lations of scarlet gilia on the San Francisco Peaks produce red and white morphs that have different flowering phenologies as well as individual plants that shift color within an inflorescence, whereas populations growing only a few kilometers away but 500 m lower in elevation do not shift color and produce only red flowers. Notably, scarlet gilia populations that do not shift color do not lose their hummingbird pollinators. In comparison, high-elevation populations that shift color do lose their hummingbird pollinators halfway through the flowering season (*8*). Since evolutionary changes in the characteristics of populations over short distances have been shown to be related to differences in microhabitat (such as competition, disturbance, and heavy metals) (*11–13*), it should also be expected that plants can become locally adapted to patterns of pollinator abundance and behavior (*14*). Color shifts within and between individual plants of *I. aggregata* permit these plants to track local changes in pollinator abundance in ecological time. Whether such local variation in flower color represents a stable polymorphism or is dynamic, favoring an even greater proportion of lighter colored flowers, remains to be determined.

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7. A standardized corolla color scale was devised with a dilution series of red and white enamel paint. Plants were grouped into one of five color categories: red (100 percent red), dark pink (75 percent red and 25 percent white), pink (50 percent red and 50 percent white), light pink (25 percent red and 75 percent white), and white (100 percent white).
8. Even in neighboring high-elevation areas with populations of *Penstemon barbatus* (a red-flowered, hummingbird-pollinated plant), hummingbirds emigrated during the same period and in the same manner as they did from Fern Mountain. Compared to low-elevation populations of *Penstemon*, high-elevation populations were generally larger and produced similar amounts of nectar per flower, yet hummingbirds still emigrated from them. It is therefore unlikely that pollinators emigrate from Fern Mountain because of local shifts in color, since *Penstemon* (which is found with *Ipomopsis*) provides an abundant and reliable nectar resource during the period of hummingbird desertion. These observations are supported by those of A. Kodric-Brown and J. H. Brown [*Ecology* **59**, 285 (1978)]. In addition, associated with hummingbird emigration is a significant switch in nectar production by red-flowered plants from diurnal to nocturnal secretion (K. N. Paige, unpublished data). Such evidence suggests that plants are responding to local patterns of pollinator abundance and behavior.
9. Since hawkmoths exhibit a negative phototactic response, a red filter was used for conducting nocturnal observations.
10. Pollinator exclosures were switched at sunset and sunrise to exclude hummingbirds and hawkmoths, respectively, throughout the flowering season, restricting overlap to only a few minutes.
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## Bovine Leukemia Virus Long Terminal Repeat: A Cell Type–Specific Promoter

Abstract. *The functional activity of the promoter unit contained within the long terminal repeat (LTR) of bovine leukemia virus (BLV) was examined by monitoring transient expression of a heterologous gene placed under its control. Various cell lines were transfected with recombinant plasmids carrying the bacterial chloramphenicol acetyltransferase (CAT) gene coupled to the BLV LTR (pBL-cat). Transient expression of CAT activity directed by the BLV LTR was observed only in the established BLV-producer cell lines derived from fetal lamb kidney (FLK) cells and bat lung cells. The amount of CAT activity transiently expressed in FLK-BLV cells was decreased approximately tenfold by deletion of LTR sequences located within a region 100 to 170 nucleotides upstream of the RNA start site. Surprisingly, removal of the region 50 base pairs downstream of the RNA initiation site to the 3′- end of the LTR reduced the expression of CAT activity by 87 percent. The BLV LTR thus appears to be an unusual promoter unit, functioning in a cell type–specific manner and possessing sequences on both the 5′ and 3′ sides of the RNA start site that influence gene expression.*

