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Germ Line Integration of a Murine Leukemia Provirus into a Retroviruslike Sequence

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Nucleotide sequence analysis of the cellular sequences flanking the integrated ecotropic (mouse-infectious) murine leukemia provirus of BALB/c mice indicated that the murine leukemia provirus is integrated in opposing transcriptional orientation within a solo long terminal repeat (LTR) of the VL30 family of endogenous retrovirus-related sequences. To quantify the effect of this integration event on the ability of the ecotropic provirus to be expressed, we constructed recombinant molecules that carried the chloramphenicol acetyltransferase (*cat*) gene and various viral LTRs and determined the CAT activity induced by these constructs after transfection of NIH 3T3 cells. Our results indicate that the BALB/c ecotropic LTR is about 10-fold more active than the VL30 LTR. The presence of the VL30 LTR did not affect the transcriptional activity of the ecotropic LTR in the context of the integration event. Our results also indicate that the LTRs of the BALB/c provirus are less transcriptionally active than are the proviral LTRs of AKR murine leukemia virus and the Harvey murine sarcoma virus.

Endogenous murine leukemia viruses (MuLV) and MuLV-related sequences are integrated in abundant numbers with no apparent target-site preference throughout the germ line of inbred mice (3, 36). Endogenous ecotropic MuLV vary in copy number and pattern of expression among inbred strains. Low-leukemic inbred strains, such as BALB/c and C57BL/6, express little or no ecotropic virus until late in life, whereas high-leukemic strains, such as AKR, express ecotropic virus before birth and throughout life (27, 30, 32, 33, 35, 36, 38, 39). Likewise, embryo cells derived from mice of high leukemic strains express endogenous ecotropic virus with greater frequency than do cells derived from mice of low-leukemic strains (27–29). The molecular bases for these differences in viral phenotype are incompletely understood. Despite the observation that ecotropic proviruses recovered from unrelated mouse strains differ from one another in less than 0.5% of their sequenced nucleotides (6, 17), various mutations that inhibit viral functions have been identified in the proviruses carried in several low-leukemic mouse strains (1, 4, 5, 17). Results of restriction enzyme and DNase digestion experiments suggest that methylation patterns of proviral and proximal cell sequences, chromosomal position of proviruses, and chromatin structure also affect provirus expression in *cis* (2, 10, 11, 14, 21, 44).

Recently, two independent molecular clones of the endogenous ecotropic provirus of BALB/c mice have been isolated and characterized for their biological activity in transfection experiments (17). The specific infectivities of the BALB/c-derived proviral clones were 1/10 of that of the AKR-derived proviral clone p623 (25). Moreover, virus recovered after transfection of cells with the BALB/c-derived clones grew to 100-fold lower titers than did virus recovered from cells transfected with the AKR clone. The BALB/c-derived virus was incapable of inducing syncytia formation of XC cells, a hallmark of ecotropic retroviruses. The XC-negative phenotype of the BALB/c ecotropic virus was shown to be determined by a single amino acid substitution at the proteolytic processing site of the envelope precursor protein $Pr85^{env}$ (17).

Nucleotide sequence analysis of the BALB/c proviral long terminal repeats (LTRs) indicated that the virus contained a single copy of the enhancer-containing sequences repeated twice in the LTRs of the AKR clone and that other regulatory signals, such as the viral promoter, were identical to those found for the AKR-derived virus (12, 17). To determine the relative ability of the BALB/c viral LTRs to direct transcription of a linked gene, we compared their ability to promote the transcription of the bacterial chloramphenicol acetyltransferase (*cat*) gene with that of other MuLV LTRs. We also noted that the cell DNA immediately proximal to the BALB/c provirus was homologous to the LTRs of a retrovirus-related element, VL30. Analysis of the sequence data indicated that the BALB/c endogenous ecotropic provirus is integrated within the R region of a VL30 LTR, in opposite transcriptional orientation. To quantify the effect of the VL30 LTR sequences on the expression of BALB/c ecotropic virus, we used several *cat*-gene constructs containing these linked VL30 sequences.

MATERIALS AND METHODS

DNA cloning and sequencing. The cloning of the BALB/c endogenous ecotropic provirus (p7D) has been previously described, as have the methods used for the acquisition of nucleotide sequence data (17). Briefly, fragments of the BALB/c proviral clones were subcloned into M13mp10 or M13mp19 and sequenced by the dideoxy chain termination method of Sanger et al. (40). [α-^{35}S]dATP and 6% acrylamide–8.3 M urea sequencing gels (thickness, 0.4 mm) were used (17).

