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

(Table 1). These results show that the BLV transcriptional elements do not function as a transcriptional promoter in these cells.

The ability of the BLV LTR sequence to function as a transcriptional element in BLV-infected and uninfected fetal lamb kidney (FLK) cells was examined. The CAT activity directed by the BLV LTR sequences was not detected in the uninfected cells, suggesting that the BLV LTR was inactive in these cells. In contrast, a high level of CAT activity was detected upon transfection of the BLV-infected producer cells (Table 1 and Fig. 2). The infected cell line used for this experiment produced BLV virions as demonstrated by high reverse transcriptase levels (data not shown). Similar results were obtained with additional uninfected and BLV-infected matched cell lines. The cell lines used were uninfected bat lung cells (CCL88) and a clonal isolate of the CCL88 line that produced BLV (*8*), and FLK cells that were infected with BLV 6 to 8 days prior to transfection (Table 1 and Fig. 2).

From these experiments we conclude that *trans* acting factors, either encoded by the virus or virally induced, activate BLV LTR-controlled gene expression. This effect probably occurs at the level of transcription (*3, 7*).

Gene expression directed by the LTR sequences of HTLV types I, II, and III is augmented by *trans* acting factors present in HTLV-infected cells (*3*). We tested if these factors could activate the BLV transcriptional control sequences. We have previously shown that *trans* acting factors present in HTLV-II–infected cells activate HTLV-1 LTR–directed CAT gene expression.

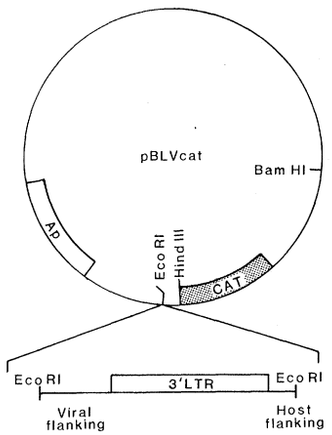

Fig. 1. Construction of pBLVcat. The BLV LTR sequences used in this study were those of the LTR subclone T15-4, obtained from an Eco RI tumor DNA fragment of a bovine lymphosarcoma (*14*). Subclone T15-4 was digested with Eco RI and the 890-base-pair fragment containing the BLV LTR was isolated from a low-melting agarose gel and ligated into the Eco RI site of plasmid pBR322. DNA was transfected into the *Escherichia coli* strain HB101 and ampicillin-resistant colonies were screened for the presence and orientation of the BLV insert. A positive clone was cleaved with Hind III–Bam HI and the vector fragment was isolated. The Hind III–Bam HI fragment of plasmid pSV2cat containing the CAT coding sequence and SV40 polyadenylation signals was ligated to this BLV fragment. The construction of the final plasmid, pBLVcat, was confirmed by extensive restriction enzyme analysis. Plasmid DNA was purified by banding in CsCI. All recombinant DNA techniques were according to established procedure (*17*). Enzyme digestions were done according to the manufacturers' specifications.

As indicated in Table 1, the *trans* acting factors present in the HTLV-I, HTLV-II, and HTLV-III infected cell lines were unable to activate BLV LTR–directed CAT gene expression. Similarly, factors present in the BLV-infected cells were unable to increase CAT gene expression directed by the different HTLV-LTR sequences. This suggests that the *trans* acting factors for HTLV-I, -II, and -III are not functionally interchangeable with those factors that activate the BLV LTR sequences; this is not surprising given the lack of primary sequence homology between the HTLV and BLV LTR sequence (*9, 10*).

The major determinant for efficient function of the BLV transcriptional elements may be the presence of BLV-induced *trans* acting factors, because CAT gene expression directed by the plasmid that contains the BLV LTR was only observed in BLV-infected cells. In this respect BLV resembles HTLV-II rather than HTLV-I because the efficient activity of the HTLV-II LTR appears to be restricted, for the most part, to HTLV-II–infected cells (*3*).