Bovine leukemia virus (BLV) is a B-cell lymphotropic retrovirus associated with a disease complex termed "enzootic bovine leukosis" (*1*). Unlike most other avian and mammalian retroviruses, BLV transcripts have not been detected in tumors or lymphocytes of infected animals (*2*). In addition, BLV displays a highly restricted infectivity in vitro (*3, 4*). Nucleotide sequence data have revealed that BLV, like the human T-cell leukemia viruses types I and II (*5*) (HTLV-I and HTLV-II) possesses an unusual long terminal repeat (LTR) structure (*6, 7*). The LTR's bordering retroviral proviruses contain sequences required for viral integration, replication, and expression (*8*), and determine to a large extent the pathological consequences of infection. Because the LTR encompasses a promoter unit analogous to that controlling cellular gene expression, we suspected that the restricted expression and infectivity of BLV were functional manifestations of an atypical LTR. To examine this possibility, we tested the ability of the BLV LTR to promote the expression of a heterologous gene placed under its control in various cellular environments.

The BLV-infected fetal lamb kidney (FLK-BLV) cell line is one of only a few lines characterized that produce significant amounts of BLV (*3*). The four BLV proviruses harbored by FLK-BLV cells were cloned into bacteriophage lambda L47 (*9*). Restriction fragments containing each of the four proviral 5′ LTR's, as well as a single 3′ LTR, were inserted into the plasmid pSV0cat (*10*) as outlined in Fig. 1. The transcriptional utilization of the BLV LTR's were assessed by comparing the levels of CAT activity transiently expressed in cells transfected with pBL-cat and pRSVcat plasmids. The latter plasmid contains a CAT gene controlled by the Rous sarcoma virus LTR (*10*). Results from typical transfection experiments are shown in Table 1 and Fig. 2. In monkey kidney (CV-1), bovine kidney (MDBK), mouse fibroblast ($LTK^-$), or human rhabdomyosarcoma (RD4) cell lines, CAT activity was not detected after transfection with pBL-9cat or the promoterless pSV0cat. In these same cell lines, CAT activity was easily detected after transfection with pRSVcat, indicating that these cells were competent to take up DNA. Similar results were obtained in transfections of primary fetal lamb kidney (FLK) cells and a bat lung cell line, Tb1Lu (Table 1). In contrast, the BLV LTR directed high levels of CAT activity in the productive-

ly infected cell lines FLK-BLV and BLV-bat$_2$ (Table 1 and Fig. 2). These cells were previously established after infection of FLK and Tb1Lu cells with BLV (*3, 4*). The plasmid pBL-9cat yielded levels of enzyme activity that were tenfold higher in FLK-BLV cells and approximately 30-fold higher in BLV-bat$_2$ cells than the levels obtained with pRSVcat. The results obtained with the plasmids pBL-1cat, pBL-8cat, pBL-13cat, and pBL-3′ cat (Fig. 1) were very similar to pBL-9cat in the above experiments (data not shown). These data suggest that the productively infected cell lines express unique factors that regulate promoter activity by interacting with specific sequences in the BLV LTR.

Table 1. Comparison of CAT expression in mammalian cells transfected with pBL-9cat, pRSVcat, and pSVOcat. All cells were at low passage number and were maintained in Temin's modified minimum essential medium supplemented with 10 percent fetal calf serum. Plasmid DNA was transfected onto cells as a calcium phosphate coprecipitate exactly as described previously (*10, 11*). DNA (10 μg) was applied to $6 \times 10^5$ cells grown in 10-cm culture dishes. Forty-eight hours after transfection the cells were collected and extracts were prepared and assayed for CAT activity (*10*). The CAT reaction mixtures contained in a volume of 180 μl: 100 μl of 0.25*M* tris-HCl, *p*H 7.5; 5 μl of $^{14}$C-labeled chloramphenicol (1 mCi/ml, 49 mCi/mmol); 20 μl of 10 m*M* acetyl coenzyme A; and cell extract. The volumes of cell extract and the incubation times were varied for each cell line to ensure that measurements of CAT activity were in the linear range of the reaction. CAT activities were determined in at least two transfection experiments and, in most instances, with different preparations of a plasmid. The data are expressed as the percentage of the total radioactivity comigrating with monoacetate products with 20 μl of the 100-μl cell extracts. Incubation times were: LTK$^-$, 1 hour; CV-1, BLV-bat$_2$, and RD-4, 2 hours; MDBK, 5 hours; FLK and Tb1Lu, 6 hours. For FLK-BLV cell extracts the incubation time was 10 minutes, with 2 μl of cell extract.