Plasmids used for preparation of *cat*-gene constructs were derivatives of two previously reported clones, pSV2Cat and pA10Cat2 (8, 24). pJE120 and pJE119 are plasmids containing the Harvey murine sarcoma virus (Ha-MSV) LTR (clone pH1) upstream of the bacterial *cat* gene and a *cat* gene cloned into pUC12 lacking transcriptional control sequences, respectively. pJE120 and pJE119 were kindly provided by Janet Embretson and Howard Temin, University of

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Wisconsin, Madison. The restriction endonuclease cleavage sites used in cloning segments from the BALB/c ecotropic provirus (p7D) and adjoining VL30 sequences, referred to as VLeco, are diagramed (see Fig. 2). The pAKR$^{+/-}$ *cat*-gene constructs contain a 541-base-pair (bp) fragment of p623 (25) extending from a *Pst*I site in the U3 region of the proviral LTR to a *Sma*I site in the R region, cloned in both orientations upstream of the *cat* gene. The pBALB/c$^{+/-}$ *cat*-gene constructs contain an analogous 442-bp fragment from p7D cloned in either orientation upstream of the *cat* gene. The pVLeco$^{+/-}$ *cat*-gene constructs carry a 350-bp *Sac*I-*Xba*I fragment containing the VLeco U3 and R sequences cloned in both orientations upstream of the *cat* gene. To prepare pB$^+$V$^-$ and pB$^-$V$^+$, a 1,500-bp *Xba*I fragment was recovered from the 3′ end of p7D. This fragment extends from the 3′ end of the BALB/c ecotropic provirus through the ecotropic LTR and most of the oppositely oriented VLeco LTR. The *Xba*I fragment was cloned in both orientations 5′ to the *cat* gene. Each of the above *cat*-gene constructs uses simian virus 40 early-region polyadenylation signals inserted at the 3′ terminus of the bacterial *cat* gene.

The pCAT-BALB/VL$^+$ constructs contain the regulatory signals of the BALB/c provirus and adjoining VLeco sequences. The *Xho*I site in the VLeco U5 region is the 5′ boundary of these constructs, and the *Xba*I site in the VLeco U3 region is the 3′ boundary. Ecotropic viral sequences between the *Bgl*II site at nucleotide 316 and the *Bgl*II site at position 7417 were deleted. The *cat* gene was inserted in either orientation as a *Hin*dIII fragment after removal of the p7D *Bgl*II sites and replacement with *Hin*dIII linkers. To prepare pCAT-BALB/VL$^-$, a 350-bp *Xba*I-*Sac*I fragment encompassing the VLeco LTR sequences proximal to the 3′ end of the BALB/c provirus was deleted from pCAT-BALB/VL$^+$. These three constructs are presumed to use polyadenylation signals encoded in the BALB/c ecotropic 3′ LTR.

Computer-assisted nucleotide sequence analysis. Sequence data were compiled and analyzed by using the programs of Staden (43) and additional programs provided by the University of Wisconsin Genetics Computer Group. Searches for sequence homology were performed on the National Institutes of Health GenBank sequence database.

