Two Elements in the Bovine Leukemia Virus Long Terminal Repeat That Regulate Gene Expression

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The bovine leukemia virus, like the human T-cell leukemia viruses (HTLV-I and HTLV-II), are unusual biologically in that viral transcripts are not detected in tumors or infected tissues. The bovine leukemia virus long terminal repeat (BLV LTR) functions as a transcriptional promoter only in cell lines productively infected with BLV. Deletion mapping indicated that at least two regions of the LTR, on the 5′ and 3′ sides of the RNA start site, influenced gene expression. An analysis has now been made of the effects of coupling sequences from these LTR regions to a heterologous core promoter derived from the SV40 early promoter unit. Through the use of the transient expression of the bacterial chloramphenicol acetyltransferase (CAT) gene to monitor transcriptional activity in vivo, two independent, regulatory elements were identified in the BLV LTR. One was present in a fragment of 75 base pairs derived from the U3 region of the LTR and behaved much like other enhancer elements. It may be a major determinant of BLV expression in productively infected cell lines, since it enhanced transcription controlled by the heterologous core promoter only in these cells. The second element was contained in a 250-bp fragment derived from LTR sequences in the R region, located downstream from the RNA start site. Its activation of CAT expression was not dependent on BLV infection and was evident only when the fragment was located immediately downstream from the RNA start site. BLV expression thus appears to be regulated in part by a cell-specific enhancer element upstream from the core promoter and a novel sequence downstream from the RNA initiation site in the viral LTR.

EXPRESSION OF A RETROVIRAL PROvirus is governed by the interaction of the promoter unit contained within the viral long terminal repeats (LTR's) with the cellular transcription apparatus (*1*). Because these viruses rely on the host transcription system, their promoters must be analogous to those of cellular genes, thus providing excellent models with which to study the regulation of eukaryotic gene expression. A fundamental component of the viral promoter unit is the "enhancer," which controls promoter utilization in a particular cellular environment and consequently mediates such biological phenomena as virus growth rate, tissue tropism, and disease spectrum (*2*).

While the enhancers from several retroviruses have been shown to activate transcription in a wide variety of cell types (*3*), others are much more specific. For example, the LTR's derived from bovine leukemia virus (BLV) and the related human T-cell leukemia viruses (HTLV-I and HTLV-II) act as transcriptional promoters in a highly restricted range of cells (*4–7*). The transcriptional promoters contained in these LTR's were most active in cell lines that were already productively infected with the respective virus. Furthermore, it has now been found that expression of the *pX* genes located at the extreme 3′ ends of HTLV-I (*8*), HTLV-II (*9*), and BLV (*10*) confer upon uninfected cells the ability to efficiently utilize the cognate LTR. It is not known whether the factors that interact with sequences in the LTR to activate transcription are identical with or induced by the *pX* gene products. As an initial step in characterizing the BLV promoter and its interactions with transcription factors, we identified two sequence elements that regulate viral expression in productively infected cell lines.

Table 1. Activities of native and hybrid promoters in uninfected cell lines. The plasmids depicted in Fig. 2 were introduced into RD-4, CV-1, and MDBK cells (*4*). CAT assays were performed as described (*12*) 44 to 48 hours after transfection. The CAT activities are expressed as the percentage of the total chloramphenicol converted to monoacetate products. Plasmid pΔE-ΦX-H was constructed by adding synthetic III linkers on the ends of a 270-bp ΦX174 Hae III fragment and inserting it into the Hind III site of pSVΔEcat. In enzyme assays 20 μl of cell extract was incubated for 30 minutes (RD-4) or 2 hours (CV-1 and MDBK).

Plasmid	Conversion (%)		
	RD-4	CV-1	MDBK
pSVΔEcat	0.3	0.06	<0.01
pSV2cat	27.8	15.1	8.95
pΔE75Bg-1	0.05	<0.01	<0.01
pΔE75H-1	0.06	<0.01	<0.01
pΔE75Ba-1	0.06	0.2	<0.01
pΔE250Bg-1	1.0	<0.01	0.05
pΔE250H-1	13.8	0.74	7.0
pΔE250H-2	8.3	0.86	6.0
pΔE250Ba-1	1.1	<0.01	<0.01
pSV2-250H-1	94.7	54.4	50.0
pSV2-250H-2	98.0	61.6	47.6
pΔE75-250H-1	18.9	1.2	8.0
pΔE75-250H-2	16.4	1.4	7.5
pΔE-ΦX-H	0.15		0.10