| Cell type | Conversion (%) | | |
|---|---|---|---|
| | pBL-9cat* | pRSVcat | pSVOcat |
| CV-1 | 0.2 | 11.0 | 0.9 |
| MDBK | 0.2 | 4.2 | 0.2 |
| RD-4 | 0.2 | 18.8 | 0.2 |
| LTK$^-$ | 0.4 | 7.6 | 0.8 |
| FLK | 0.2 | 10.4 | 0.4 |
| Tb1Lu | 0.2 | 7.5 | 0.2 |
| FLK-BLV | 25.0 | 2.2 | 0.2 |
| BLV-bat$_2$ | 32.2 | 0.9 | 0.2 |

*The other BLV LTR–containing CAT plasmids shown in Fig. 1 were also examined in RD-4, LTK$^-$, and FLK-BLV cells. These plasmids gave negligible levels of CAT activity in RD-4 and LTK$^-$ cells. When tested on FLK-BLV cells in the same experiment shown for pBL-9cat above, the plasmids pBL-1cat, pBL-8cat, pBL-13cat, and pBL-3′ cat yielded levels of CAT activity which gave, respectively, 28.0, 21.5, 23.2, and 20.4 percent conversion.

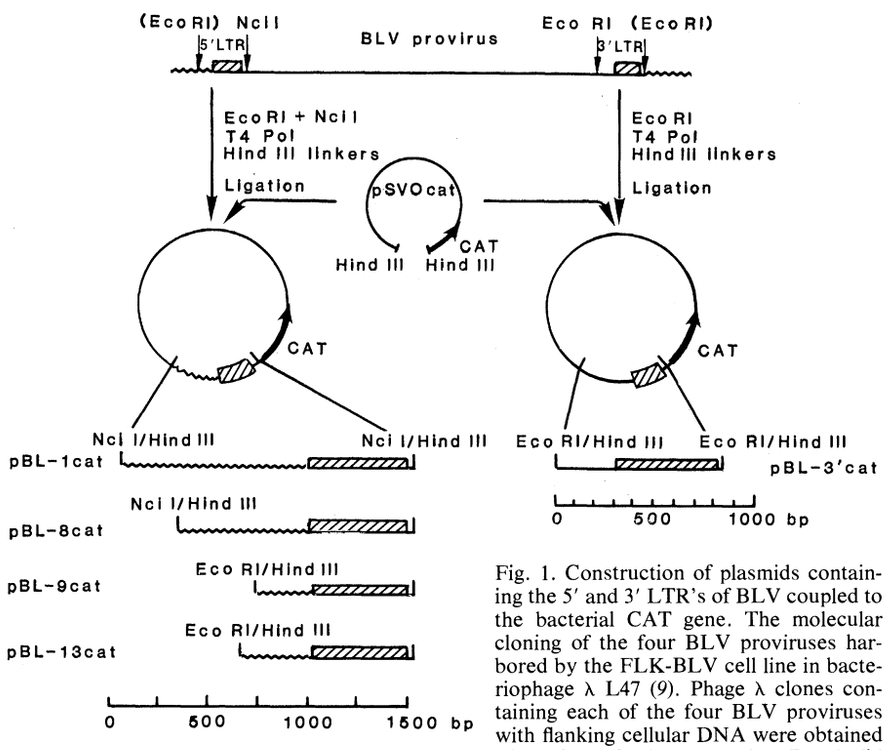


Fig. 1. Construction of plasmids containing the 5′ and 3′ LTR's of BLV coupled to the bacterial CAT gene. The molecular cloning of the four BLV proviruses harbored by the FLK-BLV cell line in bacteriophage λ L47 (*9*). Phage λ clones containing each of the four BLV proviruses with flanking cellular DNA were obtained and are identified B1, B8, B9, and B13. At the top of the figure is shown provirus B13 (solid lines) bounded by its LTR's (hatched boxes) and flanked by cellular DNA (wavy lines). The Eco RI sites in parentheses refer to sites unique to the flanking cellular DNA of proviral clone B13. The Nci I site is located 16 bp downstream of the 5′ LTR. Restriction enzyme fragments containing 5′ or 3′ LTR's derived from each proviral clone were purified by polyacrylamide gel electrophoresis then modified by filling in the ends and adding synthetic Hind III linkers. The fragments were then ligated to Hind III–digested and bacterial alkaline phosphatase–treated pSVOcat (*10*). The resulting plasmids thus contain CAT coding sequences whose expression in eukaryotic cells is controlled by transcriptional regulatory signals present in the BLV LTR. For the plasmids shown here, the viral RNA start site is located approximately 370 bp upstream of the first AUG codon in the CAT sequence.

