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Bovine Leukemia Virus Transcription Is Controlled by a Virus-Encoded *trans*-Acting Factor and by *cis*-Acting Response Elements

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Bovine leukemia virus (BLV) gene expression is exquisitely regulated at multiple levels, including a transcriptional control effected by virus-encoded *trans*-acting factors and *cis*-acting target sequences. Like the human T-cell leukemia viruses type I and type II, but unlike other RNA tumor viruses, BLV contains several open reading frames at the 3′ end of its genome. A subgenomic mRNA which encodes two overlapping reading frames from this region could produce proteins of 38 and 18 kilodaltons (kDa). A series of *cis-trans* experiments using transfected virus gene constructs in different combinations revealed that expression of the 38-kDa protein was both necessary and sufficient to activate, in *trans*, the BLV promoter. This activation was specific for the BLV long terminal repeat, as a variety of related retroviral promoters were not responsive to the expression of the 38-kDa protein p38(XBL). Deletion analysis and construction of chimeric promoters identified a 75-base-pair long terminal repeat region which functions like a p38(XBL)-dependent enhancer element.

Bovine leukemia virus (BLV) is an RNA tumor virus which infects B lymphocytes of cattle, often causing persistent lymphocytosis and less frequently lymphoma (for reviews, see references 1 and 12). BLV and human T-cell leukemia viruses type I and type II (HTLV-I and HTLV-II) constitute a unique subgroup within the retrovirus family, characterized by a distinct genetic content, organization, and strategy for gene expression. In general, these viruses are not expressed at detectable levels in lymphocytes or tumors of the infected host (9, 19, 20). Paradoxically, circulating antibodies to virus proteins are found in reasonably high titers (29), and when lymphocytes are transferred from the infected host to in vitro cell cultures, virus production often ensues (9, 27, 46). To understand this unusual pattern of virus gene expression, experiments have focused on the transcriptional activity of the viral promoters with the aim of characterizing the *cis*-acting promoter control sequences and the *trans*-acting factors with which they interact.

The retroviral promoter is contained within the proviral long terminal repeats (LTRs). These structures are derived via a fusion of the 5′ and 3′ ends of viral RNA during replication (47, 48). The transcriptional activity of the BLV LTR has previously been examined in transient expression assays by using the chloramphenicol acetyltransferase (CAT) system (4, 30). Those experiments revealed that the BLV LTR was a functional promoter only in cells productively infected with BLV. In a similar way, the HTLV-I and HTLV-II LTRs were found to be active promoters only in cells producing HTLV-I and HTLV-II (3, 10, 44). Deletion analysis of the BLV LTR and construction of chimeric promoters containing BLV LTR sequences led to the identification of an LTR region that mediates this specific activation phenomenon (5). Because the BLV sequences were active only in cells expressing virus, it was hypothesized that BLV might produce the *trans*-acting factor which stimulates its own promoter.

The BLV genome contains an approximately 1,800-base-pair (bp) stretch, termed the X region, located between the envelope gene and the 3′ LTR (33). Within this region are encoded three open reading frames, X-I, X-II, and X-III. A subgenomic mRNA has been identified in BLV-infected cells that contains the overlapping open reading frames X-I and X-II. This mRNA is generated by a double-splice event which joins three genetic regions: 100 nucleotides from the R region of the 5′ LTR, a 220-nucleotide exon surrounding the 5′ end of the envelope gene, and a 1,360-nucleotide exon containing the extreme 3′ end of the virus RNA genome (25, 32). Translation of the alternative, overlapping reading frames, X-I and X-II, encoded in this unique transcript would yield proteins of approximately 38 and 18 kilodaltons, respectively. Although both proteins have been immunologically identified in BLV-infected cells (31; Nancy Rice, personal communication), no function for either has yet been shown.

The present studies were undertaken to determine whether expression of the BLV pX genes results in the transcriptional activation of the cognate LTR and, further, to identify whether one or both of the open reading frames functions in the activation process. In addition, an examination of the LTR was made to identify the *cis*-acting target(s) or response element(s) that interact with the *trans*-acting factors. These problems were addressed by simultaneously introducing two types of plasmids into mammalian cell lines. The first contained an assayable gene or dominant selectable marker controlled by BLV promoter elements; the second contained BLV pX open reading frames coupled to various transcriptional promoters. The transient and long-term cotransfection experiments revealed that expression of p38(XBL) was necessary and sufficient to effect *trans*-activation of the BLV promoter. Furthermore, deletion analyses and construction of hybrid promoters led to the identification of a 75-bp LTR region containing the p38(XBL) response elements.