***cat*-gene assays.** NIH 3T3 cells were transfected with *cat*-plasmid constructions by the methods of Graham and Van der Eb (9), as modified by Hopkins et al. (15). Briefly, various quantities of plasmid constructions without carrier DNA were suspended in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline and raised to 0.125 M in $CaCl_2$. DNAs were added to duplicate plates of chloroquine-treated (26) NIH 3T3 cells seeded the previous day at 5×10^5 cells per 100-mm dish in 10% bovine serum–Dulbecco medium supplemented with 1 mM sodium pyruvate and antibiotics. After 6 h of incubation at 37°C, cells were rinsed with growth medium and shocked for 4 min with 15% glycerol. After incubation for 48 h, cell lysates were prepared and assayed for CAT activity, as described previously (8). CAT reaction mixtures contained 0.025 ml of cell lysate, 0.020 ml of 4 mM acetyl coenzyme A, 0.071 ml of 1 M Tris (pH 7.8), 0.001 ml of [^{14}C]chloramphenicol (0.1 μCi; 45.5 mCi/mmol; 0.05 mCi/0.5 ml; New England Nuclear Corp., Boston, Mass.), and 0.035 ml of H_2O. Reaction mixtures containing purified bacterial CAT (0.05 U; P-L Biochemicals, Inc., Milwaukee, Wis.) in place of cell lysates were performed in parallel as positive controls. Reactions were performed for 15, 30, or 60 min at 37°C and were terminated by the addition of 1 ml of ethyl acetate. Reaction mixtures were vortexed and centrifuged, and the organic phase was dried in vacuo and suspended in 10 μl of ethyl acetate before being spotted onto thin-layer chromatography plates (Polygram Sil G/UV; Macherey Nagel & Co., Düren, Federal Republic of Germany). Samples were subjected to ascending chromatography in $CHCl_3$-methanol (95:5) at room temperature and were then air dried. Thin-layer chromatography plates were sprayed with En^{3}Hance (New England Nuclear) and were exposed overnight to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen (Lightning-Plus; Du Pont Co., Wilmington, Del.) at −70°C. After the film was developed, samples were scraped into scintillation vials, radioactive chloramphenicol and acetylated derivatives were solubilized by the addition of 1 ml of ethyl acetate, and radioactivity was determined. The percentage of input chloramphenicol acetylated in each reaction was calculated by determining the total number of counts appearing as acetylated derivatives and dividing by the total number of spotted sample counts. Samples were normalized for equal numbers of cells or equal concentrations of protein. Activity in each lysate was shown to be linearly dependent on time of incubation.

RESULTS

Nucleotide sequence analysis and structure of the proviral integration site. The single, endogenous ecotropic provirus of BALB/c mice has also been inherited by other inbred strains of mice related in pedigree (16, 22, 27). To determine whether a mouse gene of interest may have been interrupted by the integration of the ecotropic provirus into the germ line of the progenitors of BALB/c-related mice, we sequenced 600 bp of cell DNA flanking the 3′ terminus of the BALB/c provirus and 200 bp flanking the 5′ terminus. A search for sequence homology within the National Institutes of Health GenBank Sequence Database indicated that the DNA immediately flanking each end of the BALB/c ecotropic provirus contained significant homology to the LTR of VL-3, a proviral clone of the polymorphic, retrovirus-related element VL30 (19). The VL30 LTR-related sequence that we identified was designated VLeco because of its close proximity to the endogenous ecotropic provirus of BALB/c mice. The LTR sequences of VL30 clones Bvl-1 (13), VLS-1 (37), NVL-3 (31), and VL11 (20) are 85 to 93% homologous with the VLeco LTR sequence outside of gaps inserted to facilitate maximal alignment. Figure 1 shows a nucleotide sequence comparison between the VLeco LTR and the LTR of VL30 proviral clone VL11 (20). The sequences in Fig. 1 are 93% homologous if gaps inserted for computer sequence alignment are excluded from consideration. The VLeco sequence contains putative transcriptional control elements analogous to those described for retroviral LTRs (boxed regions in Fig. 1). Putative promoter signals, CAAGTT and TATATAA boxes, are found in the U3 portion of the VLeco LTR, and the potential polyadenylation signal TATTAAA is positioned within the VLeco R region. In addition, sequences similar to those described as enhancer elements may be found within direct repeats in the VLeco U3 region. The VLeco sequence is characterized by imperfect inverted repeats at the termini of the element and by several imperfect direct repeats within the VLeco U3 and R regions. Integration of the BALB/c provirus within the VLeco LTR occurred within one of two 40-bp imperfect repeats in the VLeco R region (stippled box in Fig. 1).

Restriction enzyme mapping of the DNA flanking the BALB/c ecotropic proviral clones suggested that the VLeco

LTR sequences were not linked to VL30 structural gene sequences. Southern blots of restriction enzyme digests of the BALB/c proviral clones were hybridized with a genome-length VL30 probe, clone VL-3, to confirm that the VLeco element is a solo LTR sequence (19). Consistent with the solo LTR structure of the VLeco element, hybridization was observed only for those cell DNA restriction fragments proximal to the BALB/c provirus (data not shown). Further sequence analysis confirmed the structure of the VLeco element by identifying a 4-bp direct repeat of cell DNA, TGGG, that flanks the termini of VLeco, presumably generated during the integration of the VLeco element into flanking cell DNA (Fig. 2). Additional sequencing has also established that LTR-proximal, VL30-related sequences

