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trans-Acting Regulation of Bovine Leukemia Virus mRNA Processing

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Bovine leukemia virus (BLV) and the human T-cell leukemia virus types I and II comprise a unique retrovirus subfamily which has evolved complex strategies for the regulation of gene expression. A transcriptional control circuit has been characterized in both human and bovine systems in which *cis*-acting promoter control elements are responsive to *trans*-acting factors encoded in the pX region of the virus. The BLV pX mRNA encoding the transcriptional *trans*-acting factor is translated in an alternate reading frame to produce an 18-kilodalton nuclear phosphoprotein, p18. A function for this protein was revealed in cotransfection experiments using mutated BLV proviruses in combination with pX expression plasmids. These experiments indicated that p18 was required for the accumulation of viral mRNAs representing full-length (genomic) and single-spliced (*env*) transcripts. In contrast, synthesis of the double-spliced pX mRNA was not influenced by p18 expression. Large regional deletions and substitutions of provirus sequences localized elements essential for p18 regulation to the 3′ long terminal repeat. Furthermore, sequences within a 250-nucleotide region between the AATAAA signal and poly(A) site were found to be essential for efficient virus mRNA 3′-end processing and response to p18 regulation.

Bovine leukemia virus (BLV) is the etiologic agent associated with a disease complex termed enzootic bovine leukosis, often characterized by persistent lymphocytosis and development of B-cell lymphomas after long latent periods (2, 11). Like human T-cell leukemia virus type I (HTLV-I), BLV is not produced at detectable levels in the lymphocytes or tumors of the infected host but is often expressed when these cells are cultured in vitro (10, 16, 17). The molecular basis for this restricted expression can be attributed in part to the complex regulation of virus transcription. The viral components of this regulatory pathway have recently been defined for BLV and include *cis*-acting response elements in the long terminal repeats (LTRs) and a virus-specified *trans*-acting factor (4–6, 15, 22). The interaction of these components and the mechanism of transcriptional activation are still poorly understood.

The BLV transcriptional *trans*-acting factor is a 38-kilodalton (kDa) protein, referred to here as p38, encoded in the X region of the virus between the *env* gene and the 3′ LTR (4). As a result of a double-splice process, pX mRNA contains X-region coding sequences joined to translation initiation codons derived from the 5′ end of the envelope gene (19, 21, 23, 24). In addition to p38, pX mRNA is translated in a different reading frame to produce an 18-kDa nuclear phosphoprotein of unknown function (21). In previous studies it was shown that p18 was not required for transcriptional activation of the viral promoter (4). To define a function for p18, complementation experiments were designed in which transcription directed by pX-deficient proviruses was monitored in the presence or absence of pX expression plasmids. These transient expression experiments revealed a regulatory pathway in addition to transcriptional activation wherein p18 expression modulates the accumulation of full-length and single-spliced mRNAs. Furthermore, it was found that p18 probably acts at the level of the 3′-end processing reactions.

MATERIALS AND METHODS

Plasmid construction. The BLV provirus clone pBLV913 has been described (4) and was the progenitor of all proviral mutants. p913B-P and p913C-P contain frameshift mutations in the X region produced by inserting octanucleotide *Pst*I linkers into the *Bal*I site or the DNA polymerase-filled *Cla*I site, respectively. p913ΔRS was generated by deleting a 725-base-pair (bp) X-region fragment bounded by the *Sst*I site, located 50 bp upstream of the pX splice acceptor, and the *Eco*RI site. p913BG10 was made by replacing the 600-bp region between the *Cla*I site and the *Eco*RI site in the X region with a 420-bp fragment containing the rabbit β-globin coding sequences derived from the plasmid pRSV-β-globin (12). p913ΔBg was prepared by deleting a 3,720-bp *Bgl*II fragment (nucleotide positions 2580 to 6300) containing *pol* and *env* sequences. p913ΔRB is deleted of a 1,620-bp region between the *Bgl*II site at the end of the *env* gene and the *Eco*RI site in the X region. pUC-gag was made by first cloning the 5′ LTR and *gag* region of BLV into pUC19 as an *Eco*RI-to-*Bam*HI fragment; into the *Bam*HI site, downstream of the BLV *gag* gene, was inserted the *Bgl*II-to-*Bam*HI fragment from pRSV-β-globin containing the simian virus 40 polyadenylation signals. The plasmid designated pBGRS-0 was prepared by inserting the rabbit β-globin gene as a *Hin*dIII-to-*Bgl*II fragment from pRSV-β-globin into the *Hin*dIII and *Bam*HI sites of pUC19; the Rous sarcoma virus promoter (4) was then inserted into the *Hin*dIII site. Derivatives of pBGRS-0 were generated by inserting portions of the 3′ terminus of the BLV provirus downstream of the β-globin gene. pBGRS-1 contains the 3′ LTR with 270 bp of flanking viral sequences. pBGRS-2 contains a 3′ LTR lacking 10 bp of its 5′ end, and pBGRS-3 has a 3′ LTR lacking 90 bp of its 5′ end. pBGRS-5 contains the 3′ LTR as in pBGRS-3 except that it has a 100-bp internal deletion between two *Sst*I sites.