Using the transient expression of the bacterial chloramphenicol acetyltransferase (CAT) gene to monitor transcriptional activity, investigators previously observed that the BLV LTR was an active promoter only in productively infected cells (*4, 7*). Furthermore, deletion of LTR sequences on the 5′ or 3′ side of the RNA start site significantly reduced CAT expression in these cell lines (*4*). Deletion of sequences between the Hae II and the more downstream Pvu II sites in the 5′ end of the LTR (Fig. 1A) 100 to 175 base pairs upstream from the RNA start site reduced transcriptional activity 90 percent. To our surprise, removal of the LTR region between the Sac I site proximal to the RNA start site and the 3′ end of the LTR (Fig. 1A) reduced expression of the heterologous gene 87 percent. Unlike the precipitous decreases in expression resulting from progressive deletions at the 5′ end of the LTR, activity declined gradually over a broad region on removal of the downstream sequences.

On the basis of these observations, we isolated two LTR subfragments encompassing the potentially active regions; the first was a 75-bp fragment derived from the U3 region and the second was a 250-bp fragment containing most of the R region of the LTR (Fig. 1A). The ability of either fragment to *cis*-activate a heterologous core promoter unit was assessed in BLV-infected and uninfected cells. The core promoter was derived from the SV40 early promoter element by removing most of the 72-bp repeats that constitute the natural enhancers (Fig. 1, A and B). The enhancerless core promoter was contained in the plasmid pSVΔEcat (Fig. 1B), which is analogous to pA-10CAT2 (*11*) and pSV1cat (*12*), previously shown to be incapable of expressing significant levels of CAT enzyme activity in transfected cells unless complemented by the covalent attachment of activating sequences. The pSVΔEcat plasmid shown in Fig. 1B has three cloning sites used in this study. The first is situated immediately 5′ of the core promoter (Bgl II), the second is 60 bp downstream from the RNA CAP site of the core promoter (Hind III), and the third is a distal Bam HI site 1634 bp downstream (or 3400 bp upstream on the circular plasmid) from the Hind III site. The 75-bp U3-region fragment and the 250-bp R-region fragment were inserted in both orientations at each of these three sites. The effects of the

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BLV sequences on the heterologous core promoter were assessed by monitoring the expression of CAT activity in transfected cells. The cells utilized included BLV-infected fetal lamb kidney (FLK-BLV) and bat lung (BLV-bat2) cells, in which the BLV LTR is an active transcriptional promoter. The uninfected cell lines included RD-4, human rhabdomyosarcoma; CV-1, African green monkey kidney; and MDBK, bovine kidney, in which the BLV promoter is not active (*4*).

The relative inactivity of the enhancerless core promoter in the plasmid pSVΔEcat is revealed by the low levels of CAT activity expressed in FLK-BLV and BLV-bat2 cells (Fig. 2) and in RD-4, CV-1, and MDBK cells (Table 1). Addition of the SV40 72-bp repeats to the core promoter, as in the native SV40 early promoter contained in pSV2cat, resulted in high levels of CAT expression in all cell lines examined (Fig. 2 and Table 1).

The effect of the 75-bp BLV U3-region fragment on the activity of the core promoter was first analyzed in the BLV-infected cell lines (Fig. 2). When inserted at the Bgl II site immediately upstream from the core promoter, the 75-bp LTR fragment activated CAT expression; this hybrid promoter yielded levels of enzyme activity comparable to those directed by pSV2cat. The activation of transcription observed was independent of the orientation of the 75-bp fragment, since pΔE75 Bg-1 and pΔE75 Bg-2 (which harbor the 75-bp fragment in native or inverted orientations, respectively) gave comparable levels of enzyme activity. When present at the Bgl II site of pSVΔEcat, the 75-bp BLV fragment is in a location similar to that where it or the SV40 72-bp repeats are located in their native promoters (Fig. 1B). An enhancement of gene expression by the 75-bp BLV fragment was also observed when it was inserted at the distal Bam HI site of pSVΔEcat (pΔE75 Ba-1 and -2) (Fig. 2); the effect on transcription from this location, however, was approximately ten times lower than when located at the Bgl II site. Sequences in the 75-bp LTR fragment had a negligible effect on the transcriptional activity of pSVΔEcat when placed at the Hind III site immediately downstream from the RNA CAP site (pΔE75H-1 and -2) (Fig. 2). This is not surprising, since the SV40 72-bp repeats do not function as enhancers at this location (*13*).

