter in CCL64 cells. A *PstI*-*MboI* fragment of the 5' portion, which contains the protein-binding motifs of the enhancer segment as well as the upstream LTR sequences, showed moderate enhancer activities in CCL6 cells but was virtually inactive in NIH 3T3 cells and HT1080 cells; addition of this fragment to the ecotropic LTR-CAT constructs depressed CAT expression. Further analyses using chimeric LTR constructs located the presence of a strong negative regulatory element within the region containing the 5' portion of the enhancer and the immediate upstream sequences in the MuLV-related LTRs.

In chromosomal DNA of laboratory mice, 30 to 50 dispersed proviral sequences can be detected with a molecular probe of murine leukemia viruses (MuLVs). The majority of these are not the proviruses of ecotropic or xenotropic MuLVs (8, 23, 32, 49, 60) but rather belong to two classes of distinct proviruses in the mouse germ line (51, 59, 61). Each class possesses characteristic restriction enzyme sites in the DNA structure and a specific length of the long terminal repeat (LTR); these two proviral classes are both different from ecotropic and xenotropic MuLV proviruses by containing mink cell focus-forming (MCF) virus determinants in the *env* gene (10, 29, 61), the *Fv-1* B-tropism determinant within the *gag*-p30 region (3), the primer binding site for the major or minor UAG-suppressor glutamine tRNAs (47, 51), and some distinct structural features in the LTR (28, 51). The biological significance of these endogenous noncancerous, nonxenotropic proviral classes is indicated by their potential to recombine with ecotropic MuLVs and generate retroviruses of modified, if not increased, leukemogenic and pathogenic properties, such as the MCF and polytropic viruses (21), spleen focus-forming viruses (12), and B-tropic MuLVs (3, 5). Also, the glutamine tRNA primer binding site has been found in an infectious recombinant virus isolate of Moloney ecotropic MuLV (13). Despite such functional observations for three of the four distinguishing features and also documentation of full-length and apparently intact LTR-*gag-pol-env*-LTR structures by molecular cloning (1, 46), no

retroviruses with RNA genomes directly corresponding to these endogenous proviral structures has been detected.

The complete or major nucleotide sequences of the LTRs in various molecular clones of the two MuLV-related proviral classes have been reported previously (28, 46, 51, 61). Although similar to those of ecotropic MuLVs (15, 22, 42, 56, 62) in having precisely the same terminal 11-nucleotide inverted repeats as well as the basic U3-r-U5 structure with the TATA box, cap site, and polyadenylation signal at similar relative positions, these endogenous MuLV-related LTRs are different from the MuLV LTRs principally in three aspects of their structures. The most distinct feature, which is not detected in the MuLV LTRs, is a segment of 190 to 200 base pairs (bp) inserted between the apparent enhancer and the apparent promoter in the U3 region (28, 39, 51), which we will hereafter call IS. Also, sequence divergence is present in the enhancer segment and its immediately adjacent sequences. Furthermore, at the 5' side of the TATA box, there is an CAGCCC duplication, which leads to a 6-bp displacement of the presumed CAT box, CCAAC, further upstream. The presence of the additional distinct IS makes these LTRs larger than those of ecotropic and xenotropic MuLV proviruses. The LTRs of the two endogenous MuLV-related proviral classes, called polytropic and modified polytropic (61), are about 700 and 750 bp long, respectively, with the latter containing an extra 37- to 40-bp sequence immediately upstream of the 200-bp IS segment (28, 61).

In view of these distinct LTR features, and also since there has been no previous report of a recombinant virus carrying these specific LTR sequences, we have investigated

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FIG. 1. LTR structures (□) and adjacent leader region (—) of ecotropic MuLV and related mouse chromosomal proviral sequences used for the construction of LTR-CAT clones. The two ecotropic MuLV LTRs (4) are nearly identical, except that a duplicated 53-bp direct repeat is present within the enhancer domain of pWNB5. Solitary LTRs of pRFM7 and pRFM8 (33) show sequence homology values of 95 to 96% to xenotropic MuLV LTRs, 83 to 87% to ecotropic MuLV LTRs, and 81 to 87% to the chromosomal MuLV-related LTRs. Corresponding nucleotide sites (----) sequence duplications (▨) and extra sequence segments in LTR structures are shown. The distinct 190- to 200-bp IS (■) located in-between the enhancer and the promoter domains of the U3 region in MuLV-related LTRs is also shown. Also indicated are major restriction sites used for molecular construction. The *Mbo*I and *Bgl*II sites in parentheses, present in pAL10 but absent in pRFM6 and pRFM16 of the same 700-bp LTR (polytropic) class, were used in the experiments of Fig. 3 and 4 to study the transcriptional function of IS.

their LTR transcriptional activity in order to understand the apparent inability of the two endogenous MuLV-related proviral classes to be expressed directly into retroviruses. In this study, the chloramphenicol acetyltransferase (CAT) gene assay system (19, 35) was employed to analyze the possible functions of the LTR in representative clones of the two endogenous proviral classes. The analysis was also made on three separated LTR fragments that correspond approximately to the promoter, the 200-nucleotide internal segment, and the enhancer domains. Two clones of ecotropic MuLVs as well as a xenotropiclike solitary LTR clone were included for comparison. The CAT expression assay was performed in the NIH 3T3 mouse embryo cell line and the CCL64 mink lung fibroblast line, which have been commonly employed for MuLV and MCF virus studies (5, 21), as well as in the HT1080 human fibrosarcoma cell line.