MATERIALS AND METHODS

Plasmid constructions. The plasmids pSV2cat, pRSVcat, and pSV0cat (14, 15) were originally obtained from Bruce Howard. The structures of pBLH2cat, pBLS1cat, pSVΔEcat, pΔE75Bg-1, and pΔE75Bg-2 have been described previously (4, 5). Deletions of 5'-terminal BLV LTR sequences were made by digesting the LTR with the enzymes *Hae*II, *Pvu*II, *Rsa*I, or *Hph*I, followed by digestion with *Sac*I and end repair with DNA polymerase. The resulting fragments were ligated to *Hin*dIII linkers and inserted into the *Hin*dIII site of pSV0cat. pEI-1cat contains the equine infectious anemia virus LTR in pSV0cat as described before (7). pHT2cat contains the HTLV-II LTR, derived from the plasmid pH6R0.8 provided by Irvin Chen (38), inserted into pSV0cat. pHTLV-7cat contains the HTLV-I LTR fragment from the pCR-1 clone (18) provided by Leonard Seigel. pSV0neo was prepared by replacing the *Hin*dIII-to-*Bam*HI fragment of pSV0cat with the *Hin*dIII-to-*Bam*HI fragment of pSV2neo (obtained from the American Type Culture Collection, Rockville, Md.). pSV0neo is identical to pSV0cat except for the bacterial drug resistance genes. pBL9neo was constructed by transferring the *Hin*dIII fragment containing the BLV LTR and flanking sequences from the plasmid pBL9cat (4) into pSV0neo. pBLV913 is a plasmid subclone of a BLV provirus cloned from the productively infected fetal lamb kidney cell line FLK-BLV (James Casey, unpublished data). It contains a complete provirus with 250 bp of 5' cellular flanking sequence and 3 bp of 3' flanking sequence cloned into *Kpn*I-modified *Pvu*II sites of pUC9. pHK-3' is a subclone of the 3' half of pBLV913; the 4.6-kbp *Hin*dIII-to-*Kpn*I fragment was cloned into the *Hin*dIII and *Kpn*I sites of pUC19. pXB was derived from pHK-3' by first deleting the 1,975-bp region between the two *Bam*HI sites, followed by deleting the region upstream of the *Xho*I site by digestion with *Xho*I, repair with DNA polymerase, ligation to *Hin*dIII linkers, and recircularization after removal of the 430-bp *Hin*dIII-*Xho*I fragment. The BLV LTR from pBLH2cat was inserted into pXB to produce pXB-BL. The simian virus 40 (SV40) early promoter was obtained as a *Pvu*II-to-*Hin*dIII fragment from pSV2cat; the *Pvu*II end was converted to a *Hin*dIII end with synthetic linkers and then inserted into pXB, yielding pXB-SV. The Rous sarcoma virus (RSV) promoter was removed from pRSVcat as a 390-bp *Sau*96-1-to-*Hin*dIII fragment; after modification of the *Sau*96-1 end with *Hin*dIII linkers, the LTR fragment was coupled to pXB to give pXB-RS. Frameshift mutations were introduced into pXB-RS by digestion with *Cla*I in the presence of DNA polymerase or with *Bal*I followed by ligation to the synthetic octanucleotide, GCTGCAGC, which contains the *Pst*I recognition site. The plasmid pMB-RS was derived from pXB by partial digestion with *Sau*3A1 (*Mbo*I isoschizomer). The *Sau*3A1 overhang was filled by using DNA polymerase and then ligated to *Hin*dIII linkers and digested with *Hin*dIII, followed by recircularization of the plasmid. This deletes the 310-bp *Hin*dIII-to-*Sau*3A1 fragment at the 5' end of BLV sequences in pXB. The RSV promoter was transferred from pXB-RS to the new *Hin*dIII site to give pMB-RS. All plasmids were grown in *Escherichia coli* JM83 or HB101, extracted by alkaline lysis, and banded twice in cesium chloride density gradients.

Cells and transfections. The human B lymphocyte cell line Raji was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Transfections were done by suspending 10^7 cells in 3 ml of RPMI 1640 containing 10 μg of plasmid DNA, 250 μg of DEAE-dextran per ml, and 50 mM Tris hydrochloride (pH 7.3). Cells were incubated for 60 min at 37°C, pelleted, washed twice with complete medium, and then suspended in 10 ml of complete medium. The human rhabdomyosarcoma cell line RD-4 and its derivatives were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Transfections of plasmids for transient expression assays were done with 5×10^5 cells per 6-cm-diameter culture dish. Supercoiled plasmid DNA (10 μg) was applied as a calcium phosphate precipitate as previously described (15). Transfections of plasmids containing the dominant selectable marker, *neo*, were done using 10^6 RD-4 cells per 10-cm-diameter culture dish. Plasmid DNA (20 μg), previously linearized by digestion with specific restriction endonucleases, was applied as a calcium phosphate precipitate. At 48 h after transfection, cells were transferred to medium containing 500 μg of geneticin (G418) per ml. Individual G418-resistant colonies were isolated by the cloning cylinder method and expanded for further studies.