The phenomenon of *trans* activation and the presence of a LOR region distinguish both BLV and HTLV from other nonacute retroviruses. Circumstantial evidence suggests that *trans* activation of the HTLV LTR is mediated by the protein product of the HTLV LOR region (*3, 11*). Because the genome of BLV contains a LOR region 3′ to its envelope gene (*2*), we propose that the BLV LOR protein product mediates transcriptional

Table 1. Relative CAT activity in transfected cells. Non-lymphoid and lymphoid cell lines were transfected by modifications of the $CaPO_4$ and DEAE dextran coprecipitation techniques, respectively (*6*). Preparation of cellular extracts and CAT assays were as described in the legend to Fig. 2. Plasmid pU3R-III contains the LTR of HTLV-III (*3*). To control for differences in the ability of different cell types to take up and express foreign DNA, we normalized the percentage conversion of chloramphenicol to its acetylated forms against the percentage conversion in similar cells transfected with pSV2cat taken as 1.0. Thus, the values represent the percentage acetylation per hour relative to that directed by pSV2cat. Plasmid pSV2cat and the other plasmids used are described in the legend to Fig. 2; ND, not done.

Cell line	Description	pSV2cat	pBLVcat	pU3R-I	pU3R-II	pU3R-III	pRSVcat	Reference
NIH 3T3	Murine fibroblast cell line	1.0	<0.05	0.9	ND	ND	ND	
Hela	Human cervical carcinoma line	1.0	<0.05	2.2	ND	ND	1.6	
HUT 78	HTLV-negative human T lymphocyte	1.0	<0.05	4.5	ND	ND	2.1	*18*
Raji	Human B lymphocyte immortalized by EBV	1.0	<0.05	6.2	ND	2.5	ND	*19*
FLK, uninfected	Fetal lamb kidney cell	1.0	<0.05	0.9	<0.05	1.9	1.9	
FLK BLV-producer	BLV-producing fetal lamb kidney cells	1.0	7.6	1.6	<0.05	2.5	1.3	*20*
CCL 88	Bat lung cells	1.0	<0.05	1.1	<0.05	ND	3.6	*8*
CCL 88 BLV-producer	BLV-producing bat lung cells	1.0	3.8	1.6	<0.05	ND	2.2	*8*
C 8166/45	HTLV-I immortalized nonproducer	1.0	<0.05	75	ND	2.8	ND	*21*
C3-44	HTLV-II producer T-cell line	1.0	<0.05	140	95	1.7	ND	*18*
MT2	HTLV-I immortalized producer T-cell line	1.0	<0.05	180	ND	1.4	2.5	*21*
H9	HTLV-negative human T lymphocyte	1.0	<0.05	2.5	<0.05	3.6	ND	*22*
H9/HTLV-III	H9 T-lymphocytes infected with HTLV-III	1.0	<0.05	3.2	<0.05	1,160	ND	*22*

activation of the BLV LTR. A 2-kilobase message capable of encoding such a protein has been detected in BLV-infected cells (*12*).

One question arising from these studies is how viral transcription proceeds after infection if the BLV LTR is nonfunctional in uninfected cells. A possible explanation might be that either small amounts of LOR protein or message are packaged in the virions and serve to initiate transcription upon virus entry into the cell. Alternatively, transcription may be occurring in the uninfected cells, but at a level too low to be detected in our assay. In any event, virus-induced *trans* acting transcriptional activation of the LTR should result in an autostimulatory effect, thus increasing the rate of BLV transcription and replication.

Bovine lymphosarcoma, the disease induced by BLV, is characterized by a long latency often preceded by persistent lymphoblastosis, absence of chronic viremia, and, most important, no site-specific viral integration (*13, 14*). These characteristics are different from the disease patterns associated with the murine and avian retroviruses yet are similar to the disease induced by HTLV-I (*14, 15*).

We have proposed that cellular immortalization by HTLV-I is mediated either directly or indirectly by the HTLV LOR product, possibly via *trans* activation of cellular genes involved in lymphocyte growth control. Because the disease characteristics, together with the phenomenon of *trans* activation, distinguish BLV and HTLV from other nonacute retroviruses, we suggest that the product of the BLV LOR region also plays an important role in the transformation process.