Using the permissive FLK-BLV cell line as the host for transfections, we next sought to identify regions in the BLV LTR required for optimal expression of the heterologous gene. FLK-BLV cells accumulated nearly identical levels of CAT activity after transfections with pBL-1cat, pBL-8cat, pBL-9cat, pBL-13cat, or pBL-3′cat (data not shown), indicating that all of the transcriptional control sequences are contained entirely within the LTR; 5′-host or virus sequences flanking the LTR's in these constructs did not affect promoter activity. As shown in Fig. 3, sequences from the 5′ and 3′ ends of the LTR were deleted at the indicated restriction enzyme sites and the remainder of the LTR was then inserted into pSVOcat. Removal of 25 bp from the 5′ end and 30 bp from the 3′ end of the LTR did not adversely affect promoter function manifested as CAT activity (pBL-H2cat, Fig. 3). In pBL-P2cat, 61 bp has been deleted from the 5′ end of the LTR, resulting in a 68 percent reduction of CAT activity compared to levels produced by pBL-9cat. The removal of an additional 26 bp from the 5′ end (that is, deletion of nucleotides 1 through 88 in the LTR) caused a 91 percent decrease in the expression of CAT activity (pBL-P1cat, Fig. 3). It thus appears that sequences contained approximately between nucleotides numbered 30 to 100 in the BLV LTR (Fig. 3), 100 bp to 170 bp upstream of the RNA start site, are essential for optimal transcriptional activity. The position of this region, relative to the site of RNA initiation, is similar to where enhancer sequences have been identified in several other LTR's (*12, 13*).

Although this region contains several short (8 to 10 bp) direct and inverted repeats (*6, 7*), there is no extensive homology with other enhancer sequences. Weiher has proposed the existence of a core enhancer sequence, 5′-GTGG$^{AAA}_{TTT}$-3′, which is found near a number of promoter units but whose functional significance is still unknown (*14*). Several related sequences are present in the region 100 to 170 bp upstream of the BLV RNA start site. As shown by the nucleotide numbering of the LTR in Fig. 3,

these sequences are: 5′-CTGGTGA-3′ (nucleotides 74 to 80), 5′-GTGGCTA-3′ (nucleotides 93 to 99), and, in inverted form, 5′-AAACCAG-3′ (nucleotides 44 to 50). Delineation of functionally significant sequences within this area of U3 must await more extensive analysis.