MATERIALS AND METHODS

Plasmids. A total of 11 LTR-containing recombinant plasmid clones were used. The eight clones derived from RFM/Un mouse chromosomal DNA (4, 33, 46, 47) were of three classes: the 700-bp LTR (polytropic) class represented by pRFM6 and pRFM16; the 750-bp LTR (modified polytropic) class represented by pRFM1, pRFM3, pRFM9, and pRFM17; and the solitary LTR of presumed xenotropic MuLV origin represented by pRFM7 and pRFM8. The three clones of BALB/c mouse origin were pAL10 of the 700-bp LTR class (51), pWN41 of the N-ecotropic WN1802N MuLV and pWNB5 of the B-ecotropic WN1802B MuLV (4). The characteristic structural features of these LTRs, as well as the restriction sites used for molecular construction, are depicted in Fig. 1. The CAT gene-containing plasmids (35), including pSV2-CAT, pA₁₀CAT₂ for assaying enhancer sequence function, pCAT-3M for assaying promoter or pro-

motor-enhancer function, and pMSV-CAT, were kindly given by the late George Khoury of the National Cancer Institute, Bethesda, Md. All plasmids were in pBR322 or pUC vectors and maintained in *Escherichia coli* host strains HB101, C600, or RR1. The plasmid DNA was prepared from spectinomycin-treated host bacteria by standard isolation procedures (44).

Construction of recombinant clones. Three major series of LTR-CAT recombinant clones were constructed in the cassette manner that allows easy replacement of homologous LTR sequences (Fig. 2; also see Fig. 5A). In the first series, a pUC19 subclone of pWN41, called p19A1, which carries the 5' *Pst*I-*Bam*HI fragment of WN1802N MuLV genome spanning from the 5' end of LTR to the 5' end of *gag*-p30, was used as a starting material. The *Bgl*II-*Bam*HI fragment (leader to *gag*-p30) of p19A1 was replaced by the *Bgl*II-*Bam*HI fragment of pCAT-3M, which includes the total CAT gene and the simian virus 40 (SV40) polyadenylation segment, to form the master clone of this pX-CAT series, p41-CAT. The *Pst*I and *Bgl*II sites in p41-CAT were utilized to replace its LTR-leader sequence with the homologous *Pst*I-*Bgl*II fragments of other proviral classes. In some cases, the unique *Spe*I site immediately 5' to the *Bgl*II site was used for fragment exchange. In the second recombinant clone series (Fig. 2B), the *Sma*I-*Sma*I fragment containing pAL10 LTR and 5' cellular flanking sequence was first subcloned into pUC9 so that it could be subsequently isolated as a *Pst*I-*Bam*HI fragment (with the *Bam*HI site derived from the polylinker of the vector) and used to replace the *Pst*I-*Bgl*II LTR-leader fragment in p41-CAT. The resulting clone, called p10L-CAT, served as the master clone in which the *Pst*I-*Sma*I LTR fragment of AL10 could be replaced by intact or IS-deleted *Pst*I-*Sma*I fragments of various proviral class LTRs. The third, called pE-CAT₂

FIG. 2. Schemes for construction of two LTR-CAT recombinant clone series. (A) pX-CAT series. X represents the *Pst*I-*Bgl*II fragment of an individual proviral clone that includes the whole LTR (minus 38 bp at the 5' end) and the 163-bp leader sequence immediately downstream. p19A1 is a pUC19 plasmid carrying a pWN41-derived 1.4-kbp insert that spans from the *Pst*I site at the 5' end of LTR to the *Bam*HI site at the 5' end of *gag*-p30. The *gag*-carrying *Bgl*II (*G*)-*Bam*HI fragment of p19A1 was replaced with the CAT-poly(A) (SV40) *Bgl*II-*Bam*HI fragment of pCAT-3M (35), thereby generating the master clone of this series, p41-CAT. Homologous fragment replacements were performed by using *Pst*I-*Bgl*II fragments to generate pB5-CAT and pRF6-CAT/pRF16-CAT and by using *Pst*I-*Spe*I fragments (*Spe*I site being 16 bp upstream of the *Bgl*II site) to generate pAL10-CAT, pRF1-CAT/pRT9-CAT, and pRF17-CAT. (B) pL-CAT series. L represents the LTR fragments spanning from the *Pst*I site at the 5' end of the LTR to the *Sma*I site located in the middle of the r region. This LTR *Pst*I-*Sma*I fragment of pAL10 proviral clone was subcloned into the *Sma*I sites of pUC9 and subsequently recovered as a *Pst*I-*Bam*HI fragment that carried the 16-bp *Sma*I-*Bam*HI segment of the pUC9 polylinker sequence. With the use of this *Pst*I-*Bam*HI fragment to substitute the LTR-leader *Pst*I-*Bgl*II fragment in p41-CAT, a master clone of the pL-CAT series, p10L-CAT, was constructed. Homologous fragment replacement at the *Pst*I and *Sma*I sites was performed by using the LTR *Pst*I-*Sma*I fragment of various proviral clones. Restriction enzyme abbreviations: G, *Bgl*II; B, *Bam*HI; P, *Pst*I; S, *Sma*I.

series (see Fig. 5A), employed the pA₁₀CAT₂ that carries the SV40 promoter without the enhancer sequence (35). With the use of the *Bgl*II-*Bam*HI fragment of pA₁₀CAT₂ to replace the *Bgl*II-*Bam*HI fragment in p19A1 to generate the master clone of p41-A₁₀CAT₂, various *Pst*I-*Bgl*II or *Pst*I-*Mbo*I (*Sau*3A) fragments representing the candidate enhancer sequences of different LTRs could be used for replacement and thus linked to the SV40 early promoter and CAT gene sequences. Standard recombinant DNA procedures (44) were used in all the molecular construction experiments. Reaction conditions for the restriction enzymes, ligase, and phosphatase were according to the commercial supplier (New England BioLabs, Bethesda Research Laboratories, and Boehringer Mannheim Biochemicals). The authenticity of a recombinant plasmid clone was assessed by restriction fragment analysis and gel blot hybridization and, in some cases, firmly established by direct DNA sequencing of critical regions in the molecule.