Cells and transfections. The canine, primary osteogenic sarcoma cell line D17 (ATCC CCL 183) was maintained in

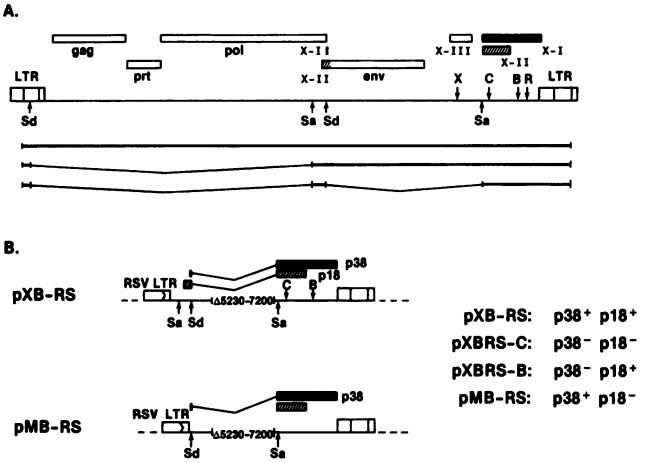

FIG. 1. (A) Genetic organization and transcription pattern of BLV. The genetic regions encoding group-specific antigens (*gag*), protease (*prt*), reverse transcriptase (*pol*), envelope glycoprotein (*env*), and X-region open reading frames X-I (p38), X-II (p18), and X-III are shown. Restriction enzyme sites used to construct proviral mutants: X, *Xho*I; C, *Cla*I; B, *Bal*I; R, *Eco*RI. The splice sites used in the formation of the three major viral mRNAs, which are shown below the genome, are designated Sd for splice donor and Sa for splice acceptor. The full-length transcript is translated to give *gag* and *pol*, the single-spliced message yields *env*, and the double-spliced mRNA gives pX proteins p38 and p18. The latter proteins are encoded in overlapping but different reading frames joined to two initiation codons derived from the 5′ end of the *env* gene. The nucleotide sequences surrounding both initiation codons predict a low efficiency of translation (4), permitting occasional bypass of the upstream AUG codon and thus dual protein synthesis. (B) Structures of pX expression plasmids. pXB-RS was constructed from the 3′ half of the BLV provirus clone pBLV913 by deleting most of the *env* region (nucleotides 5230 to 7200 on the BLV genome) and placing the resulting BLV fragment under the control of a Rous sarcoma virus promoter (4). pMB-RS was derived from pXB-RS by deleting 5′-terminal BLV sequences, including the AUG codon for p18, to a point 10 bp upstream from the p38 AUG codon. Frameshift mutants pXBRS-C and pXBRS-B were prepared by linker insertions at the *Cla*I (C) or *Bal*I (B) sites, respectively. The ability of each of these expression plasmids to produce p38 or p18 in transfected cells is summarized at the right.