To assess the cell specificity of the effects of the 75-bp U3 region fragment, the hybrid plasmids were transfected onto RD-4, CV-1, and MDBK cells (Table 1). In these cell lines, which cannot utilize the BLV LTR as a transcriptional promoter, the 75-bp BLV fragment was unable to significantly activate the core promoter unit. Although CAT expression directed by pΔE75Bg-1 and pΔE75Ba-1 was in some cases greater than that directed by pSVΔEcat, the levels of CAT activity obtained were far below those directed by pSV2cat. These results are in marked contrast to those obtained in the BLV-producer cells, where the 75-bp U3-region fragment behaved like a typical enhancer element with an activity similar to the SV40 72-bp repeats. The 75-bp BLV fragment thus appears to contain sequences that mediate, at least in part, the specific transcriptional activity of the virus in the infected cell lines. It is noteworthy that, while the 75-bp LTR fragment can restore promoter activity to a level comparable to that elicited by the SV40 72-bp repeats in BLV-infected cell lines, the levels of expression obtained with pΔE75Bg-1 are only a fraction of those directed by the intact BLV LTR (Fig. 2). This indicates that, besides the 75-bp BLV enhancer fragment, other LTR sequences are required to achieve maximal expression.

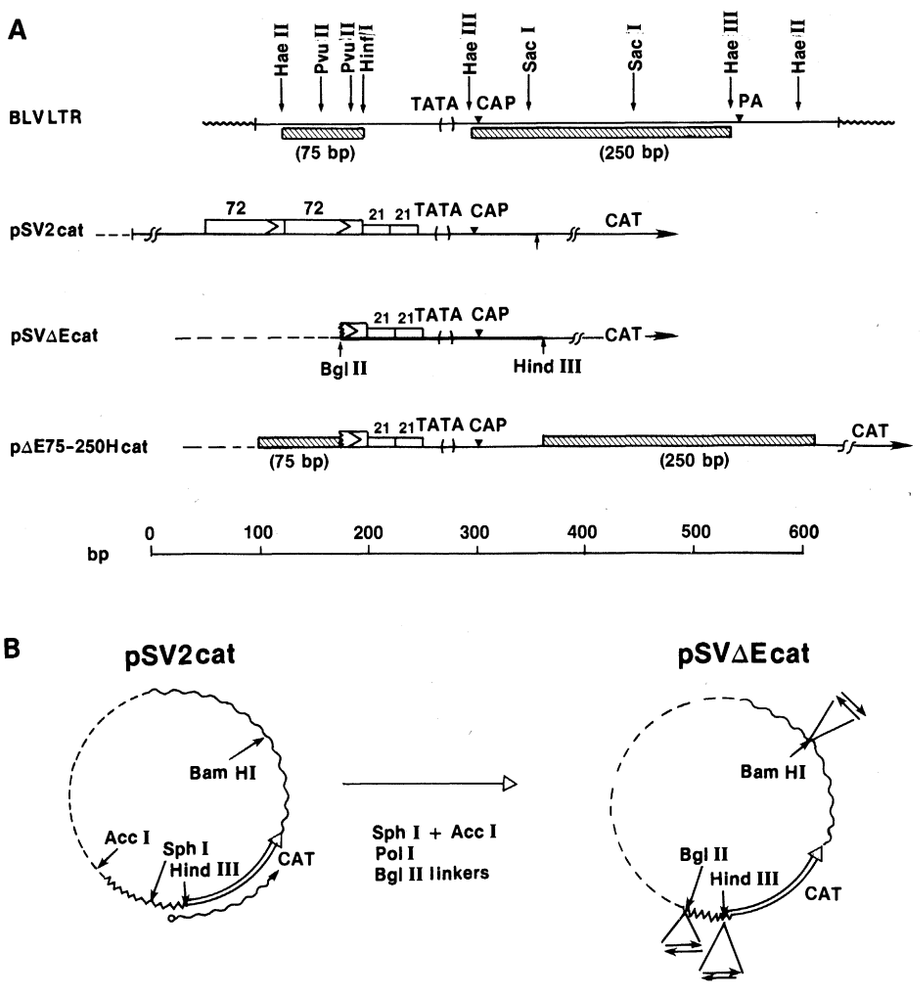

Fig. 1. (A) Top line shows the 531-bp BLV LTR (solid lines), with relevant restriction enzyme sites, TATA box, RNA start site (CAP), and polyadenylation site (PA) indicated. The hatched boxes below the LTR illustrate the 75-bp Hae II–Hinf I and 250-bp Hae III fragments used in subsequent constructions. The early promoter unit of SV40 present in pSV2cat (*12*) is depicted on the second line, and the truncated version, lacking most of the 72-bp repeat sequences, present in pSVΔEcat is shown on the third line. The 75- and 250-bp LTR fragments were purified by polyacrylamide gel electrophoresis, and, where necessary, the ends were repaired with T4 DNA polymerase. Fragment ends were converted to Hind III or Bam HI ends by ligation to synthetic linkers. The 75-bp fragment with Bam HI ends was inserted into the Bgl II or Bam HI sites of pSVΔEcat to give pΔE75Bg-1 and -2 and pΔE75Ba-1 and -2, respectively (the numbers 1 and 2 signify the orientation of the LTR fragment; Fig. 2). The Hind III–ended 75-bp fragment was inserted into the Hind III site of pSVΔEcat to give pΔE75H-1 and -2. The 250-bp LTR fragment was similarly cloned into pSVΔEcat at the Bgl II, Bam HI, and Hind III sites. The bottom line shows an example of a hybrid promoter unit and reveals the locations of the LTR fragments with respect to positions of the elements in their native promoters. (B) Plasmid pSVΔEcat was derived from pSV2cat by deletion of sequences between the Acc I and Sph I sites shown, followed by DNA polymerase I (Klenow fragment) end filling and insertion of a synthetic oligonucleotide containing a Bgl II site. The unique restriction enzyme sites used in these studies for insertion of BLV LTR fragments are shown.