Cell culture. The mouse embryo NIH 3T3 cell line was obtained from G. J. Todaro in the early 1970's (4); the mink lung cell line CCL64 was from the American Type Culture Collection, Rockville, Md.; and human fibrosarcoma cell line HT1080 was originally from R. M. McAllister of Los Angeles Children's Hospital, Los Angeles, Calif. Large pools of these cell lines have been stored in liquid N₂ freezers immediately after their receipt in this laboratory. These cultured cells were grown in Eagle minimal essential medium containing 4% fetal calf serum, 4% calf serum, 100

μg of streptomycin per ml, and 100 U of penicillin per ml in a 5% CO₂ incubator at 37°C. Subculture was performed every 3 to 4 days for NIH 3T3 cells and every 4 to 5 days for the other two cell lines. DNA transfection assay of the CAT recombinant constructs was usually performed with these cells within less than 10 passages from the frozen cell stocks.

DNA transfections and CAT assays. Transient expression of CAT activity was performed by DNA transfection and enzyme measurement, essentially by the procedures previously published (19, 35). Briefly, duplicate 100-mm-diameter dishes seeded 24 h previously with 1.5×10^6 cells were each inoculated with 10 μg of plasmid DNA precipitate in 1 ml of calcium-phosphate-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, which was applied in drops evenly on the cells through the culture medium. After 16 h, the DNA-containing medium was removed and replaced with fresh culture medium. No glycerol shock treatment was applied, as this step gave various degrees of cytotoxicity. Cells were harvested 48 h after DNA treatment and lysed by three cycles of freezing and thawing with liquid N₂. Protein content of cell extracts was measured by a dye-binding assay (Bio-Rad protein assay), using bovine serum albumin as the standard. Samples containing equal amounts of protein from the transfected cells were used in 50 μl of reaction mixture (19) for measuring acetylation of ¹⁴C-labeled chloramphenicol (45 mCi/mmol; Dupont, NEN Research Products). After incubation at 37°C for 60 min, the reaction mixture was extracted three times each with 0.6 ml of ethyl acetate. The

extracts were combined, evaporated to dryness, and taken up in 30 μ l of ethyl acetate to dissolve chloramphenicol and its acetylated derivatives. Ten microliters was used for chromatography. After separating in ascending thin-layer chromatography on plastic-based silica gel plates (J. T. Baker Chemical Co.), chloramphenicol and its two monoacetylated and one diacetylated derivative were revealed by autoradiography on X-ray film. With the autoradiogram as a guide, the precise spots of chloramphenicol and its three acetylated derivatives were cut from thin-layer plates and placed in a toluene-based scintillation fluid, and the radioactivity was measured in a liquid scintillation counter. Samples of reactions with untransfected or pSV2-NEO-transfected cell extracts were chromatographed in parallel, and their corresponding spots were measured to serve, respectively, as background counts of acetylated derivatives and as a reference for total initial chloramphenicol substrate in the reaction. The CAT activity was expressed as the percentage of chloramphenicol converted to the acetylated forms on the basis of radioactivities recovered in the four chromatographic spots. Our preliminary experiments showed that the enzymatic reaction was virtually linear until about 70% of the chloramphenicol substrate was acetylated. Also, with various amounts of plasmid DNA used for the transfection, linearity of CAT expression was obtained with the addition of up to 20 μ g of pSV2CAT and up to 10 μ g of pB5-CAT.

RESULTS

Common and specific features of LTR structures. Summarized in Fig. 1 are the LTR structures of seven endogenous MuLV-related proviral clones, in comparison with two ecotropic MuLV LTRs and two xenotropiclike solitary LTRs (33). Nucleotide sequences of these 11 LTRs are all known (46, 51; also see Fig. 8). The 527-bp LTR of pWN41 is virtually identical (i.e., with only a few base changes) to the LTR of endogenous ecotropic MuLV isolated from the RFM mouse (42); the pWNB5 LTR is the same, except for a tandem 53-bp duplication in the enhancer region (4). Of the seven MuLV-related clones, pAL10 (51), pRFM6 (46), and pRFM16 proviruses belong to the polytropic class while pRFM1, pRFM3, pRFM9, and pRFM17 are of the modified polytropic class by the classification of Stoye and Coffin (61). Sequences at the two ends of all 11 LTR structures are virtually identical or at least 90% homologous; these include the 5' 90-bp sequence, a common *Pst*I site 38 nucleotides from the 5' end, the 3' 180-bp sequence, and a common *Sma*I site 113 nucleotides from the 3' end. It is apparent that sequence divergence among the four endogenous proviral classes occurs mainly within the U3 portion of the LTR structure, where the transcriptional elements are located. The class-specific features are all contained within the sequences between the *Pst*I site and the *Sma*I site. Our molecular constructions of LTR-CAT clones were therefore performed mainly for comparative studies of these *Pst*I-*Sma*I fragments.

The approximately 85-bp enhancer sequences with six segments of protein-binding motifs and a common *Eco*RV site (58) can be identified in the 750-bp LTRs of the four modified polytropic MuLV-related proviral clones (i.e., pRFM1, pRFM3, pRFM9, and pRFM17) as well as in those of ecotropic MuLVs (i.e., pWN41 and pWNB5) and solitary (pRFM7 and pRFM8) LTRs. The 700-bp LTRs of three polytropic class proviral clones (i.e., pAL10, pRFM6, and pRFM16) contain nearly the same enhancer sequences as the

750-bp LTRs and the solitary LTRs, except for the *Eco*RV site and the *Lvb* motif. Divergence of the enhancer sequence observed in the four proviral classes appears to be not significantly higher than that obtained by comparing each with the Moloney leukemia and sarcoma proviruses (15, 56, 62). The major class-specific differences are found at both the 5' and the 3' adjacent regions of the enhancer sequence. A CAGTTAAAGATTAA tandem duplication at the immediate 5' side and a distinct 190- to 200-bp IS at the 3' region specify the two MuLV-related proviral classes; the differences of ecotropic MuLV and solitary LTRs involve base changes and deletions or additions. The 750-bp LTR of the modified polytropic class differs from the 700-bp LTR of polytropic proviral class in possessing an extra sequence of about 40 bp located between the enhancer and their 200-bp IS.

Two specific features are noted for the LTR promoter signals. In the two MuLV-related proviral classes and the xenotropiclike solitary LTRs, a CAGCC tandem duplication at the immediate 5' side of the TATAA box shifts the other LTR sequences on its 5' side 5 bp further upstream. Also, a CCAAC box is present in MuLV-related LTRs in contrast to a CCAAT box in the ecotropic MuLV and solitary LTRs.