Dulbecco minimal essential medium supplemented with 10% fetal bovine serum. Samples of 1.5×10^6 cells were plated in 10-cm culture dishes 1 day before transfections. Plasmid DNAs were introduced into cells as calcium phosphate coprecipitates (4). Cells and DNA were incubated for 8 h, followed by a single wash with phosphate-buffered saline and replacement with fresh medium. Approximately 40 h later, cells were washed twice with cold phosphate-buffered saline, collected by scraping, transferred to 1.5-ml Microfuge tubes, and pelleted by centrifugation for 15 s. Cells were suspended in 0.2 ml of ice-cold 10 mM Tris hydrochloride (pH 7.5)–0.15 M NaCl–1.5 mM $MgCl_2$–0.5% Nonidet P-40 and placed on ice for 5 min. Nuclei were pelleted, and the cytoplasmic supernatants were transferred to new tubes containing 0.2 ml of 7 M urea–0.35 M NaCl–10 mM EDTA–1% sodium dodecyl sulfate and extracted twice with phenol-chloroform. Total cytoplasmic RNA was then ethanol precipitated and stored at −20°C.

Poly(A)$^+$ mRNA and Northern (RNA) blotting. Poly(A)$^+$ mRNA was prepared by oligo(dT)-cellulose chromatography. Half of each sample (approximately 2 μg of mRNA) was applied to lanes of a 1.5% agarose–2.2 M formaldehyde gel. RNAs were transferred to nitrocellulose or nylon (BioTrace RP; Gelman Sciences Inc.) membrane filters and hybridized as previously described (4).

RESULTS

Role of both pX proteins in BLV *gag* and *env* mRNA synthesis. The genetic organization and transcription pattern of BLV are shown in Fig. 1A. As for HTLV-I and HTLV-II, but unlike other RNA tumor viruses, a small transcript arising via a double-splice event and representing part of the X region is synthesized in infected cells (19, 21, 24). For BLV this bicistronic mRNA encodes both the 38-kDa *trans*-activator (p38) and, in an alternate overlapping reading frame, an 18-kDa nuclear phosphoprotein (p18) (21, 23). To define a role for this protein and other viral sequences, a series of proviruses were constructed that contain frameshift mutations, deletions, or substitutions in the X region. The proviral mutants were defective in pX expression but possessed all normal splice sites, thus permitting one to follow the synthesis of all three viral transcripts. A second series of plasmids, designed to express p38 and p18, were cotransfected with the altered proviruses to complement potential defects in expression. These pX expression plasmids (Fig. 1B) include pXB-RS (p38$^+$ p18$^+$), pXBRS-C (p38$^-$ p18$^-$), pXBRS-B (p38$^-$ p18$^+$), and pMB-RS (p38$^+$ p18$^-$). Transient RNA synthesis was monitored by Northern blot analysis of poly(A)$^+$ mRNA isolated from the transfected cells.

In the first set of experiments an intact proviral clone, pBLV913, was cotransfected with the pX expression plasmids into D17 cells (Fig. 2A, lanes 1 through 5). When pBLV913 was introduced into cells with plasmids that were unable to supply the transcriptional activator, p38, barely detectable levels of full-length (genomic) or single-spliced (*env*) viral mRNAs were observed (Fig. 2A, lanes 1, 4, and 5). In contrast, cotransfection of pBLV913 with either pXB-RS (p38$^+$ p18$^+$) or pMB-RS (p38$^+$ p18$^-$) resulted in the accumulation of viral transcripts at high levels (Fig. 2A, lanes 2 and 3). In this experimental system, transcription of the provirus requires expression of p38 from an independent source. Apparently a mechanism exists such that the provirus is unable to synthesize enough functional p38 to sustain a self-activation cycle. Next, provirus X-region mutants were examined. The mutated proviruses p913C-P and p913ΔRS are defective in both p38 and p18 due to a frameshift mutation or deletion of pX coding sequences, respectively. When these mutants were introduced into cells with the plasmid pXB-RS, viral mRNA synthesis directed by both mutants was abundant (Fig. 2A, lanes 6 and 10). However, when cells were transfected with pMB-RS, which produces p38 but not p18, viral mRNAs were produced at very low levels (Fig. 2A, lanes 7 and 11). It should be noted that virus RNAs were not detected in cells transfected with the mutated proviruses alone (not shown). Thus, p18 was required for the accumulation of BLV genomic and *env* mRNAs.