CAT assays. Cells were harvested 48 h after transfection, and cell extracts were prepared as previously described (4, 15). CAT assay reaction mixtures of 100 μl contained 0.15 M Tris hydrochloride, pH 7.8; 56 μM [^{14}C]chloramphenicol, ca. 50 mCi/mmol; 1 mM acetyl coenzyme A; and cell extract. Reactions were incubated at 37°C; reaction times and amounts of cell extract varied depending on the cell line and the promoter directing *cat* expression to ensure a linear correlation to CAT enzyme levels.

DNA and RNA blot hybridizations. Total cellular RNA was isolated by guanidine thiocyanate disruption of cells and pelleting through a CsCl layer as previously described (26). Polyadenylated mRNA was prepared by oligo(dT) cellulose column chromatography. mRNA was fractionated on 1% agarose–formaldehyde gels and transferred to nitrocellulose filters. High-molecular-weight cellular DNA was isolated either by sodium dodecyl sulfate-proteinase K disruption of cells or concomitant with the preparation of RNA by the guanidine thiocyanate-CsCl method. In both methods, DNA was extracted with phenol-chloroform, ethanol precipitated, dissolved, and extensively dialyzed. DNAs were digested with restriction endonucleases, fractionated by electrophoresis on 1% agarose gels, and transferred to nitrocellulose filters. Both Southern and Northern (RNA) blots were hybridized with probes prepared by nick translating DNA fragments encompassing the region at the 3' end of the BLV genome. Hybridizations were done at 37°C for 48 h in 50% formamide, 1 M NaCl, 50 mM PIPES (piperazine-*N*,*N*'-bis[2-ethanesulfonic acid]) (pH 6.4), 200 μg of calf thymus DNA per ml, 10 mM EDTA, 0.1% *N*-lauroylsarcosine, 0.1% bovine serum albumin, 0.1% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), and 0.1% polyvinylpyrrolidone. The DNA probe was present at a concentration of 30 ng/ml (10^8 cpm/μg). Blots were washed in a 0.1× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) and 0.1% *N*-lauroylsarcosine, at 50°C and then exposed to X-ray film.

RESULTS

Transient expression of the X region. To determine whether BLV encodes a gene(s) whose expression either directly or indirectly activates its own promoter, cotransfection experiments were done. In these experiments, the BLV LTR was coupled to the *cat* gene in the plasmid pBLH2cat. No CAT enzyme activity is produced when this plasmid is introduced into most cell lines, since the BLV LTR is transcriptionally

active only in cells productively infected with the virus (4, 30). CAT activity would be transiently expressed in uninfected cells, however, if pBLH2cat was cotransfected with a plasmid that produced the factors that transcriptionally activate the BLV LTR. This approach was initially tested by cotransfecting pBLH2cat with a plasmid containing the entire BLV provirus, pBLV913, onto the human rhabdomyosarcoma cell line RD-4 or the human B lymphocyte cell line Raji. CAT activity was not detected in either cell line when pBLH2cat was introduced alone; in the presence of pBLV913, a low level of activity was measured in RD-4 cells but not in Raji cells (Table 1). In view of later results, this negligible level of CAT activity, and thus transactivation, reflects the inability of the transfected provirus to either initiate or sustain gene expression. It was reasoned that to examine the effects of expression of the BLV X region it would be necessary to eliminate extraneous sequences and to place these genes under the control of strong promoter elements.

This rationale led to the series of plasmids designated pXB (Fig. 1). The genetic organization of BLV as well as the transcription and processing events which lead to the generation of pX mRNA is shown in Fig. 1A. pXB plasmids (Fig. 1B) were derived from the 3′ half of the genome present in the plasmid pHK-3′; the envelope and post-envelope sequences were deleted, leaving a 400-bp intron in the pX coding domain. Promoter units were then inserted at the *Xho*I site (modified with *Hin*dIII linkers) located about 100 bp upstream of the second pX exon, thus relieving the requirement for the usual upstream splice event. The SV40 early promoter, RSV LTR, and BLV LTR were inserted into pXB to give the plasmids pXB-SV, pXB-RS, and pXB-BL, respectively. The ability of these constructs to *trans*-activate the BLV promoter was tested by cotransfection with pBLH2cat in a number of cell lines; results of such experiments in RD-4 and Raji cells are summarized in Table 1. Clearly, cotransfection with pXB-RS or pXB-SV, in which pX expression is controlled by strong promoters, results in high levels of CAT activity directed by the BLV LTR in both cell lines. In RD-4 cells, the levels of activity observed were approximately 15 times higher than those produced from pSV2cat (in which the *cat* gene is controlled by the SV40 early promoter). In Raji cells, cotransfection of pBLH2cat and pXB-SV produced levels of CAT activity approximately 3.5 times higher than those obtained with pSV2cat. Thus, overexpression of the BLV pX genes supplies the factor(s) that activates the BLV promoter.