Comparison of LTR transcriptional activities. The cloning strategies depicted in Fig. 2 (also see Fig. 5A) made it possible for us to compare transcriptional activities of homologous LTR fragments in the same sequence settings of the CAT gene and the pUC19 plasmid vector. Although considerable variations in the absolute CAT activity were observed in different DNA transfection experiments employing the same LTR-CAT recombinants and the same recipient cells, consistent relative activities of CAT expression by different LTR-CAT constructs were obtained from experiment to experiment. Representative results of transient CAT expression in mouse NIH 3T3 embryo cells, human HT1080 fibrosarcoma cells, and mink CCL64 lung fibroblast cells transfected with various LTR-CAT recombinants are shown in Table 1. The LTRs of the two ecotropic MuLVs were highly active in expressing CAT in the three cell lines, with pWNB5 LTR (pB5-CAT) severalfold higher than, and the pWN41 LTR (p41-CAT) about equal to, the SV40 enhancer-promoter activity (pSV2-CAT). In contrast, the LTRs of the seven MuLV-related proviral clones all showed very little, if any, activity in NIH 3T3 cells, ranging from 1/50 to 1/200 that of the reference pSV2-CAT activity; these were essentially the same as the background activity of pCAT-3M (35) that carries only the CAT structural gene without associated eucaryotic promoter or enhancer sequences. Similar results were obtained with HT1080 cells. CAT expression in CCL64 cells was significantly higher than in the other two recipient cell lines for all LTR-CAT clones; although still relatively low, the absolute CAT activities obtained with the polytropic pAL10 LTR-carrying pAL10-CAT in CCL64 were about equal to those expressed by pSV2-CAT in NIH 3T3 cells. Also, in CCL64 cells, the transcriptional activities of the polytropic class LTRs (i.e., pAL10, pRFM6, and pRFM16) were significantly higher than those of the modified polytropic class LTRs (i.e., pRFM1, pRFM9, and pRFM17), which remained at the background level of pCAT-3M.

Essentially the same relative CAT expression activities by different proviral classes were obtained with the pL-CAT series (Fig. 2B constructs) as with the pX-CAT series (Fig. 2B constructs). This indicated that the inefficient transcriptional activity of the two classes of MuLV-related proviral LTRs is due to sequence elements within the *Pst*I-*Sma*I LTR

TABLE 1. Relative activities of CAT gene expression under the control of MuLV-related LTRs^a

Expt no. and plasmid	LTR type ^b	Relative activity ^c of CAT gene expression on cell line:		
		NIH 3T3	HT1080	CCL64
Expt 1				
pSV2-CAT		100	120	1,500
p41-CAT	eco	110	120	1,000
pB5-CAT	eco	670	1,000	2,600
pAL10-CAT	poly	2	3	100
pRF1-CAT	m-poly	1	2	10
pCAT-3M		1	1	30
Expt 2				
pSV2-CAT		100	120	940
p41-CAT	eco	120	120	870
pRF6-CAT	poly	3	6	110
pRF16-CAT	poly	2	1	130
pRF17-CAT	m-poly	3	7	40
pCAT-3M		3	1	20
Expt 3				
p41L-CAT	eco	100	81	600
p10L-CAT	poly	1	0.5	48
p1L-CAT	m-poly	0.4	0.8	15
p6L-CAT	poly	0.4	0.7	75
p9L-CAT	m-poly	0.4	0.7	17
p16L-CAT	poly	0.5	0.7	69
p17L-CAT	m-poly	0.5	0.9	17
pCAT-3M		0.4	0.7	20
Expt 4				
p41L-CAT	eco	100	ND ^d	1,600
p7L-CAT	xeno	25	ND	1,500
p10L-CAT	poly	1.6	ND	88
p9L-CAT	m-poly	0.1	ND	9
pCAT-3M		0.5	ND	28

^a Cell extracts from duplicate plates of NIH 3T3, HT1080, and CCL64 cells 48 h after transfection with 10 µg of plasmid DNA were examined by protein determination, enzymatic chloramphenicol acetylation reaction, thin-layer chromatography, and liquid scintillation counting as described in Materials and Methods.

^b The classification of endogenous proviral sequences by Stoye and Coffin (61) is used. Abbreviations: eco, ecotropic MuLV LTR type; xeno, xenotropic; poly, polytropic; m-poly, modified polytropic.

^c In experiments 1 and 2, activities of various pX-CAT constructs (Fig. 2A) are shown as average values relative to the pSV2-CAT activities in NIH 3T3 cells (3.3 and 2.2 nmol of acetylated chloramphenicol per mg of protein per h of specific CAT enzyme activities, respectively), which are each expressed as 100. In experiments 3 and 4 for testing pL-CAT constructs (Fig. 2B), the reference activities (each expressed as 100) of NIH 3T3 cells transfected with p41L-CAT, were 5.1 and 1.9 nmol/mg of protein per h, respectively.

^d ND, Not determined.

fragment and not due to the remaining LTR sequences or the downstream primer tRNA binding and leader sequences, which are known to affect gene expression in other systems, such as human immunodeficiency virus in human cells (54) and MuLV in embryonal cells (43).