The effects on gene expression of coupling the 250-bp R-region fragment to the SV40 core promoter unit were analyzed first in the productively infected cell lines (Fig. 2). The 250-bp fragment stimulated CAT expression controlled by the core promoter unit in both FLK-BLV and BLV-bat2 cells, but only when inserted at the Hind III site located between the RNA start site and CAT coding sequences (pΔE250H-1) (Fig. 2). In both cell lines, the level of enzyme activity directed by pΔE250H-1 was approximately 60 times greater than that directed by pSVΔEcat. A marked orientation preference of the 250-bp fragment was observed in these cells, with the native orientation (pΔE250H-1) yielding activity five to six times higher than that of the inverse orientation (pΔE250H-2). This LTR fragment had a negligible effect when inserted at the Bgl II site of pSVΔEcat (pΔE250Bg-1 and -2 (Fig. 2), and only a slight stimulatory effect was obtained when it was located at the Bam HI site (pΔE250 Ba-1 and -2). Thus, unlike typical enhancer elements, the 250-bp fragment exhibited a maximal stimulatory effect on CAT expression when located downstream from the RNA initiation site.

In contrast to the cell-specific effects of the 75-bp enhancer fragment, the 250-bp fragment was active in both infected (Fig. 2) and uninfected (Table 1) cell lines. In RD-4, CV-1, and MDBK cells the 250-bp fragment was able to stimulate CAT expression driven by the SV40 core promoter, but again only when placed immediately downstream from the RNA start site (Table 1). In the uninfected cells, no orientation preference was observed; pΔE250H-1 and pΔE250H-2 yielded similar levels of enzyme activity (Table 1). Clearly the levels of CAT expression directed by pΔE250H-1 are significantly greater than those obtained from pSVΔEcat; however, when these levels are expressed relative to pSV2cat, a wide range of values is obtained. For example, in CV-1 cells pΔE250H-1 yielded approximately 5 percent of the CAT activity directed by pSV2cat, whereas in MDBK cells this ratio was 0.78. These variations might be due to the different abilities of the cells to utilize the SV40 early promoter in pSV2cat or the core promoter in pSVΔEcat. Alternatively, the 250-bp fragment may function differently in the various cell lines.