Placing pXB expression under the control of the BLV LTR led to some puzzling results, which may provide insights into some of the properties of the factors acting in this pathway. In RD-4 cells, pXB-BL activated expression of pBLH2cat at about half the level observed with pXB-RS or pXB-SV. In Raji cells, levels of CAT activity obtained from pBLH2cat in the presence of pXB-BL were approximately 100 times lower than with pXB-SV. A priori, one would expect that pXB-BL would either be totally inactive because of an initially quiescent promoter or highly active as a consequence of self *trans*-activation, i.e., a positive feedback loop. The results of the cotransfections of Raji cells support the first expectation; in RD-4 cells, however, pXB-BL produced an effect between the two extremes.

This phenomenon was examined further by transfecting RD-4 cells with pBLH2cat in the presence of increasing amounts of either pXB-RS or pXB-BL (Fig. 2). *cat* expression, directed by the BLV LTR, was activated when pXB-RS was present in amounts as low as 0.01 μg. The levels of activation obtained with pXB-RS increased sharply when the amount of plasmid DNA was increased between 0.01 and 0.10 μg and leveled out thereafter. In contrast, the levels of BLV promoter activation observed by using pXB-BL, whose expression is dependent on its own gene product, were well below those obtained with pXB-RS at all concentrations examined. The inability of pXB-BL to produce a very strong *trans*-activation suggests some kind of self-limitation.

TABLE 1. *trans*-Activation of the BLV-LTR in transient cotransfection experiments[a]

Transfected plasmids	Relative CAT activity of:	
	RD-4	Raji
pSV2cat	1.00	1.00
pBLH2cat + pUC19	<0.01	<0.01
pBLH2cat + pBLV913	0.21	<0.01
pBLH2cat + pXB	0.35	<0.01
pBLH2cat + pXB-RS	15.3	0.90
pBLH2cat + pXB-SV	14.5	3.48
pBLH2cat + pXB-BL	6.70	0.03

[a] Levels of CAT activity are presented relative to the level of activity obtained with pSV2cat. *cat* gene expression is directed by the SV40 early promoter in pSV2cat or by the BLV LTR in pBLH2cat. The cloning vector, pUC19, does not contain any pertinent coding information. The plasmid pBLV913 contains a complete BLV provirus from which the X region constructs were derived (Fig. 1). The pXB series of plasmids contain either no promoter (pXB), the RSV LTR (pXB-RS), the SV40 early promoter (pXB-SV), or the BLV LTR (pXB-BL). pSV2cat (10 μg) or pBLH2cat (5 μg) plus 5 μg of the indicated plasmids was transfected onto RD-4 cells as calcium phosphate coprecipitates or onto Raji cells by the DEAE-dextran method. Cells were harvested approximately 40 h after transfection and assayed for CAT enzyme activity. Assays of RD-4 transfectants used 10 μl of cell extract incubated for 10 min at 37°C; Raji assays contained 30 μl of cell extract incubated for 12 h at 37°C. In both cases, pSV2cat transfectants gave approximately 5% conversion of chloramphenicol to its monoacetate products.

Stable expression of the BLV X region. The following experiments examine whether the effects of pX expression observed in transient assays are also manifested in cells containing stably integrated copies of pXB-RS. The dominant selectable marker gene, *neo*, was coupled to the BLV LTR yielding the plasmid pBL9neo. In mammalian cells, this plasmid would produce the bacterial aminoglycoside phosphotransferase enzyme and thus allow cells to grow in the presence of geneticin (G418) (45), but only if the cells also produced the factor(s) which activates the BLV promoter. pBL9neo or pSV2neo (in which *neo* expression is directed by the SV40 early promoter) were cotransfected with a variety of plasmids onto RD-4 cells. The number of G418-resistant colonies obtained after transfection with pSV2neo was unaffected by the contents of the cotransfected plasmids (Table 2). pBL9neo transfection, on the other hand, did not produce a significant number of colonies unless introduced into cells with pXB-RS. These data qualitatively parallel what was seen in the transient-expression assays; i.e., synthesis of a gene controlled by the BLV LTR requires the concomitant expression of the BLV pX genes. Interestingly, the *trans*-acting factor(s) was not supplied in sufficient quantities by the integrated plasmids pBLV913 or pXB-BL to allow cell growth in the presence of G418.