Promoter elements in MuLV-related proviral LTRs. To understand the basis for the almost negligible transcriptional activities observed with the MuLV-related LTRs, we first examined the promoter element. The *Pst*I-*Sma*I LTR fragment of AL10 can be divided by a *Mbo*I site and a *Bgl*II site into three segments, which respectively contain the enhancer, the IS, and the promoter signals (Fig. 1). CAT recombinant clones were therefore constructed which allowed us to examine the functional activities of these three separate LTR segments. Removal of the 155 bp of the IS situated between the *Mbo*I and the *Bgl*II site (i.e., p10dIS-

CAT) caused a further decrease, rather than an increase, in the CAT expression activity by the pAL10 LTR (i.e., p10L-CAT) (Fig. 3). When chimeric LTRs were made by linking the promoter-signal-carrying *Bgl*II-*Sma*I fragment of pAL10 LTR to the enhancer-sequence-carrying *Pst*I-*Mbo*I fragment of pWNB5 (pB5EP-CAT) or pWN41 LTR (p41EP-CAT), CAT expression was enhanced 20- to 30-fold in NIH 3T3 cells and 4- to 5-fold in CCL64 cells. Similar results were obtained by using combinations of the *Pst*I-*Mbo*I segment of pWN41 and the *Bgl*II-*Sma*I segment of pRFM9 and pRFM17 of the 750-bp LTR modified polytropic class (data not shown but see also Fig. 7). The CAT expression activities of the chimeric *Pst*I-*Sma*I fragments (pB5EP-CAT and p41EP-CAT) were one-half to one-third of those obtained with pB5L-CAT and p41L-CAT, suggesting that promoter elements of the MuLV-related LTRs are competent but slightly less efficient than those of ecotropic MuLV LTRs.

Enhancer elements in the IS of MuLV-related LTRs. The experiment involving deletion of the IS (Fig. 3) suggested a possible positive effect of this segment on CAT expression. This possibility was confirmed by subsequent experiments (Fig. 4). Placed either in the same orientation (i.e., in pISP-CAT) or in the opposite orientation (in pISOP-CAT), the *Mbo*I-*Bgl*II IS fragment was able to enhance the CAT expression activity of its own promoter two- to threefold in NIH 3T3 cells and severalfold in CCL64 cells. By using pIS-CAT and pISO-CAT clones, which carry the *Mbo*I-*Bgl*II IS fragment directly linked upstream to the CAT gene in the same or opposite orientation, no increase of CAT expression was obtained over the minimal levels observed with the promoterless enhancerless pCAT-3M, indicating that the *Mbo*I-*Bgl*II IS fragment of pAL10 by itself did not serve as a transcriptional promoter.

Further analysis of the IS was carried out by construction of pE-CAT2 series (Fig. 5A) that utilized pA₁₀CAT₂, a CAT expression clone useful for testing enhancer sequence activities (35). Since the CAT gene of this clone is linked to a SV40 promoter with deletion of its associated enhancer elements, addition of an enhancer-containing sequence to this clone should increase CAT expression. To facilitate the placement of a LTR fragment upstream of the SV40 promoter and also to standardize the plasmid background, the SV40 promoter-CAT gene (*Bgl*II-*Bam*HI fragment) of pA₁₀CAT₂ was transferred into a pUC19-derived plasmid. Figure 5B and C show the results of DNA transfection experiments using mainly the pE-CAT₂ constructs. In CCL64 cells, CAT expression of pIS-CAT₂ as well as p41-CAT₂ was more than 20-fold that of pA₁₀CAT₂, indicating that the IS of pAL10 LTR was as strong as the enhancer-containing segment of pWN41 LTR in enhancing the SV40 promoter activity in these mink cells. Comparison of pIS-CAT₂ and pISO-CAT₂ showed that IS was rendered less effective by placing it in the opposite orientation with respect to the SV40 promoter. In sharp contrast, CAT expression by pIS-CAT₂ was much lower in NIH 3T3 cells and was undetectable in HT1080 cells, although the enhancer-containing *Pst*I-*Mbo*I fragment of pWN41 LTR (p41-CAT₂ clone) showed strong activity in these cells. Also, in NIH 3T3 and HT1080 cells transfected with p10-CAT₂, which contains the IS and the upstream enhancer segments of pAL10 LTR, CAT expression was significantly increased. The enhancer-containing *Pst*I-*Mbo*I segment of pAL10 LTR by itself (p10dIS-CAT₂) was, however, virtually inactive in enhancing CAT expression by the SV40 promoter in NIH 3T3 cells or HT1080 cells. These results demonstrate that the IS of pAL10 LTR possesses potential enhancer elements for

FIG. 3. Functional determination of the promoter-carrying *BglII-SmaI* sequence segment of LTR in pAL10 proviral clone. CAT expression was determined in mouse NIH 3T3 (3T3) cells and mink CCL64 cells transfected 48 h previously with plasmid DNA of the PL-CAT series recombinants (Fig. 2B). Shown on the left are *PstI-SmaI* LTR fragments: 10L, the intact fragment of pAL10 proviral clone; 10dIS, with most of the distinct 190-bp IS (■) deleted from *MboI* (M) and *BglII* (B) sites; 10P, *BglII-SmaI* segment that carries CCAAC ("C") and TATAAA ("T") boxes; B5EP and 41EP, 10P linked to enhancer-carrying *PstI-MboI* segments of pWNB5 (▨) and pWN41 (▩) (4) LTRs, respectively. Shown on the right are corresponding autoradiograms of separated chloramphenicol (C) and the two monoacetylated chloramphenicol derivatives (AC); the ascending chromatographic direction is from left to right. With the CAT activities of p10L-CAT (percentages of conversion from chloramphenicol to monoacetylated chloramphenicol derivatives shown in parentheses) assigned as 1, relative activities (R.A.) of other plasmid constructs are indicated.

the SV40 promoter but the enhancer effect is probably cell specific.