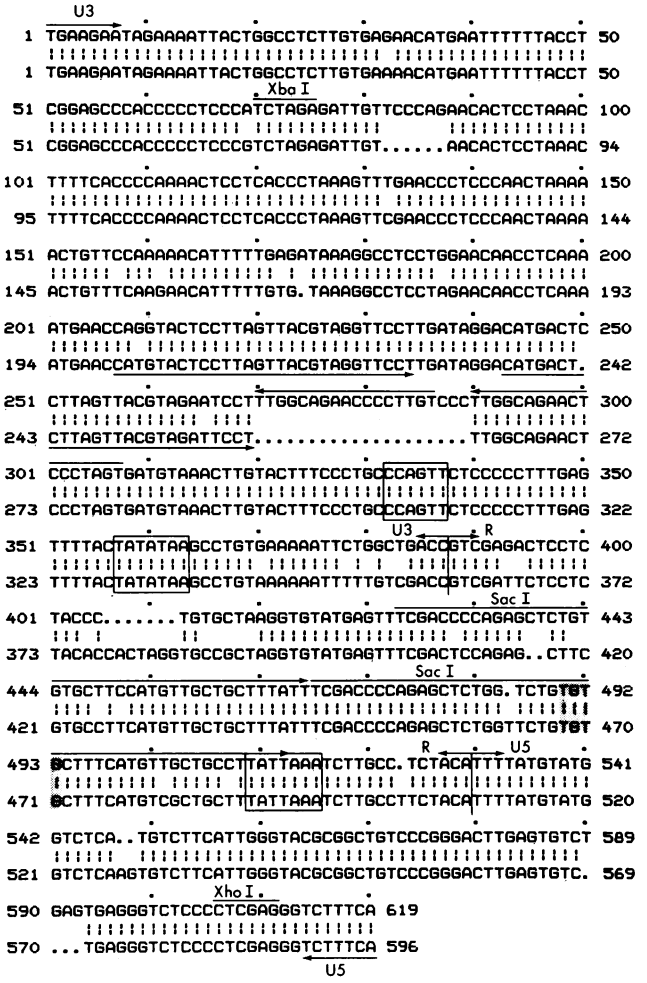

FIG. 1. Sequence comparison between VLeco and VL11 LTRs. The sequence of the VLeco LTR is shown on the top line of each row, the VL11 sequence is shown on the bottom line of each row (20). The U3, R, and U5 regions of the VL30 LTRs are illustrated, and boxes enclose putative transcriptional control sequences (CAAT and TATA boxes in U3 and polyadenylation signals in R). Imperfect inverted repeats at the termini of the VL30 LTRs are represented by opposing arrows. Imperfect direct repeats found within U3 and R regions of the VL30 LTRs are represented by coincident arrows. The site of integration of the BALB/c endogenous ecotropic provirus, indicated by darker letters, is within the R region. Gaps inserted for maximal alignment are indicated (·). In addition, several restriction endonuclease cleavage sites are indicated.

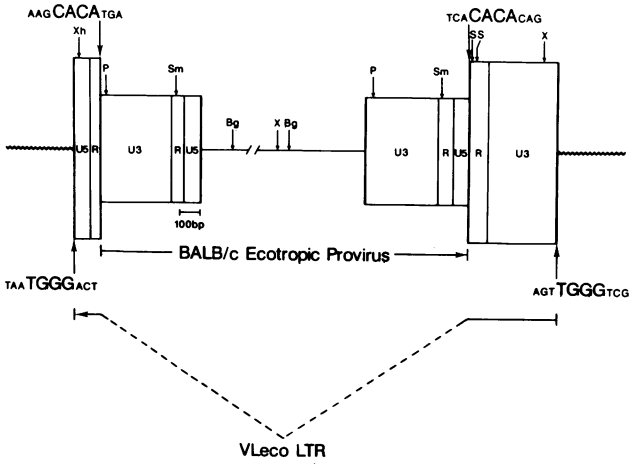

FIG. 2. Schematic illustration of the juxtaposition of BALB/c ecotropic provirus and VLeco sequences. Sequences of the BALB/c ecotropic proviral LTRs are represented by smaller boxes, and VLeco sequences are represented by larger boxes. The sequences that occur at the junction of ecotropic and VL30 sequences and the sequences that occur at the junctions of VL30 and cellular sequences are indicated. The 5′ end of the BALB/c provirus is drawn on the left side. The positions of restriction endonuclease cleavage sites used in construction of CAT-expression plasmids are indicated as follows: P, *Pst*I; Sm, *Sma*I; X, *Xba*I; Bg, *Bgl*II; Xh, *Xho*I; S, *Sac*I.

such as the retroviral primer-binding site and polypurine tract do not flank the VLeco LTR (data not shown).