To test these possibilities, the 250-bp fragment was inserted at the Hind III site downstream from the complete SV40 early promoter of pSV2cat or the hybrid promoter of pΔE75Bg-1 to yield pSV2-250H and pΔE75-250-H, respectively (Fig. 2). When placed immediately downstream from the entire SV40 early promoter unit, sequences in the 250-bp fragment increased gene expression in all cell lines examined (Fig. 2 and Table 1). The extent of the stimulation ranged from three to fivefold, as compared with pSV2cat, in all cells except BLV-bat2, where a 23-fold stimulation was observed. In all cases the stimulatory effect was independent of the orientation of the 250-bp fragment (Fig. 2 and Table 1). When placed downstream from the hybrid promoter of pΔE75Bg-1, which contains the SV40 core promoter preceded by the BLV-specific enhancer, the 250-bp fragment stimulated CAT expression approximately fourfold in FLK-BLV cells (pΔE75-250H) (Fig. 2) and 11-fold in BLV-bat2 cells.

The hybrid promoter composed of the SV40 core element and the 75-bp U3 fragment (pΔE75 Bg) was a much weaker promoter than the intact BLV LTR (pBL-H2cat) in FLK-BLV and BLV-bat2 cells (Fig. 2). However, addition of the 250-bp R-region fragment downstream from the hybrid promoter (pΔE75-250H-1) yielded levels of expression approaching those of the intact LTR; in essence, the LTR was reconstructed around the heterologous core promoter. In the uninfected cell lines, levels of CAT activity directed by pΔE75-250H were only slightly greater than those directed by pΔE250H, which lacks an upstream enhancer (Table 1), and far below those directed by pSV2-250H. Thus, the 250-bp R-region fragment can activate gene expression from a position between the site of RNA initiation and the start of the protein-coding sequences. This activity is independent of the orientation of the fragment and the cellular environment. In addition, the effect is sequence-specific and not due to a spacing effect, since the pΔE75H plasmids were inactive (Fig. 2 and Table 1) and insertion of a 270-bp fragment derived from bacteriophage ΦX174 at the Hind III site of