Negative effect of sequences in the 5' region of MuLV-related LTRs. In the previous experiments, CAT recombinant clones that carry the 5' *PstI-MboI* fragment of pAL10 LTR all showed relatively low CAT expression in CCL64 cells and virtually no activity in NIH 3T3 cells (e.g., p10dIS-CAT versus p10L-CAT in Fig. 3 and p10dIS-CAT₂ versus pA10-CAT₂ in Fig. 5B). This was not expected since five of the six similar motifs of regulatory protein-binding sequences, disclosed by Speck and Baltimore (58), are present in this *PstI-MboI* fragment of pAL10 LTR, similar to those of the pWN41 and pWNB5 ecotropic MuLV LTRs, and yet the latter two are evidently much stronger enhancers (e.g., p41-CAT₂, Fig. 5B and C). To examine this phenomenon closely, homologous segments from the 700-bp LTR of pRFM16 (that contains five of the six motifs) and from the 750-bp LTR of pRFM9 (that contains all six motifs) were isolated as *PstI-HgiAI* fragments and linked to the 5' *PstI*

site of the pWN41 LTR that contained both the enhancer and the promoter elements. This addition of the *PstI-HgiAI* MuLV-related LTR fragment in *cis* caused a significant decrease in CAT expression by the pWN41 LTR (Fig. 6). The negative effect was more marked in NIH 3T3 cells than in CCL64 cells and with linking of the sequence in the same orientation rather than in the opposite orientation. The *EcoRV* site in the middle of the enhancer region was utilized to create a chimeric LTR clone, p9V/41-CAT, in which the downstream sequences of the *EcoRV* site were from pWN41 LTR and the upstream sequences were from pRFM9 LTR. This chimeric LTR transcriptional unit showed a drastic decrease in CAT expression activity, suggesting that a strong negative effect was present in the *PstI-EcoRV* fragment of pRFM9 LTR.

The presence of apparent negative elements in the 5' region of MuLV-related LTRs was confirmed by another experimental approach, in which the internal *EcoRV-BglII* fragments of the 750-bp LTRs of pRFM9 and pRFM17 were

FIG. 4. Functional determination of the distinct 190-bp IS of pAL10 LTR. Shown on the left are *PstI-SmaI* fragments of pL-CAT series plasmids: 41L, L (see legend to Fig. 2B) segment of pWN41 LTR with the enhancer-carrying domain (▨) and the promoter-carrying domain (▩); 10L, L segment of pAL10 LTR; ISP and ISOP, *MboI-BglII* IS fragment (■) linked to promoter-carrying *BglII-SmaI* segment in the same and opposite orientations, respectively; IS and ISO, *MboI-BglII* segment of IS in the same and opposite orientations. These sequence segments, if necessary, were subcloned in pUC19 to generate *PstI* and *SmaI* ends for segment replacement in the pL-CAT series of construction (Fig. 2B). Autoradiograms are shown horizontally. Percentages of acetylated chloramphenicol (AC, %) were calculated from direct measurements of radioactive spots in thin-layer plates, as described in Materials and Methods. Restriction enzyme abbreviations: P, *PstI*; M, *MboI*; B, *BglII*; S, *SmaI*. 3T3, NIH 3T3.

FIG. 5. Determination of enhancer activity of pAL10 LTR fragments for the expression of a SV40 promoter-carrying CAT gene. (A) Scheme used for construction of pE-CAT₂ series of molecular clones. The BglII-BamHI fragment of pA₁₀CAT₂ (35), which contains an enhancerless SV40 promoter, the CAT structural gene, and the SV40 polyadenylation site, was used to replace the gag BglII-BamHI fragment of p19A1 to generate p41-A₁₀CAT₂ that served as the precursor clone of the pE-CAT₂ series. The PstI-BglII fragment in p41-A₁₀CAT₂ can be easily replaced by a PstI-BglII or PstI-MboI fragment from each LTR of various proviral clones for testing the enhancer activity of these LTR fragments. An LTR sequence segment that lacks 5' PstI, 3' BglII, or BamHI ends was first inserted into the polylinker cloning site of a pUC vector to gain these restriction ends. Restriction site abbreviations: G, BglII; B, BamHI; P, PstI; M, MboI. (B and C) Autoradiograms of thin-layer chromatographic plates of two separate experiments showing CAT activities expressed in NIH 3T3 (3T3), HT1080, and CCL64 cells transfected 48 h previously with various pAL10 LTR fragment-containing recombinant plasmids as well as two control clones p41-CAT and pAL10-CAT (Fig. 2A). Percentage of acetylated chloramphenicol (AC, %) was determined as described in the legends to Fig. 3 and 4.

each replaced by the *EcoRV*-*MboI* fragment of pWNB5 LTR. The resulting two chimeric LTRs, 9LB5E and 17LB5E, remained as inactive as their respective parental MuLV-related LTRs, 9L and 17L (Fig. 7). Since the *BglII*-*SmaI* fragments of pRFM9 and pRFM17 LTRs serve as functional promoters, when associated with the enhancer elements of an ecotropic MuLV LTR (Fig. 3) and the *EcoRV*-*MboI* fragment of pWNB5 LTR contains most of these enhancer elements, it is evident that certain sequence elements within the *PstI*-*EcoRV* fragment of pRFM9 and pRFM17 LTRs were responsible for the depressed CAT expression by the chimeric LTRs.

DISCUSSION

In this study, we have employed the experimental approach of CAT gene expression (19, 35) to assess the transcriptional functions of LTR structures in noncancerous, nonxenotropic MuLV-related proviral sequences isolated from the mouse genome. Previously, two experimental efforts in this laboratory were unsuccessful in demonstrating the functional activity of these isolated endogenous proviral sequences. No virus production was obtained in NIH 3T3, CCL64, or HT1080 cells by transfection with pRFM6, a mouse genomic DNA clone that contains a full-length and apparently intact proviral sequence (46). Also, no virus was produced in NIH 3T3 cells transfected with a recombinant DNA clone in which the LTR of pWN41, an infectious DNA clone of WN1802N ecotropic MuLV (4), was replaced by the LTR of the pAL10 polytropic proviral DNA clone (51),

indicating that the pAL10 LTR was unable to complement the viral structural genes of pWN41 to produce a recombinant retrovirus. On the basis of our present results demonstrating little or no elevation of heterologous CAT gene expression by the LTRs of all seven MuLV-related proviral clones (Table 1), it is clear that transcriptional inactivity of the LTR is the reason for the apparent lack of direct retrovirus expression by MuLV-related proviral sequences introduced into these three cell lines in vitro.