Similarities in the virion capsid protein of HTLV-I and -II and of BLV have been detected previously (*1*). Likewise, certain features of the LTR sequences of BLV resemble more closely those of HTLV-I and -II than they do the LTR regions of other retroviruses (*16*). Such features include an unusually long R region, the potential to perform a thermodynamically stable loop structure between sequences in the U3 and R regions (this loop includes the site of RNA initiation), and the absence of appropriately located polyadenylation signals (*10*). These common structural features, together with the functional (that is, transactivation) and pathological similarities of HTLV-I and BLV-induced disease, indicate that these viruses are members of a new family of retroviruses distinct from both the nonacute and the acute (oncogene-containing) transforming retroviruses.

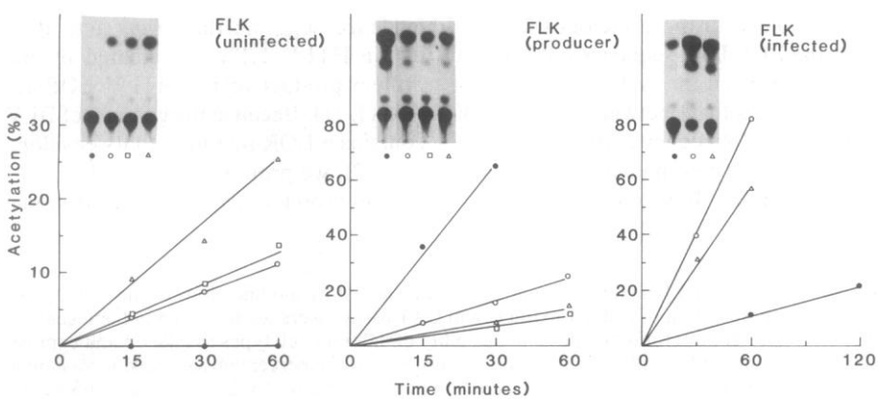

Fig. 2. Transient expression of the CAT gene directed by BLV LTR transcriptional sequences. In addition to the pBLVcat (●) plasmid several other plasmids that contained the CAT gene under control of other promoters were transfected for control purposes. These plasmids include pSV2cat (□), which contains the SV40 early region promoter sequences (*5*), pRSVCAT (△), which contains the Rous sarcoma virus LTR (*5*), and pU3R-I (○) and pU3R-II that contain the HTLV-I and HTLV-II LTR sequences, respectively (*3*). The recombinant plasmids were introduced into the fibroblast and epithelial cells by a modification of the $CaPO_4$ coprecipitation technique (*6*). Approximately 1×10^6 cells were seeded onto 100-mm dishes 24 hours prior to transfection. One milliliter of the $CaPO_4$ precipitate containing 5 μg of plasmid DNA and 30 μg of carrier salmon sperm DNA was added to the medium. After a 24-hour incubation period at 37°C the medium was removed, cells were washed once with phosphate-buffered saline (PBS), fed again with fresh medium containing 10 percent fetal bovine serum. Lymphocyte cell lines were transfected by the DEAE dextran method as described previously (*6*). Forty-eight hours after transfection, the cells were centrifuged (lymphocytes) or scraped from dishes, washed once with PBS, resuspended in 150 μl of 250 m*M* tris, *p*H 8.0, and subjected to three freeze (−70°C)-thaw (37°C) cycles. Debris was removed by a brief centrifugation and protein determinations were made on the cell extract. Acetyl coenzyme A (final concentration 4 m*M*) and ^{14}C-labeled chloramphenicol (0.30 μCi) were added to the cell extracts, and a time-course assay was performed. The percentage conversion of chloramphenicol to its acetylated forms was determined by ascending thin-layer chromotography and liquid scintillation counting of the spots cut from the thin-layer chromatography plate. The graph depicts typical time-course assays obtained from the indicated cell types. The insets show actual autoradiograms of CAT conversions obtained from one time point within the linear range of a time-course assay. All experiments were repeated a minimum of three times and relative CAT activity differed by no more than 20 percent.

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