The G418-resistant colonies which resulted from these cotransfections were individually expanded and analyzed with respect to pX integration, expression, and *trans*-activation. The cell lines derived from the infrequent G418-resistant colonies arising after cotransfections with pBL9neo and either pUC19, pXB-BL, or pBLV913 were unable to

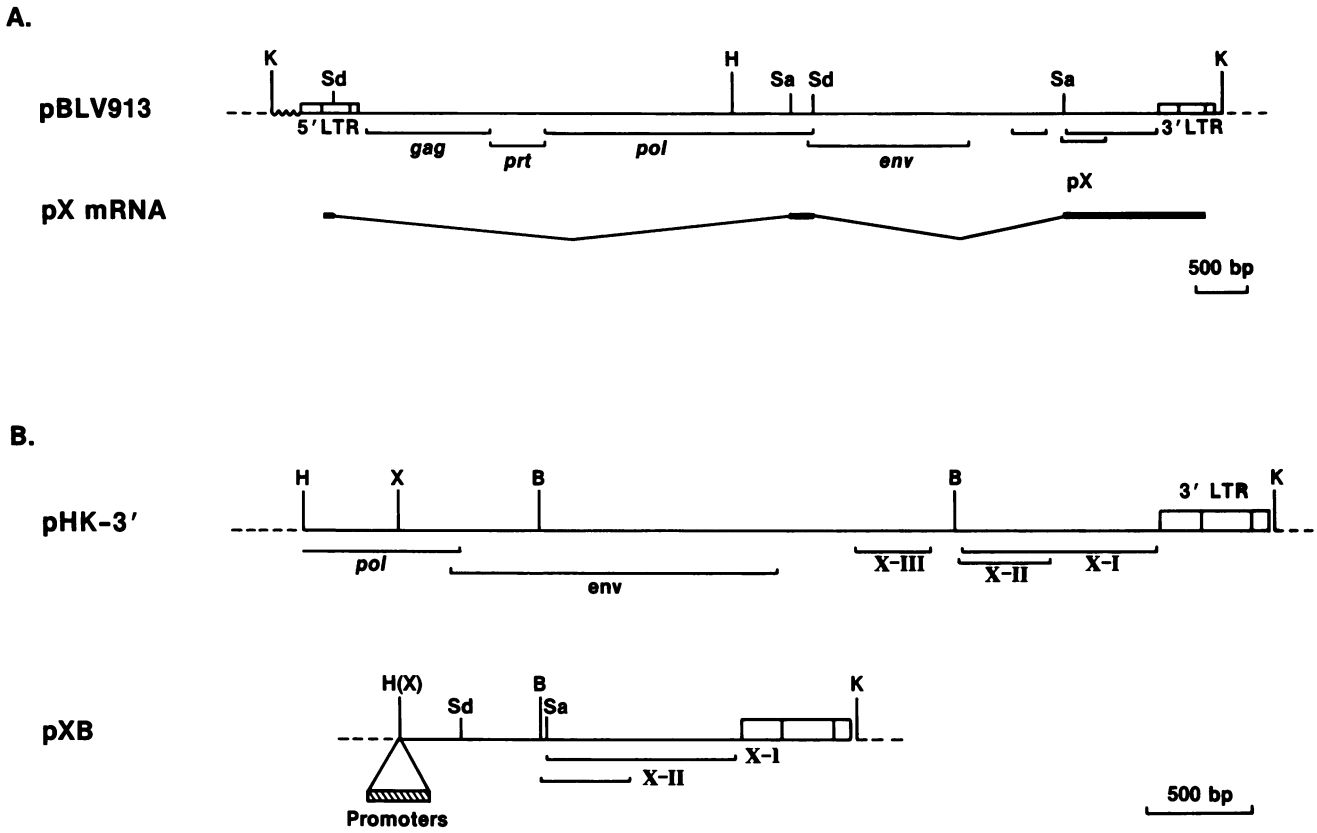

FIG. 1. (A) Genetic organization of BLV and the transcription program used to generate pX mRNA deduced from nucleotide sequence data. An intact BLV provirus was subcloned as a *Kpn*I insert into a modified pUC9 plasmid to give pBLV913. The genetic regions encoding the group-specific antigens (*gag*), protease (*prt*), reverse transcriptase (*pol*), envelope glycoproteins (*env*), and the open reading frames in the X region (X-I, -II, and -III) are shown below pBLV913. The splice donor (Sd) and splice acceptor (Sa) sites used in the synthesis of pX transcripts are shown, revealing that the mRNA contains three exons derived from (i) the R region of the 5' LTR, (ii) a region where the 3' end of *pol* and the 5' end of *env*-coding sequences overlap, and (iii) the 3' end of the provirus containing open reading frames X-I and X-II. (B) Construction of pX-expression plasmids. The 3' half of the BLV provirus in pBLV913 was subcloned into the *Hin*dIII (H) to *Kpn*I (K) sites of pUC19 to yield pHK-3'. The pXB plasmid was derived from pHK-3' by deleting the region between the two *Bam*HI (B) sites and the region upstream of the *Xho*I (X) site. The transcriptional promoters from SV40, RSV, and BLV were inserted into the *Hin*dIII site of pXB to give pXB-SV, pXB-RS, and pXB-BL, respectively. Synthesis of a functional pX message requires one splice, removing the ca. 400-bp intron between the indicated splice sites.