In contrast, the LTRs of the two ecotropic MuLV clones were highly active for CAT gene expression in NIH 3T3, CCL64, and HT1080 cells. The finding that the pWNB5 LTR was severalfold more effective than the pWN41 LTR is consistent with our previous observation that NIH 3T3 cells transfected with a MuLV proviral DNA clone carrying the pWNB5 LTR produced 5 to 10 times more viruses than NIH 3T3 cells transfected with the same proviral DNA clone carrying the pWN41 LTR (5). The nucleotide sequences of these two ecotropic MuLV LTRs are essentially the same (4; also Fig. 8), except that the pWNB5 LTR has a tandem duplication of a 53-bp sequence corresponding to protein-binding motifs of *Lva-NFI-Lbv-core-Lvc*, as defined in the Moloney MuLV LTR (58). Thus, our results of CAT gene expression with the two ecotropic LTRs are consistent with the idea that these protein-binding motifs serve as positive enhancer elements in the transcriptional mechanism (2, 11, 25, 26, 35, 38). Indeed, highly infectious ecotropic MuLVs selected by serial cell or animal passages generally show duplicated enhancer sequences (4, 11, 15, 22, 37, 56, 62, 63),

FIG. 6. Inhibitory effect of 5' sequences of two MuLV-related proviral LTRs on CAT expression activity of the LTR of ecotropic MuLV pWN41 proviral clone. Depicted on the left are specific sequence segments of various pX-CAT clones (Fig. 2A) tested: 41 is the X (see legend to Fig. 2A) of pWN41, or the *Pst*I-*Bgl*II fragment that comprises the enhancer-carrying segment (▨), the promoter-carrying segment (▤), and the downstream leader sequences of this ecotropic MuLV; 16/41 or 160/41 indicates the X of pWN41 with its 5' end linked, in the same or opposite orientation, to the *Pst*I-*Hgi*AI fragment (▤) of pRFM16 LTR (*Pst*I is 38 bp and *Hgi*AI is 256 bp from the 5' end of this LTR, and this 218-bp fragment is homologous to the enhancer-carrying 5' sequences of ecotropic MuLV; see Fig. 8 for details); 9/41 and 90/41 are the similar combinational constructs of the X of pWN41 and the *Pst*I-*Hgi*AI fragment from pRFM9 LTR (▤); and 9V/41 is derived from 9/41 by deletion of sequences between the two homologous *Eco*RV sites, thus resulting in a chimeric enhancer sequence. The 3' adjacent IS subfragments (▤) of pRFM16 and pRFM9 are also shown. The three short bars under the *Mbo*I-*Sma*I promoter segment indicate the CCAAT, TATAA, and cap sites. The downstream *Sma*I-*Bgl*II segments of these pX-CAT clones are identical and hence not depicted. The CAT expression assay was as described in the legends of Fig. 3 and 4. Restriction enzyme abbreviations: P, *Pst*I; M, *Mbo*I; S, *Sma*I; V, *Eco*RV.

whereas LTRs of newly isolated endogenous ecotropic and xenotropic MuLVs contain only a single copy of the enhancer sequence (4, 28, 33, 42). In this regard, all reported MuLV-related proviral sequences, including the seven clones studied here, show no duplication or deletion of the enhancer sequence in the LTR (28, 46, 51, 61).

Nondefective promoter and enhancer elements. Like ecotropic MuLV LTRs, the MuLV-related proviral LTRs contain apparent promoter and enhancer consensus sequences in separate domains. It is therefore possible to examine the promoter function and the enhancer function separately by employing three LTR fragments in the con-

FIG. 7. CAT expression activity of pL-CAT recombinant plasmids carrying LTR promoter and chimeric enhancer fragments of an ecotropic MuLV and two proviral clones of the 750-bp LTR modified polytropic class. Depicted on the left are *Pst*I-*Sma*I fragments of various pL-CAT clones (Fig. 2B): B5L and 41L are from pWNB5 and pWN41 ecotropic MuLV proviral DNA clones; RF7L is from pRFM7 solitary LTR (33); 9L is from pRFM9 LTR; 9LB5E is a chimeric fragment derived from 9L with its *Eco*RV-*Bgl*II segment replaced by the *Eco*RV-*Mbo*I segment of pWNB5 LTR; 17L is from pRFM17 LTR; and 17LB5E is a similar chimeric fragment from pRFM17 and pWNB5 LTR sequences. CAT expression assay was performed as described in the legends of Fig. 3 and 4. Restriction enzyme abbreviations: P, *Pst*I; V, *Eco*RV; M, *Mbo*I; S, *Sma*I; B, *Bgl*II.

FIG. 8. Comparison of nucleotide sequences of the enhancer and the adjacent 5' and 3' areas in the LTR U₃ region of two ecotropic MuLV clones, pWN41 and pWNB5 (4; our unpublished data); xenotropic-like solitary LTR of pRFM7 (33); two polytropic class MuLV-related proviral clones, pAL10 (51) and pRFM6 (46); and two modified polytropic class MuLV-related proviral clones, pRFM1 and pRFM17. Protein-binding motifs (boxed) of the LTR enhancer in Moloney MuLV (58) are also included for comparison. All nucleotides of the pWN41 sequence are shown; of the other LTR sequences, only the different nucleotides are indicated, while the same nucleotides are indicated as dots and deleted nucleotides are indicated as dashes. The tandemly duplicated 53-nucleotide sequence in pWNB5 is indicated by brackets. The characteristic 14-nucleotide direct repeats in all four MuLV-related LTRs are boxed. The restriction enzyme sites used for molecular construction are indicated and underlined.

struction of CAT recombinant clones. We have found that the promoter-signal-carrying fragment of pAL10 MuLV-related LTR in combination with the enhancer-carrying fragment of pWN41 ecotropic MuLV LTR was able to strongly support CAT expression. Thus, the two specific structural features of this sequence fragment, namely the pentanucleotide CAGCC tandem duplication immediately upstream of the TATAA box and the CCAAC box (instead of the CCAAT box), appear to cause no serious defect in the promoter function. The CAGCC duplication is also found in the pRFM7 solitary LTR (33), which was active in supporting CAT expression in NIH 3T3 and CCL64 cells (RF7L in Fig. 7).