As stated above, removal of 30 bp from the 3′ end of the LTR did not reduce CAT expression (pBL-H2 cat, Fig. 3). However, deletion of 176 bp of DNA from the 3′ end of the LTR produced a 78 percent decrease in the expression of CAT activity (pBL-S2cat) and removal of 276 bp of 3′ terminal LTR sequences, spanning the region between 50 bp downstream of the RNA start site and the 3′-end of the LTR, caused an 87 percent reduction in the transient accumulation of CAT activity (pBL-S1cat). The influence of LTR sequences located downstream of the RNA start site on heterologous gene expression has not previously been observed with other retroviral LTR's. Finally, the plasmid pBL-Hcat (Fig. 3), possessing 100 nucleotides on the 5′ side and 185 nucleotides on the 3′ side of the RNA start site, yielded a level of CAT activity that was only 3 percent of that obtained with pBL-9cat, suggesting that sequences located 5′ and 3′ to the RNA start site independently influence heterologous gene expression. It is unlikely that the 3′ deletion effect is solely the result of shortening the distance between the RNA start site and CAT coding sequences (in pBL9-cat this distance is 370 bp and in pBL-S2cat the distance is approximately 80 bp). The transcriptionally active LTR's contained within pRSVcat (*11*) and pMS-LTR2 (*12*) (carrying the Mo-MSV LTR coupled to the CAT gene) lack U5 and R sequences, and the viral RNA start sites are located 60 to 70 bp from the first AUG codon in the CAT gene. Our results indicate that the BLV LTR may contain two independent promoter control sequences (one in U3 and one in R), perhaps explaining why BLV has such an unusually long R region (*6, 7*). An alternative explanation that should not be overlooked is that deletion of U5 and parts of R may adversely affect post-transcriptional stages of gene expression.

While we anticipated that the BLV LTR would direct CAT expression in the cells known to produce BLV, it was surprising to observe a total lack of activity in all other cell lines tested. This cell type–specificity is reminiscent of the promoters controlling expression of insulin, chymotrypsin (*15*), and immunoglobulin (*16*) genes and is analogous to the cell-specific or response-specific expression previously observed with other retroviruses. Transcription of mouse mammary tumor virus is induced in response to glucocorticoids and mediated through an interaction of the activated hormone receptor with sequences in the LTR (*17*). Moloney murine leukemia virus is expressed in fibroblasts but not in F9 embryonal carcinoma (EC) cells (*18*). The transcriptional block in the latter cell line can be overcome by substituting a variant enhancer element for its normal counterpart in the viral LTR, suggesting that the permissive and nonpermissive cells express different sets of regulatory proteins that interact with specific enhancer sequences (*18*). That BLV expression is also regulated by cellular *trans*acting factors in vivo is suggested by the biological behavior of the virus. For example, viral RNA cannot be detected in lymphocytes taken directly from infected animals (*19*). However, virus is transiently produced shortly after these cells are transferred to short-term culture (*20*), implying that BLV expression is regulated by cellular fac-

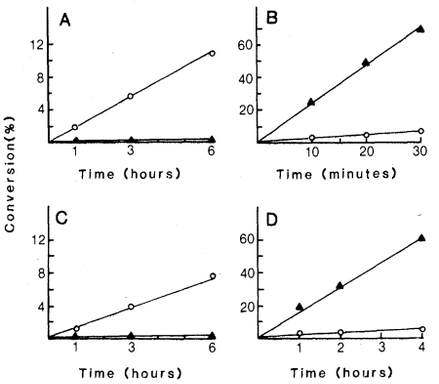


Fig. 2. Differential expression of CAT activity directed by BLV and RSV LTR's in uninfected and BLV-producer cells. Transfections and CAT assays were performed as described in Table 1. The percentage of $^{14}C$-labeled chloramphenicol converted to monoacetate products is plotted as a function of incubation time with extracts of (A) FLK cells, (B) FLK-BLV cells, (C) bat lung TB1Lu cells, and (D) BLV-bat$_2$ cells transfected with pBL-9cat (△), pRSVcat (○), and pSVOcat (baseline).