Two regions of sequence homology were identified among the LTRs of the BALB/c ecotropic provirus and VLeco. A 53-bp sequence of VLeco DNA beginning within the integration site of the BALB/c provirus is approximately 70% homologous to a similar-sized sequence within the U3 portion of the BALB/c proviral LTR (nucleotides 7891 to 7942 by the numbering system of Herr [12]; Fig. 3A). In addition, a 33-bp sequence at the U5 terminus of the VLeco LTR is approximately 70% homologous with the terminal 36 bp of the BALB/c proviral LTR (nucleotides 107 to 142; Fig. 3B). Computer-assisted randomization of these homologous sequences in six trials did not yield the degree of homology found here; therefore, it is unlikely that these regions of homology result solely from similarities of base composition.

Figure 2 shows the structure of the locus composed of the BALB/c ecotropic provirus, VLeco sequences, and flanking cell DNA. Integration of the BALB/c ecotropic virus occurred within the R region of the VLeco LTR such that the provirus and VLeco LTR elements are in opposing transcriptional orientation. The 4-bp direct repeat of VLeco DNA, CACA, that flanks the ecotropic provirus presumably results from the BALB/c proviral integration event. It is readily apparent from the structure of the BALB/c provirus-VLeco locus that transcription from the LTR of one element could affect the transcription of the other. To establish whether the putative transcriptional control elements within the VLeco LTR were capable of directing transcription and to determine their transcriptional strength relative to the BALB/c proviral LTRs, *cat*-gene constructs were prepared and tested in transfection experiments.

Quantitation of the transcriptional activity of the BALB/c proviral and VL30 LTRs. To quantify the relative transcriptional potential of regulatory signals found within the BALB/c provirus and VLeco LTRs, we used *cat*-gene

A.

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VLeco LTR 438   ACAGACCAGAGCTCTGGGGTCGAAATAAAGCAGCAACATGGAAGCACACAGAG 490
                |  | ||||||||||     | ||   |   |||  ||   ||||  |||||  |||  ||||
BALB/c LTR 7891 AAATACCAGAGCTG•ATGTTCTCAGAAAAACAAGAACAAGGAAGTACAGAGAG 7942
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B.

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VLeco LTR 591   TCTGAGTGAGGGTCTCCC•••CTCGAGGGTCTTTCA 623
                || |||||| | ||  ||   ||  | ||||||||||
BALB/c LTR 107  TCAGAGTGATTGACTGCCCAGCTTGGGGGTCTTTCA 142
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FIG. 3. Sequences of homology between the LTRs of the BALB/c endogenous ecotropic provirus and VLeco. (A) A 53-bp sequence of the ecotropic proviral U3 region is aligned with a homologous sequence in the VLeco R region. (B) A 35-bp sequence of the ecotropic proviral U5 region is aligned with a homologous sequence in the VLeco U5 region. Ecotropic viral sequences are numbered beginning with 1 at the 5′ terminus of the viral genome as suggested by Herr (12); VLeco sequences are numbered from 1 beginning at the 5′ terminus of U3.

constructs carrying these LTRs. For comparison, we also quantified the activity of regulatory signals within the AKR-MuLV and Ha-MSV LTRs. A 350-bp *Xba*I-*Sac*I fragment (Fig. 2) containing putative enhancer and promoter sequences from the VLeco U3 and R regions was subcloned in both orientations upstream from the bacterial *cat* gene. Lysates from NIH 3T3 cells transfected with these constructs were assayed for enzymatic activity (pVLeco$^+$ and pVLeco$^-$ in Fig. 4 and 5). Similar constructs containing the LTRs from Ha-MSV (pJE120), the BALB/c endogenous ecotropic provirus (pBALB/c$^+$ and pBALB/c$^-$), or the LTR of the AKR-derived provirus p623 (pAKR$^+$ and pAKR$^-$) were transfected in parallel, as were *cat*-containing plasmids containing simian virus 40-derived elements (pSV2Cat and pA10Cat2).