The provirus p913B-P has a frameshift mutation that prevents the synthesis of p38 but not p18. Unlike the other mutant proviruses, expression of p913B-P was complemented equally by pXB-RS and pMB-RS (Fig. 2A, lanes 8 and 9), suggesting that p18 could be supplied by the provirus. To show that p18, like p38, acts in *trans* to regulate the synthesis of viral mRNAs, cotransfection experiments were performed in which the two proteins were supplied independently by the plasmids pMB-RS (p38$^+$ p18$^-$) and pXBRS-B (p38$^-$ p18$^+$) or concomitantly by the plasmid pXB-RS. The altered provirus p913C-P did not direct substantial levels of mRNA synthesis in the presence of either p38 or p18 alone

(Fig. 2B, lanes 2 and 3). However, when cells were cotransfected with a combination of pMB-RS and pXBRS-B (Fig. 2B, lane 4), mRNA levels were restored to approximately the levels obtained in cotransfections with pXB-RS (lane 1). Thus, p18 acts in *trans* to regulate the production of genomic and *env* mRNAs. Since it was previously shown that p18 is not required for the transcriptional activation of the BLV promoter (4), its effects are probably exerted at posttranscriptional steps. It is apparent that the requirement for p18 in this experimental system was not absolute since low levels of virus mRNA were detected in its absence (Fig. 2A, lanes 7 and 11; Fig. 2B, lanes 2, 6, and 10).

Synthesis of pX mRNA. In the previous experiments, Northern blots were hybridized with an *env* region probe to monitor provirus expression and avoid interference from RNAs produced by the pX expression plasmids. To visualize all three BLV mRNAs, the provirus p913BG10 was constructed, in which the region downstream of the pX splice acceptor was replaced with a rabbit β-globin cDNA sequence (Materials and Methods). RNA synthesis was monitored by hybridization of Northern blots with a β-globin probe, allowing the double-spliced message from the provirus to be distinguished from that produced by the pX expression plasmids. Production of full-length and *env* mRNAs, in cells transfected with p913BG10, required both p38 and p18 (Fig. 2B, lanes 5 through 8). Longer exposures of these blots (Fig. 2B, lanes 9 and 10) revealed that the synthesis of the double-spliced message, corresponding to pX mRNA, was not dependent on p18 expression. This result suggests that processing steps utilized to generate the pX message, specifically the 3′ splice event, circumvent the p18-dependent reactions.