The most prominent feature of the LTR in endogenous MuLV-related proviral sequences is the 190- to 200-bp IS, located between the enhancer domain and the CCAAC promoter box in the U₃ region (28, 39, 51). The particular location as well as its absence in transcriptionally active ecotropic and xenotropic MuLV LTRs would suggest a negative role played by IS. On the contrary, our experimental results (Fig. 4 and 5) clearly demonstrate that IS can function as a positive enhancer element. The positive en-

hancer activity of IS, although quite marked in association with the SV40 promoter sequence and by measurement in CCL64 cells, was not as conspicuous with its own LTR promoter sequence (the *Bgl*II-*Sma*I fragment [Fig. 4]) and when assayed in NIH 3T3 and HT1080 cells. The reason for the variation of positive enhancer activity in these three cell lines is not clear. In addition to being found within the MuLV-related LTRs, IS constitutes the 5' half of the LTR of another distinct class, called LTR-IS (55), which is moderately repeated in 2,000 to 4,000 disperse copies, largely as a solitary LTR, in the mouse genome. In a recent study (31), LTR-IS was found to be unable to support CAT gene expression in NIH 3T6 cells but was active in cell line 293 that produced the adenovirus Ela protein, implying that LTR-IS requires certain *trans*-activating factor for its transcriptional function. It is therefore possible that a similar *trans*-activating factor required for the positive enhancer activity of IS, particularly in combination with the SV40 early promoter, may be present in CCL64 cells but not in NIH 3T3 or HT1080 cells. Alternatively, the latter two cells may contain a negative factor specific for controlling the

transcriptional activity of IS. These alternative possibilities remain to be investigated.

Negative regulatory element in the endogenous MuLV-related LTR. With the demonstration of positive transcriptional functions in the two fragments of the 3' portion (i.e., the promoter and IS), it became obvious that the remaining fragments from the 5' portion of the U3 region were responsible for the apparent transcriptional inactivity of the endogenous MuLV-related LTRs. Subsequent experiments (Fig. 6 and 7) indeed revealed a negative effect exerted by this LTR fragment in depressing transcriptional activity of the positive elements. Although the molecular basis of the *cis*-acting negative effect remains to be investigated, this observation is significant since it implies that the LTRs of endogenous MuLV-related proviral sequences may not be neutral or defective but rather represent functional transcriptional units possessing a negative element for controlling their expression. In this regard, they are similar to many important cellular genes, such as *c-mos* (64), *c-myc* (53), and *p1450* gene (17), the interferon genes (16, 18, 34) and the insulin gene (36, 48), all of which carry a strong negative regulatory element at the 5' region upstream of the promoter sequence. Similar specific *cis* sequence elements functional in negative control of gene expression in the absence of glucocorticoid have also been found in the LTR of mouse mammary tumor virus (45, 50, 52), in the enhancer sequence of Moloney MuLV LTR introduced into undifferentiated embryonic stem cells (20), and in the LTR of human immunodeficiency virus (54). Another well-noted example of genes under negative regulation is the yeast mating type loci, *HML* and *HMRI*, the *cis* regulatory element of which, *HMRE*, is named a silencer sequence to indicate its ability to repress transcription relatively independent of the position and the orientation with respect to the affected promoter (6). (This term is not used for the 5' fragment of MuLV-related LTRs, since this LTR fragment appeared to be not completely position independent and was more effective in the same than in the opposite orientation.) It appears that negative *cis* sequence elements are generally detected in cellular genes that are involved in adaptive responses to internal signal changes or external stimuli (e.g., growth factor and hormone exposure). Also, the regulation of gene expression in this regard is presumably mediated through interaction of the sequence of the negative element with corresponding specific *trans*-acting factor(s). Whether the negative regulatory element of endogenous MuLV-related proviral sequences utilizes a similar molecular mechanism remains to be investigated.

Our experimental results clearly imply that the negative regulatory element is located within the sequence of the *PstI-EcoRV* fragment of MuLV-related LTRs. The 128- or 123-nucleotide sequence of this fragment contains the 5' portion of the enhancer (the *Lva-NFI-Lvb* binding motif), a duplicated 14-nucleotide segment, and the upstream ≈ 90 nucleotides. Comparison with corresponding regions of ecotropic MuLV and solitary LTRs (Fig. 8) suggests the CAGTTAAAGATCAA duplication as the most likely candidate for the negative element. However, the slightly divergent enhancer protein-binding motifs located on the 5' portion of enhancer region cannot be ruled out, since a single base change has been shown to significantly alter enhancer function (e.g., 11, 43). Further work is in progress to determine the precise identity of the negative element.

One characteristic aspect of enhancer sequence function is cell or tissue specificity (for a recent review, see reference 30). In particular, the leukemogenic potential of MuLVs for the induction of specific types of lymphohematopoietic neo-

plasms has been found to be associated with the LTR function (7, 9, 14, 24, 37, 57, 65). Therefore, the biological significance of our experimental results for the MuLV-related LTRs should be limited to the three lines of cultured cells used for measuring CAT expression, which presumably represent different mesenchymal cell lineages. In NIH 3T3 cells, there are at least 30 individual copies of MuLV-related proviral sequences contained within the chromosomes, but no RNA transcripts of these proviral sequences were detected (our unpublished data). Expression of endogenous MuLV-related proviral sequences, however, has been found in certain organs or specific tissues of normal adult mice, including liver (27, 40), epididymis (39), kidney, endometrium (our unpublished data), and thymus (27). In an *in vitro* study, U3 sequences of a cDNA clone of such transcripts were shown to support CAT expression in the SSLC cultured cells of the 129 strain mouse origin (41). These observations, together with ours, indicate that regulation of endogenous MuLV-related proviral expression in the mouse is complex. This is significant since the specific *env* gene sequences of MCF viruses, known to be associated with the leukemogenic process, are presumably derived from endogenous MuLV-related proviral sequences (10, 12, 21, 24, 29, 46, 61). In this regard, whether the negative regulatory element disclosed in the present study plays a role in controlling the generation of MCF recombinant viruses should be further studied.

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