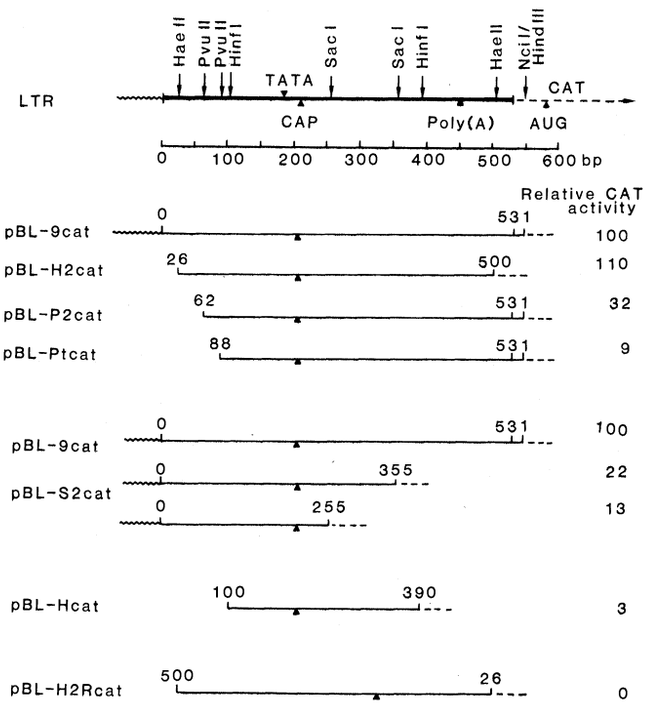


Fig. 3. Deletion mapping of LTR sequences that regulate the expression of CAT activity in FLK-BLV cells. The top line shows restriction enzyme sites in the 531-bp BLV LTR (heavy line) used to generate the deletions of the LTR sequences shown below. For reference, the viral poly(A) addition site (at nucleotide number 445) and the RNA start site (position number 205 ± 5) are shown (*6, 7*); the latter is indicated by an arrowhead in all of the deleted LTR's. The construction of pBL-9cat is described in the legend to Fig. 1. The remainder of the plasmids shown here were constructed by inserting restriction fragments into the Hind III site of pSVOcat as already outlined. The plasmids pBL-H2cat and pBL-H2Rcat were derived by cloning the 473-bp Hae II LTR fragment into pSVOcat in sense (pBL-H2cat) and antisense (pBL-H2Rcat) polarities with respect to the CAT gene. pBL-P2cat and pBL-P1cat were prepared by partial digestion of pBL-3′cat with Pvu II, followed by addition of Hind III linkers. After Hind III digestion, the 480-bp and 453-bp LTR fragments were purified by polyacrylamide gel electrophoresis and inserted into pSVOcat. pBL-S2cat and pBL-S1cat were generated from pBL-9cat by partial digestion with Sac I, end repair with T4 DNA polymerase, and addition of Hind III linkers. The 635-bp and 535-bp LTR fragments, obtained by gel electrophoresis of Hind III digests, were again inserted into pSVOcat. pBL-Hcat contains a 290-bp Hinf I LTR fragment cloned into the Hind III site of pSVOcat after Hind III linker addition. The transfections and assays were performed as described in Table 1. For the CAT assays we used 2 μl of cell extract incubated for 10, 20, and 30 minutes. Activities are expressed as percentages relative to the levels measured in extracts of cells transfected with pBL-9cat.

tors responsive to environmental stimuli.