transiently express CAT activity when transfected with pBLH2cat (data not shown). Thus, G418 resistance in these cells probably results from expression of the *neo* gene by a mechanism other than *trans*-activation of the BLV promoter. In contrast, 10 colonies which survived G418 selection after cotransfection with pBL9neo and pXB-RS, were all able to express high levels of CAT activity when transfected with pBLH2cat (Table 3), indicating that these cell lines produce the factor(s) that activates the BLV promoter. High-molecular-weight DNA prepared from these cell lines was analyzed by Southern blot hybridization using a BLV pX-specific probe (Fig. 3). All of these cellular DNAs contained one to five integrated copies of pXB-RS. There was no apparent correlation between the level of *trans*-activation observed in the CAT assays and the number of copies of pXB-RS integrated in the genome. Poly(A)$^+$ RNAs from RD-4 cells and one of the G418-resistant lines, RD-N7, were compared with RNA prepared from the BLV-infected fetal lamb kidney cell line FLK-BLV by Northern blot analysis (Fig. 4). The hybridization probe, specific for the BLV pX region, revealed significant levels of pX mRNA in the RD-N7 cells (lane 2) but none in RD-4 cells (lane 1). The pX mRNA detected in RD-N7 cells was approximately equal in size to the pX message made in a fetal lamb kidney cell line (lane 3) which was productively infected with BLV (FLK-BLV) (49). The sizes of these mRNAs are predicted to be 1,700 bases in FLK-BLV cells and 1,740 bases in RD-N7 cells [excluding the poly(A) tracts]. Thus, an integrated copy of pXB-RS produces a mature pX mRNA whose expression results in the transcriptional activation of the BLV LTR.

***trans*-Activation results from p38(XBL) expression.** The BLV pX message has a dual coding potential; it possesses two alternative, overlapping open reading frames, each joined to a different translation initiation codon (Fig. 5A) (31). The upstream ATG initiation codon, also used for envelope (*env*) gene translation, with 16 codons from the amino-terminal region of *env*, is spliced to the pX open reading frame for p18(XBL). Approximately 50 nucleotides downstream of the *env*/p18(XBL) initiation codon is a second ATG codon which forms part of the splice donor site used to join this methionine codon to the pX open reading frame for p38(XBL). As both the p18(XBL) and p38(XBL) polypeptides are apparently synthesized from the same mRNA, it was necessary to determine whether expression of one or both is required for the *trans*-activation phenomenon.

Three plasmids were constructed by modifying pXB-RS

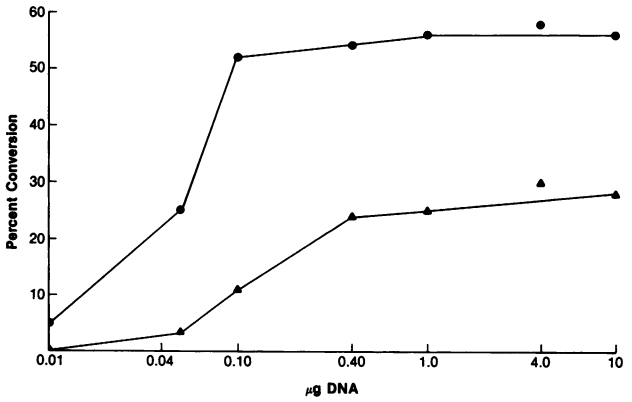

FIG. 2. Relationship between the concentration of pXB-RS or pXB-BL cotransfected onto RD-4 cells and the levels of CAT activity produced from pBLH2cat. pBLH2cat (5 μg) was cotransfected with increasing amounts of pXB-RS (●) or pXB-BL (▲). The total amount of plasmid DNA was maintained at 15 μg per transfection by the addition of pUC19. Plasmid DNAs were transfected onto RD-4 cells as calcium phosphate coprecipitates, and CAT assays were done as described in Materials and Methods. The data are expressed as the percentage of the total chloramphenicol converted to monoacetate products versus the amount of pXB plasmid introduced.

so that the syntheses of p38(XBL) and p18(XBL) were either individually or simultaneously obstructed (Fig. 5). Frameshift mutations were introduced at the *Cla*I or *Bal*I sites of pXB-RS to produce the plasmids pXBRS-C and pXBRS-B, respectively (Fig. 5A). The mutation in pXBRS-C obstructs the synthesis of both proteins, whereas pXBRS-B can produce a normal p18(XBL) only. Deletion of the translation initiation codon for p18(XBL) resulted in the plasmid pMB-RS (Fig. 5B), which can produce only p38(XBL). The ability of each of these plasmids to activate the BLV promoter was compared with the ability of pXB-RS in transient CAT assays (Fig. 5C). The inactivity of the plasmids pXBRS-C and pXBRS-B, which cannot produce p38(XBL), indicates that this protein is necessary for the activation of the BLV promoter. That expression of p38(XBL) is also sufficient to effect *trans*-activation is revealed by the activity of pMB-RS, which produces p38(XBL) exclusively.

p38(XBL)-mediated activation is specific for the BLV LTR.