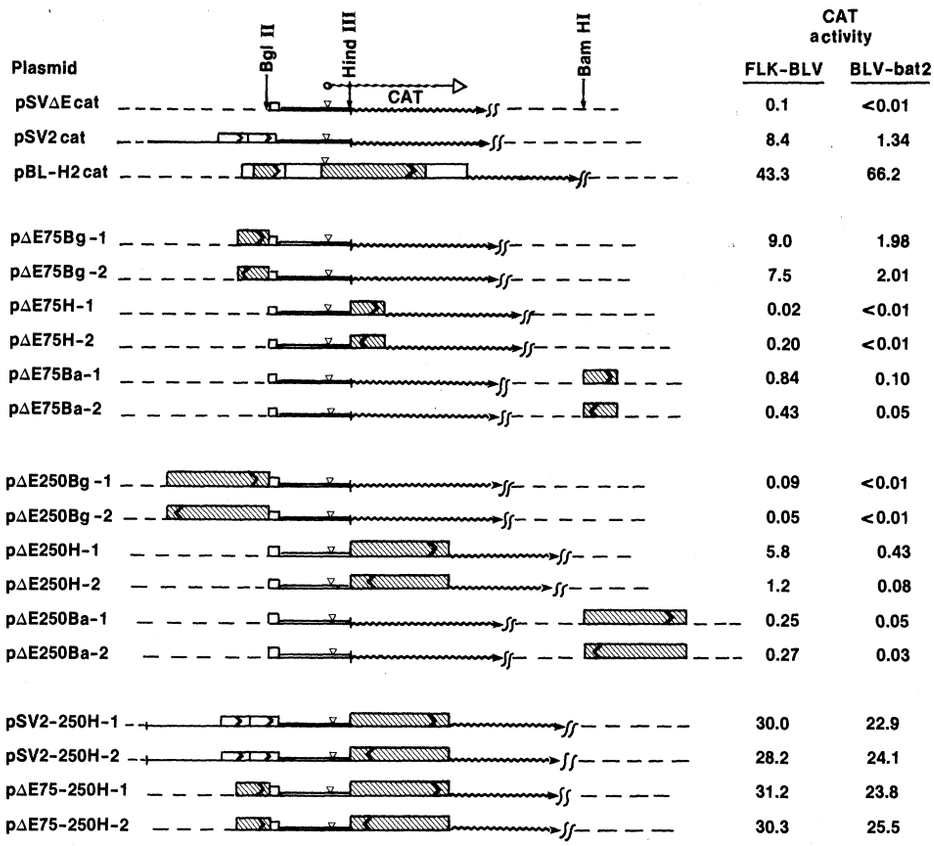

Fig. 2. Levels of CAT activity directed by native and hybrid promoter units compared in FLK-BLV and BLV-bat2 cells. The core promoter unit (bold lines), CAT coding sequences (wavy lines), and cloning sites used for insertion of LTR fragments in pSVΔE cat are shown at the top. Plasmid pBL-H2cat (line 3) contains the 480-bp Hae II BLV LTR fragment (boxed region) linked to CAT coding sequences (wavy line) (*4*). The 75-bp and 250-bp LTR subfragments are shown as hatched regions and their orientations are denoted by internal arrowheads. The open triangles indicate the RNA start sites. Plasmid DNA's were introduced into FLK-BLV and BLV-bat2 cells as calcium phosphate coprecipitates (*12*). Forty-eight hours after transfection cells were collected and CAT assays were performed. Transfections and assays were done at least four times by using two different preparations of each plasmid with a variation in CAT activities between experiments of less than 25 percent. The data on the right express the percentage of the total chloramphenicol present in the reaction that was converted into monoacetate products.

pSVΔEcat had no stimulatory effect (pΔE-ΦX-H) (Table 1).

Several viral and eukaryotic genes have complex promoter units controlled by multiple regulatory elements. For example, Rous sarcoma virus LTR (*14*) and polyoma virus (*15*) promoters contain several enhancer domains. The HTLV-III LTR appears to contain several regulatory elements (*16*) and immunoglobulin heavy chain gene expression is controlled by sequence elements upstream and downstream from the promoter (*17*). Based on deletion analyses and examinations of the effects of LTR regions on a heterologous promoter unit, two elements were identified that regulate BLV gene expression. The first is in the U3 region of the BLV LTR 100 to 175 bp upstream from the RNA start site. It behaves like a typical enhancer element, but its activity is restricted to cells productively infected with BLV; it thus appears to be a major determinant of virus expression in these cell lines. The nucleotide sequence (*18*) shows several short, direct, and inverted repeats in the active 75-bp region and three short sequence units related to the proposed core enhancer sequence $GTGG^{TTT}_{AAA}G$ (*19*). It is probable that transcription factors unique to the productively infected cell lines interact with this sequence element to regulate transcription initiation. The data do not allow us to conclude whether these factors are identical with the *trans*-acting factors induced or encoded by the *pX* or *X-Lor* gene products or whether sequences in the 75-bp U3 fragment are necessary and sufficient to respond to *trans*-activation resulting from *pX* gene expression. This point is made because the productively infected cell lines are exceptions to the more general phenomenon of BLV infection followed by provirus formation and, later, cessation of viral production (*20*). Preliminary data suggest that the 75-bp region is necessary but not sufficient to activate transcription in uninfected cells expressing the BLV *pX* gene (*10*).

A second sequence element was required for maximal gene expression; it resides in a 250-bp region encompassing the unusually long R region of the LTR. There is no extensive homology between the 75-bp and 250-bp fragments. The 250-bp region contains five core enhancer–related sequences, all located 100 to 200 bp downstream from the RNA start site, suggesting that this region might act independently or in cooperation with 5′ enhancer elements to increase RNA polymerase affinity or loading at the promoter. However, the unique position dependence exhibited by this fragment sets it apart from the prototypic enhancers and suggests that alternative mechanisms underlie its activity. Although an effect on translation cannot be ruled out, preliminary evidence indicates that the 250-bp fragment acts by increasing the steady-state level of CAT messenger RNA. Whether the R-region fragment influences messenger RNA synthesis by accelerating the rate at which RNA polymerase progresses from the "closed" (inactive) to the "open" (transcriptionally active) complex after binding to the promoter, or functions posttranscriptionally, that is, at the level of RNA processing or stabilization, remains to be ascertained. It will be of interest to see whether sequences in the R regions of the LTR's of the related viruses HTLV-I and HTLV-II as well as other retroviruses have an effect on gene expression. Sequence elements located downstream from both viral and eukaryotic promoters may prove to be a means of modifying and regulating gene expression.

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