Transcription directed by the VLeco U3-R-region construct is weak relative to that of the simian virus 40- or Ha-MSV-derived constructs (Fig. 4), typically ranging from 1 to 3% of the activity of pJE120 and about 10% of pSV2Cat (Fig. 5). As would be predicted, the VLeco-derived *Xba*I-*Sac*I fragment in reverse orientation was shown to be negative in CAT assays (data not shown). The BALB/c proviral LTR construct, pBALB/c$^+$, exhibited a relatively high level of transcription of the linked *cat* gene, 24 or 28% of that seen for Ha-MSV or AKR-derived LTRs, respectively (Fig. 5). Transcription from the pAKR$^+$ construct approximates that of pJE120, attaining about 85% of pJE120 activity (Fig. 5). As mentioned above, the major structural difference between the LTRs of the BALB/c- and AKR-derived proviruses is that the BALB/c proviral LTRs contain a single copy of the enhancer-containing sequences repeated twice in the LTRs of p623 (12, 17).

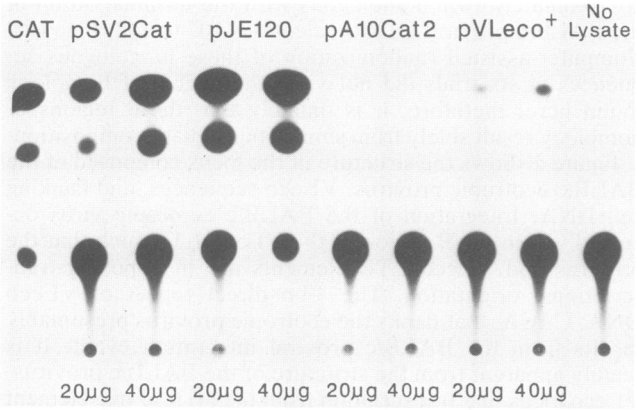

FIG. 4. Autoradiogram of a typical CAT assay performed with lysates prepared from transfected NIH 3T3 cells. Plasmids were transfected into NIH 3T3 cells, and lysates were tested for CAT activity, as described in Materials and Methods. The origin of each plasmid is described in Materials and Methods, and a diagram of each plasmid is presented in Fig. 5. The construct pa10CAT lacks simian virus 40 enhancer sequences (24). The amount of DNA used to transfect each 100-mm-diameter plate from which the lysate was prepared is indicated below each lane. Control CAT reactions containing purified bacterial CAT enzyme and no cell lysate are indicated (CAT and No Lysate, respectively).

Quantitation of gene activity in the context of converging LTRs. Although pVLeco$^+$ was weakly capable of directing transcription of a linked *cat* gene, its transcriptional ability might be influenced by the presence of nearby ecotropic MuLV enhancer-containing sequences. To establish whether the results obtained with the pVLeco$^+$ construct accurately reflect the transcriptional strength of the VLeco

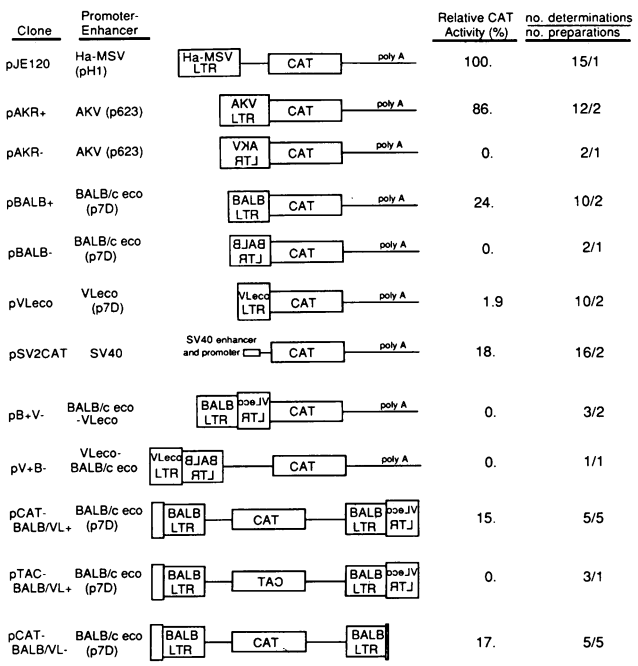

FIG. 5. Transcriptional activities associated with ecotropic and VL30 LTRs. The names, sources of the 5′ regulatory regions, and structures of the transfected plasmids are indicated. CAT acetylation values are standardized to the value found for 20 μg (5×10^5 cells) of pJE120 in each experiment. The number of plasmid preparations of each construction tested is indicated as is the number of independent transfections performed per plasmid construct. The CAT value is the mean of all preparations, and the range of relative values did not deviate more than 20% from the mean value.