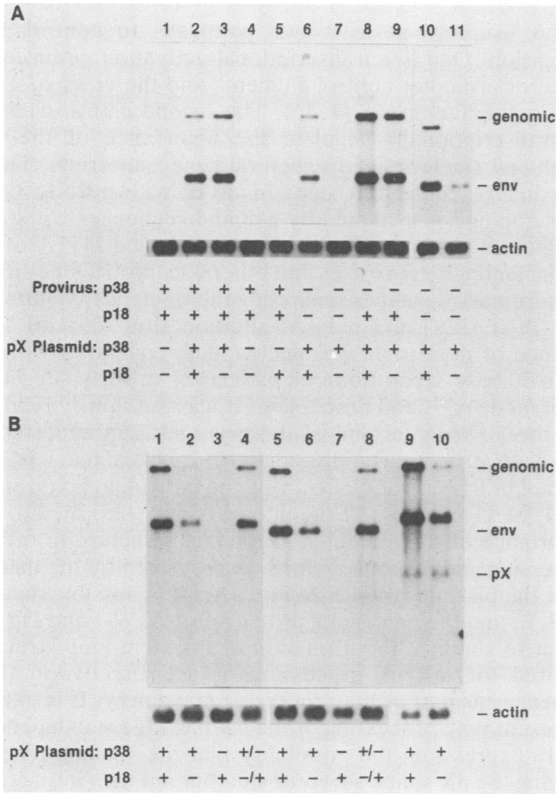

FIG. 2. Northern blot analysis of transiently expressed poly(A)$^+$ mRNAs from cotransfected cells. (A) D17 cells were cotransfected with the provirus clone pBLV913 and either pUC19 (lane 1), pXB-RS (lane 2), pMB-RS (lane 3), pXBRS-C (lane 4), or pXBRS-B (lane 5). Proviral mutants p913C-P (lanes 6 and 7), p913B-P (lanes 8 and 9), or p913ΔRS (lanes 10 and 11) were cotransfected with pXB-RS (lanes 6, 8, and 10) or pMB-RS (lanes 7, 9, and 11). The blots were hybridized with a 2-kilobase probe representing BLV *env* and post-*env* sequences that were not present in the pX expression plasmids (i.e., from positions 5230 to 7200) or with an actin probe (lower inset). Unspliced (genomic) and single-spliced (*env*) mRNAs are indicated. The ability of each cotransfected provirus and pX expression plasmid to produce p38 or p18 is summarized below each lane. (B) Provirus clones p913C-P (lanes 1 through 4) and p913BG10 (lanes 5 through 10) were introduced into D17 cells with pXB-RS (lanes 1, 5, and 9), pMB-RS (lanes 2, 6, and 10), pXBRS-B (lanes 3 and 7), or pMB-RS plus pXBRS-B (lanes 4 and 8). All transfections utilized 15 μg of provirus DNA and 2 μg of pX expression plasmid DNA. Blots were hybridized with the BLV *env* probe as described above (lanes 1 through 4) or with a rabbit β-globin probe (lanes 5 through 10). Hybridization with the actin probe is shown in the lower inset. Lanes 9 and 10 are longer exposures than the rest to bring up the double-spliced (pX) message.

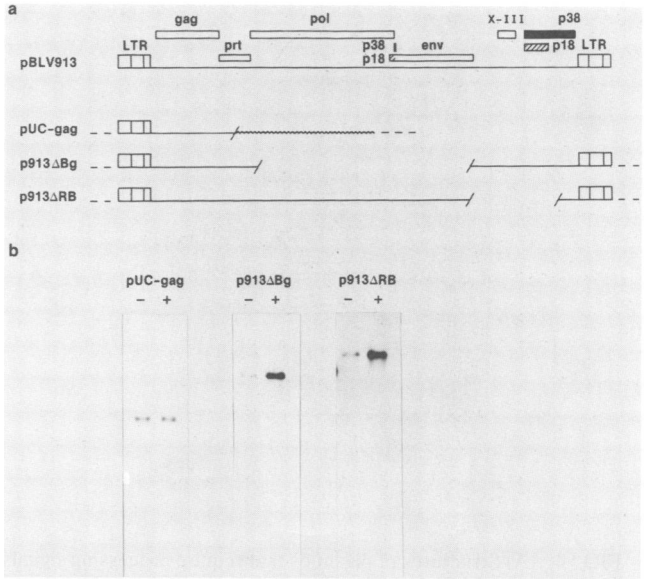

FIG. 3. (a) Structures of deleted proviruses used to locate p18 responsive sequences. pBLV913 is the complete BLV provirus clone used to generate the deletion constructs. pUC-gag contains the 5′ LTR and *gag* region of BLV fused to the polyadenylation sequences of simian virus 40. p913ΔBg was deleted of a 3,720-bp *Bgl*II fragment containing *pol* and *env* sequences; p913ΔRB lacks a 1,620-bp fragment between the 3′ end of *env* and the *Eco*RI site in the X region. (b) Northern blot analysis of transiently expressed mRNAs. Cotransfected DNAs contained 15 μg of the indicated provirus construct and 2 μg of pMB-RS (lanes –) or 2 μg of pXB-RS (lanes +). Blots were hybridized with a 450-bp BLV *gag* region probe (positions 1090 to 1550).