TABLE 2. *trans*-Activation of the BLV LTR in long-term cotransfection experiments[a]

Cotransfected plasmids	No. of G418^r colonies in expt:	
	1	2
pSV2neo + pUC19	36	40
pSV2neo + pXB-RS	30	ND[b]
pSV2neo + pBLV913	26	ND[b]
pBL9neo + pUC19	0	1
pBL9neo + pXB-RS	18	38
pBL9neo + pXB-BL	1	0
pBL9neo + pBLV913	1	1

[a] G418-resistant colonies arising after cotransfections of RD-4 cells. Data from two separate experiments are shown. pSV2neo or pBL9neo (2 μg), in which the dominant selectable marker is controlled, respectively, by the SV40 early region promoter or the BLV LTR, was linearized with *Eco*RI and cotransfected with 18 μg of *Kpn*I-digested pUC19, pXB-RS, pXB-BL, or pBLV913 DNA. At 48 h after transfection, cells were placed in medium containing 500 μg of G418 per ml. The number of colonies arising in each culture dish were scored at 20 days after transfection.
[b] ND, Not done.

TABLE 3. Transcriptional activity of the BLV LTR in cells constitutively producing BLV pX genes[a]

Cell line	CAT activity		pBLH2cat/pSV2cat ratio
	pSV2cat	pBLH2cat	
RD-4	3.2	<0.01	
RD-N1	2.7	28.0	10.4
RD-N2	4.6	37.0	8.0
RD-N3	3.7	37.2	10.0
RD-N4	2.0	29.2	14.6
RD-N5	3.4	9.5	2.8
RD-N6	3.2	36.2	11.3
RD-N7	4.9	65.9	13.4
RD-N8	2.6	36.4	14.0
RD-N9	4.8	36.8	7.7
RD-N10	2.4	20.8	8.7

[a] Levels of CAT activity transiently expressed in the G418-resistant cells containing integrated pXB-RS. The RD-4 cell line and its G418^r derivatives were transfected with 10 μg of either pBLH2cat, pSV2cat, or pSV0cat and assayed 44 h later for CAT enzyme expression. Levels of CAT activity are expressed as the percentage of the total chloramphenicol converted to monoacetate products.

Because BLV, HTLV-I, and HTLV-II are structurally and functionally related, it is conceivable that p38(XBL) might activate the heterologous LTRs. The effects of p38(XBL) expression on other retroviral LTRs was examined in RD-4

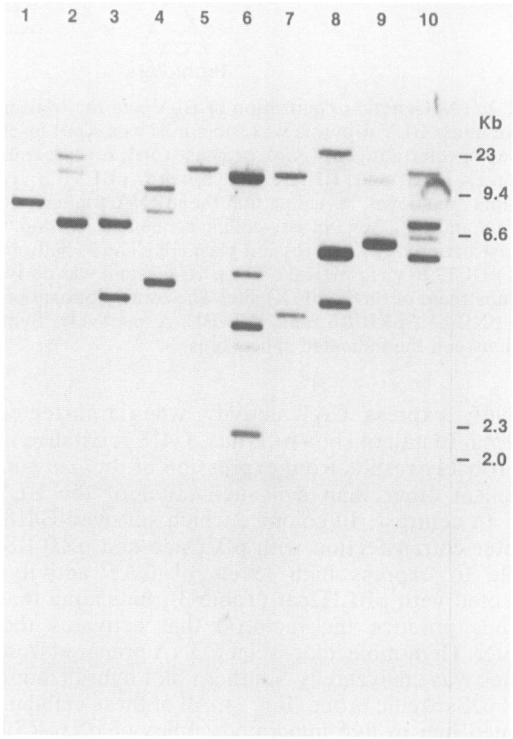

FIG. 3. Southern blot analysis of cellular DNAs prepared from RD-4 cells cotransfected with pBL9neo and pXB-RS. Subsequent to transfection, G418-resistant colonies were expanded and high-molecular-weight DNA was purified. Lanes 1 to 10 correspond to cell lines RD-N1 through RD-N10. DNAs were digested with *Bam*HI, fractionated on a 1% agarose gel, and transferred to nitrocellulose paper. Blots were hybridized with a 1.2-kbp, nick-translated, BLV pX-region probe encompassing the region located between the *Bam*HI site and a *Sac*I site (in the middle of the 3′ LTR) shown in Fig. 1B. Strongly hybridizing bands correspond to integrated copies of pXB-RS.

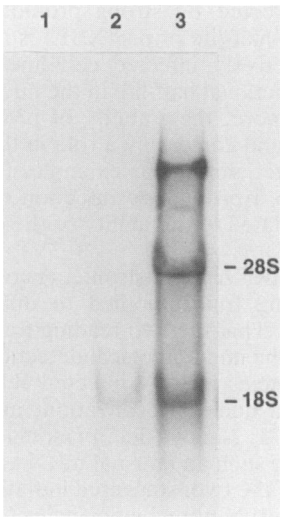

FIG. 4. Northern blot analysis of pX mRNA synthesis in infected and transfected cells. Poly(A)$^+$ mRNA (5 μg) was fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose paper. RNAs were purified from RD-4 cells (lane 1), RD-N7 cells (lane 2), and FLK-BLV cells (lane 3). The blot was hybridized with the BLV pX-specific probe described in the legend to Fig. 3.