Recently, the LTR's derived from HTLV-I and HTLV-II were examined with respect to promoter function (*21, 22*). In a manner similar to the BLV LTR, the HTLV-I and HTLV-II LTR's functioned at high levels only in cell lines known to express HTLV information. According to an alternative interpretation of this phenomenon, virus expression is activated by a viral gene product (*22*). In this model, a low-frequency transcriptional event would initiate a positive feedback scheme resulting in the production of virus at a high and uncontrolled level. For BLV, at least, this model seems inconsistent with the biologic properties of the virus in nature. We are inclined to believe that the events and factors that initiate the primary transcription of BLV are identical to those which allow its sustained expression in the producer cell lines. This raises the question of how the productively infected cell lines in our experiments acquired the ability to utilize the BLV promoter when the parental FLK and bat lung cells could not. This question is related to the problem of establishing cell lines productively infected with BLV, an apparently rare occurrence (*3, 4*). One explanation consistent with both of these observations is that the original cell populations contain rare variants that express the factor or factors that allow BLV promoter function and, thus, productive infection. Regardless of the type or origin of these regulatory factors, they probably interact with specific sequence elements in the LTR's.

We have observed that the BLV LTR functions as a highly restricted promoter unit and possesses sequences 5′ and 3′ to the RNA start site that influence gene expression. The expression of BLV in vivo is probably restricted to a specific cell type in the B-cell lineage or to a specific state of response of that cell to environmental stimuli. Characterization of the unique transcriptional regulatory factors present in the productively infected cell lines, which confer activity on the BLV promoter unit, should clarify the mechanisms of restricted viral expression seen in nature. Retroviral LTR's contain promoters that must be analogous to those of cellular genes, and cellular gene expression is highly regulated by a variety of mechanisms. Thus it is not surprising to observe tissue-specific or response-specific (hormonally regulated) retrovirus expression. BLV and HTLV are unusual biologically and structurally compared with all other RNA tumor viruses and it seems probable that they belong to a unique class of retroviruses whose other members have not yet been identified because of their highly restricted infectivity and expression.

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## *Trans* Activation of the Bovine Leukemia Virus Long Terminal Repeat in BLV-Infected Cells

Abstract. *The transcription initiation signals for retroviruses lie within the long terminal repeat (LTR) sequences that flank the integrated provirus. This study shows that factors present in cells infected with bovine leukemia virus (BLV) mediate transcriptional* trans *activation of the BLV LTR. This phenomenon is similar to that reported for the human T-cell leukemia virus LTR and establishes both structural and functional criteria for inclusion of BLV and human T-cell leukemia viruses in the same family of transforming retroviruses.*

The genome of bovine leukemia virus (BLV) contains the long terminal repeat (LTR), *gag*, *pol*, and *env* sequences characteristic of all retroviruses (*1*). In addition, like the human T-cell leukemia virus (HTLV), the BLV genome has a long open reading frame (LOR) region of approximately 1600 nucleotides 3′ to the envelope gene (*2*). We have previously proposed that the HTLV LOR region protein mediates transcriptional *trans* activation of the HTLV LTR (*3*). Therefore, it was of interest to determine whether factors present in BLV-infected cells mediate *trans* activation of the BLV LTR.

The transcription initiation signals for retroviruses lie within the long terminal repeat (LTR) sequences that flank the integrated provirus (*4*). To test the transcriptional capabilities of the BLV LTR sequences, we constructed plasmids in which the BLV LTR was placed 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 1) (*5*). To assay for transcriptional activity, we introduced the recombinant plasmids into eukaryotic cells via transfection (*6*). Levels of CAT enzymatic activity are closely correlated with levels of CAT messenger RNA, thereby providing a measure of the ability of the sequences 5′ to the CAT gene to promote transcription (*5, 7*).

The ability of the BLV LTR sequences to function as transcriptional elements in uninfected murine fibroblasts and human epithelial lines was tested. Parallel experiments were done with several other plasmids in which the CAT gene was under control of other promoter sequences. Upon transfection these additional plasmids all yielded appreciable levels of CAT activity (Table 1), indicating that the transfection procedures used resulted in efficient uptake and expression of DNA. No CAT activity was detected in extracts prepared from those cells that were transfected with the plasmid containing the BLV LTR sequences. Similar results were obtained after transfection of human T and B lymphocytes