Location of p18-responsive sequences. To define possible sites of action for p18, the transcription patterns of proviruses with large regional deletions (Fig. 3a) were examined. The first plasmid in this series, pUC-gag, contains the 5′ LTR and *gag* gene of BLV fused to simian virus 40 polyadenylation sequences. Levels of mRNA produced from pUC-gag were the same in the presence or absence of p18 expression (Fig. 3b), indicating that sequences that mediate p18 control had been deleted. This result argues against regulatory mechanisms directly involving perturbations of transcription initiation or RNA elongation. The plasmids p913ΔBg and p913ΔRB (Fig. 3a) together lack sequences encompassing *pol*, *env*, and most of the X region. Accumulation of mRNA directed by these plasmids was dependent

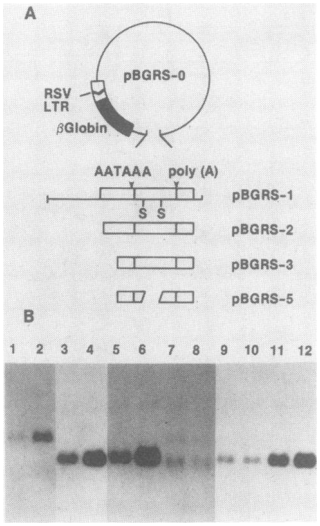

FIG. 4. (A) Structures of plasmids that require processing signals supplied by the BLV 3′ LTR. The parent plasmid for this series, pBGRS-0, was constructed by inserting the rabbit β-globin gene and Rous sarcoma virus promoter into pUC19. Derivatives of pBGRS-0 contain portions of the 3′ terminus of BLV placed downstream of the β-globin coding sequences. The LTR fragments consist of: pBGRS-1, a 3′ LTR with 270 bp of flanking viral sequence; pBGRS-2, a 3′ LTR lacking 10 bp of its 5′ end; pBGRS-3, a 3′ LTR lacking 90 bp of its 5′ end; and pBGRS-5, the 3′ LTR of pBGRS-3 with a deletion of a 100-bp *Sst*I (S) fragment between the poly(A) addition signal (AATAAA) and addition site. (B) Northern blot analysis of transient β-globin expression. Samples (5 μg) of pBGRS-1 (lanes 1 and 2), pBGRS-2 (lanes 3 and 4), pBGRS-3 (lanes 5 and 6), or pBGRS-5 (lanes 7 and 8) were transfected with 5 μg of either pXBRS-C ($p38^-$ $p18^-$; lanes 1, 3, 5, and 7) or pXBRS-B ($p38^-$ $p18^+$; lanes 2, 4, 6, and 8). In addition, all transfections contained 5 μg of pUC19 DNA. Lanes 9 to 12 represent transfections of 5 μg of pBGRS-3, 5 μg of pUC19, and 0, 0.1, 0.5, and 5.0 μg of pXBRS-B, respectively. pXBRS-C was added where necessary to adjust the total DNA concentration to 15 μg and to maintain a constant amount of the Rous sarcoma virus promoter. Blots were hybridized with a rabbit β-globin probe.

on the expression of p18 (Fig. 3b), revealing that the *cis*-acting sequences responsive to p18 must be located in the 3′ terminus of the virus.

Effects on 3′-end processing reactions. To more precisely define the p18-responsive sequences, plasmids were constructed that contained a rabbit β-globin coding domain controlled by a Rous sarcoma virus promoter (pBGRS-0, Fig. 4A). 3′-End processing signals were required for β-globin mRNA synthesis in transfected cells (data not shown) and were supplied by inserting BLV fragments, derived from the 3′ end of the provirus, into pBGRS-0. Based on densitometric analyses, levels of β-globin mRNA directed by pBGRS-1, 2, and 3 (containing various portions of the 3′ terminus of the virus) were from 10 to 20 times higher when cells were cotransfected with the p18 expression plasmid pXBRS-B than with pXBRS-C ($p18^-$) (Fig. 4B, lanes 1 through 6). As the amount of pXBRS-B cotransfected with pBGRS-3 was increased, a parallel increase in the levels of β-globin mRNA was observed (Fig. 4B, lanes 9 through 12). A 100-bp deletion in the R region of the BLV LTR, present in pBGRS-5, does not alter the known 3′-end processing signals but would prevent formation of a potential secondary structure (25, 29). pBGRS-5 was unable to efficiently direct the production of β-globin mRNA in transfected cells (Fig. 4B, lanes 7 and 8), indicating that these deleted sequences are essential for mRNA processing. Furthermore, the low levels of β-globin RNA that were observed were not influenced by p18 expression, suggesting that the p18-responsive sequences had been removed. Together, these data suggest that p18 acts in *trans* with sequences located within the 3′ LTR either to increase the rates of mRNA processing reactions, increase message stability, or alter the subcellular localization of specific transcripts. As in the preceding experiments, the requirement for p18 was not absolute and some RNA processing occurred in its absence, perhaps as a result of overloading the system with a disproportionate amount of RNA.