element when in close proximity to the BALB/c proviral LTR, two additional *cat*-gene constructs were tested (pB^+V^- and pB^-V^+; Fig. 5). A 1,500-bp *Xba*I fragment containing all of the 3′ ecotropic proviral and VLeco sequences of interest was cloned upstream of the bacterial *cat* gene in both orientations. In one construct (pB^+V^-), the ecotropic 3′ LTR is in the sense orientation relative to the *cat* gene, and antisense VLeco sequences lie between the proviral LTR and the *cat*-gene translational start site. In the opposite construct (pB^-V^+), the proviral 3′ LTR is in an antisense orientation relative to the *cat* gene and lies between the VLeco LTR and the *cat*-gene translational start site. Transcription by pB^+V^- would be expected to be directed by the BALB/c proviral LTR and that of pB^-V^+ by the VLeco LTR. When transfected into NIH 3T3 cells, neither plasmid was capable of inducing detectable quantities of acetyltransferase activity (Fig. 5). Although it is not possible to interpret these negative results without evidence that RNA synthesis was initiated by either construct, it is apparent that the juxtaposition of these LTRs is not functionally equivalent to either LTR alone. The inability of pB^+V^- or pV^+B^- to direct *cat*-gene expression could result from effects on RNA transcription, stability, or translation, possibilities which are under investigation.

To determine whether the VLeco LTR could regulate expression of the BALB/c ecotropic provirus in the context in which it occurred in vivo, i.e., at the 3′ terminus of the ecotropic provirus, the following constructions were prepared. A pair of *cat*-gene-containing defective retroviruses were constructed such that each contained the BALB/c ecotropic proviral 5′ and 3′ LTRs, and most of the viral structural gene sequences had been replaced with the *cat* gene. In addition, one construct contained, in opposite transcriptional orientation, the VLeco LTR adjacent to the proviral 3′ LTR. If transcription directed from the VLeco LTR was capable of interfering with the 5′ LTR-initiated *cat*-gene message, one would predict a resulting diminished quantity of CAT protein in cells transfected with the construct containing VLeco sequences. The activity of these two constructs is not significantly different when the average for five independent plasmid preparations is calculated (Fig. 5). Therefore, we conclude that the presence of a VL30 LTR at the 3′ terminus of the BALB/c ecotropic provirus does not have a significant effect on transcriptional activity in these DNA transfection experiments.

DISCUSSION

Analysis of 800 bp of cellular DNA proximal to the single, endogenous ecotropic provirus of BALB/c mice showed that the provirus is integrated within a retroviruslike, repetitive sequence of mice, a VL30 LTR. Other VL30 sequences such as the viral primer-binding site, polypurine tract, and structural gene sequences were not found to be linked to the VLeco LTR; therefore, VLeco is a member of a previously described family of VL30 solo LTRs (37). The BALB/c provirus was found to be integrated within the R region of the VLeco LTR, resulting in ecotropic and VL30 sequences juxtaposed in opposing transcriptional orientations. The apposition of VLeco and ecotropic proviral sequences suggested that transcription of one element could interfere with expression of the other. To test this possibility, we quantified the relative ability of the VLeco and BALB/c ecotropic LTRs to direct the transcription of a linked *cat* gene. Our results indicate that the BALB/c ecotropic LTR is approximately 10-fold more efficient at directing expression of a linked *cat* gene than is the VLeco LTR in transient assays in NIH 3T3 cells. The VLeco LTR weakly stimulated the synthesis of a linked *cat* gene to levels comparable to those of other VL30 LTRs of similar sequence (E. Keshet, personal communication). In our experiments, VLeco was not able to modulate the expression of a distant 5′ LTR, and hence, it is unlikely that the integration of the BALB/c provirus within VLeco results in a significant diminution of proviral specific infectivity, at least in the cell type tested.