DISCUSSION

BLV employs at least two pathways to control gene expression. One is a transcriptional activation circuit comprised of promoter control elements and the virus-specified *trans*-acting factor p38 (4, 15). The second pathway acts to posttranscriptionally regulate the abundance of the full-length and single-spliced structural gene transcripts. This is most likely achieved at steps involved with mRNA 3′-end formation and is mediated by p18 and sequences within the R region of the 3′ LTR. BLV, HTLV-I, and HTLV-II are unique among retroviruses and other eucaryotic genes in the extraordinary distances separating their polyadenylation signals (AATAAA) and poly(A) addition sites (25, 26). This distance of more than 250 nucleotides, compared with the 10- to 30-base separation in most genes, strongly suggests a novel mode of 3′-end processing. It was previously reported that the 3′ RNA termini of these viruses can adopt stable stem-loop structures (approximately −154 kcal [ca. −643 kJ] per mol in BLV) that would bring the polyadenylation signal (AATAAA) and poly(A) site into proximity (25, 26, 29). The importance of this potential secondary structure in mRNA processing and response to p18 was revealed by the inactivity of the plasmid pBGRS-5 (Fig. 4A). It is possible that p18 in BLV, and the analogous proteins in HTLV-I and HTLV-II, act to stabilize the formation of the stem-loop structure required for mRNA processing or act directly on these sequences as part of the processing machinery. It is unclear at present why processing of the pX message is independent of p18 expression; the possibility that the splicing reaction utilizing the pX splice acceptor site (but not the *env* acceptor site) permits 3′-end processing in the absence of p18 is currently being tested. Control of gene expression through 3′-end processing reactions has been reported in several other systems: the pattern of adenovirus late gene transcription was shown to be controlled at the level of 3′-end selection and splicing (20), and the two immunoglobulin mu gene mRNAs, encoding membrane-bound and secreted forms of the protein, represent utilization of alternate polyadenylation sites (1, 7). However, the *cis*- and *trans*-acting regulatory components have not yet been identified in these systems.

The biological purpose of the two regulatory pathways employed by BLV is not clear since little is known about BLV expression in vivo. Similar mechanisms are used by certain DNA viruses to temporally regulate and coordinate the pattern of gene expression. It is possible that the pathways described here insure a rapid burst of virus production after some initiating event. For example, immediately after induction, virus transcription would predominantly yield the double-spliced pX mRNA, since its synthesis is independent of p18. Translation of the pX

message and subsequent accumulation of p38 and p18 would result in both an amplification of virus transcription and a switch to the processing events allowing expression of virus structural genes.

The synthesis and actions of *trans*-regulatory proteins seem to unify BLV, HTLV, and the distantly related human immunodeficiency virus. HTLV-I, which is structurally related to BLV, was recently shown encode proteins of 40 and 27 kDa that are functionally analogous to p38 and p18 of BLV, respectively (3, 9, 13, 14, 18, 27). Interestingly, human immunodeficiency virus also produces two regulatory proteins, *Tat* and *Art/Trs*, translated from a bicistronic, multiply spliced mRNA (8, 28); the *Art/Trs* protein appears to regulate the abundance of structural gene products. Although the genetic arrangements, locations of *cis*-acting elements, and mechanisms of control may differ among these viruses, they all utilize transcriptional and posttranscriptional controls. It will be of interest to determine whether the two *trans*-regulatory proteins of BLV interact with one another, are regulated by posttranslational modifications, or have activities in addition to those described here.

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