In a previous report (17), we demonstrated that the specific infectivity of the BALB/c endogenous ecotropic provirus is approximately 1/10 that of a prototype ecotropic provirus derived from AKR mice, p623. This diminished infectivity presumably reflects both the determinants of expression of the BALB/c proviral clone and the ability of expressed virus to replicate in culture and be detected. In this report, we show that the BALB/c endogenous ecotropic LTR is only one-third as effective at promoting gene activity as is an LTR derived from the AKR provirus p623. Recent results of RNase protection experiments performed in our laboratory have demonstrated that there is also a threefold difference in the ability of the AKR and BALB/c viral genomes to be transcribed (B. Berson and R. Risser, unpublished observations). As such, the threefold difference in the ability of the two viral LTRs to promote transcription is likely to be a major contributor to the lessened infectivity of the BALB/c provirus. Each LTR of the BALB/c provirus contains a single copy of the enhancer-containing sequences repeated twice within each LTR of the AKR provirus (12, 17). Transfection experiments done by other researchers studying the Moloney MSV LTR indicate that LTRs with repeated enhancers are approximately threefold more efficient at promoting transcription than are LTRs with a single enhancer (23), and our results are entirely consistent with those observations. Our results also indicate that the endogenous-derived AKR LTR is nearly as effective as the exogenous-derived Ha-MSV LTR in promoting expression in NIH 3T3 cells, although their nucleotide sequences are only 80% conserved. This observation indicates exogenous and endogenous MuLV LTRs do not differ greatly in their promoter-enhancer strengths, unlike LTRs of exogenous and endogenous avian retroviruses (7).

Although the diminished activity of the BALB/c proviral LTRs to express linked genes is important for the lessened specific infectivity of the BALB/c provirus, other viral and cellular determinants are likely to contribute to the overall levels of ecotropic virus expression in BALB/c and related animals. Sequence analysis of the BALB/c ecotropic provirus indicated that it suffered a mutation at the site of envelope precursor processing, and metabolic studies of envelope gene expression in cells has confirmed that the envelope precursor $Pr85^{env}$ of the BALB/c provirus is processed much less efficiently than that of other MuLVs (E. Freed and R. Risser, unpublished observations). In addition, the BALB/c ecotropic provirus is N-tropic (17) and is thus inhibited in its ability to replicate in *Fv-1*^{b} BALB/c mice (34). The expression and spread of ecotropic virus from the BALB/c endogenous provirus in BALB/c mice is thus diminished by at least three viral determinants: lessened levels of RNA expression, N-tropism, and defective processing of viral envelope proteins.

Itin and Keshet (18) have described a number of unusual VL30 genomes in the germ line of BALB/c mice that carry internal MuLV-related sequences. These recombinant genomes presumably arose during some phase of reverse transcription and then integrated into the cellular genome.

The integration event we documented may represent another mechanism by which recombinant MuLV-VL30 genomes can be generated. Integration of MuLV proviruses within complete VL30 elements in the same orientation followed by deletion events could lead to the recombinant MuLV-VL30 genomes previously reported. Integration of the BALB/c provirus may have taken place during the integration of VLeco preproviral sequences, or it may have taken place within the resident VLeco element. Precedents exist for autointegration of homologous retroviruses during preproviral DNA synthesis, although such molecules have not been shown to integrate into cellular chromosomes (42). Whether the integration of the BALB/c ecotropic provirus occurred concomitantly with the loss of VL30 sequences linked to the VLeco LTR is unknown. In this respect, it would be of interest to isolate the segment of mouse DNA containing VLeco from the genome of an inbred strain lacking this ecotropic provirus. The presence of a genome-length VLeco element at this locus would suggest that excision and integration could have occurred concomitantly.

It is interesting to note that there is a 53-bp region of homology between the site of integration within VLeco and a sequence 50 bp from the termini of the ecotropic provirus. Because target-site homology has not been found in other integration events in somatic cells (41), this observation may be simply fortuitous. Alternatively, it may indicate that particular regions of the genome carrying homologous sequences are more favored in germ line integrations. It may also be that actively transcribed VL30 sequences and other retroviruslike repetitive sequences are more accessible as potential integration sites than are other sequences in the germ line. Characterization of cellular DNA immediately proximal to other cloned endogenous retroviruses should provide insight into the general significance of the BALB/c proviral integration event.

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