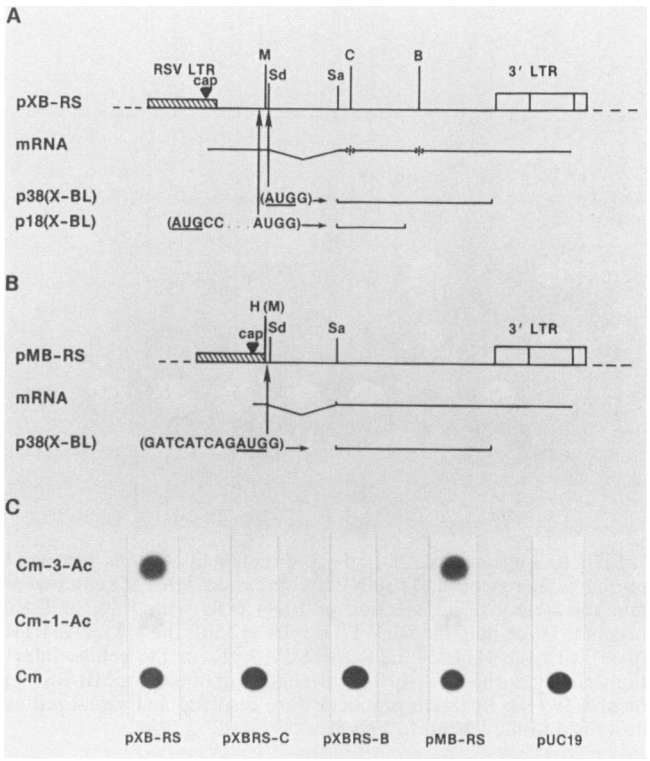

FIG. 5. (A) Introduction of frameshift mutations into pXB-RS. An octanucleotide containing the *Pst*I recognition sequence was inserted either at the *Cla*I site (C) or the *Bal*I site (B) of pXB-RS to give pXBRS-C or pXBRS-B, respectively. Below the pXB-RS map are shown the pX mRNAs, with the positions of the frameshift mutations indicated by asterisks, and the open reading frames encoding p38(XBL) and p18(XBL). The translation initiation codons for each reading frame are underlined. The *Cla*I frameshift in pXBRS-C is coincident with codons number 26 of p38(XBL) and number 42 of p18(XBL). The *Bal*I frameshift in pXBRS-B occurs at codon number 157 of p38(XBL) but is outside the coding domain of p18(XBL). Sd, Splice donor site; Sa, splice acceptor site. (B) Deletion of the 5′ end of the p18(XBL) reading frame. A *Sau*3A (*Mbo*I) site, indicated as M, is located 8 bp upstream of the initiation codon for p38(XBL). The region 5′ of this site was deleted from pXB-RS and replaced with a *Hin*dIII site to give pMB-RS. This plasmid can thus produce only the p38(XBL) message. (C) Stimulation of CAT activity directed by the BLV LTR by the modified pX plasmids. pBLH2cat (5 μg) was cotransfected with 5 μg of each of the indicated plasmids onto RD-4 cells. At 40 h after transfection, cell extracts were prepared and CAT assays were done as described in Materials and Methods. Reaction products were resolved by thin-layer chromatography and visualized by autoradiography. Cm, chloramphenicol; Cm-1-Ac and Cm-3-Ac, monoacetylated chloramphenicol.

cells cotransfected with pMB-RS and plasmids containing the *cat* gene controlled by the HTLV-I LTR (pHTLV-7cat), the HTLV-II LTR (pHT2cat), and the equine infectious anemia virus LTR (pEI-1cat). Each of these plasmids directed the expression of CAT enzyme activity at high levels in cell lines infected with the respective virus (data not shown). However, none of these LTRs was responsive to p38(XBL) expression (Fig. 6). The sensitivity of these assays would have allowed the detection of a positive effect even if it was 3 orders of magnitude lower than the levels directed by the BLV LTR. Thus, the *trans*-activation resulting from p38(XBL) expression is specific for sequences (response elements) in the BLV LTR.

Targets of p38(XBL)-mediated *trans*-activation. Deletion analyses of BLV LTR sequences were done as a first step toward identifying the targets or response elements required for p38(XBL)-mediated *trans*-activation. The parent plasmid used for these deletions, pBLS1cat, contains the *cat* gene linked to a truncated BLV LTR; the entire U_3 region and 50 bp of the R region are present (Fig. 7A). Sequences were progressively deleted from the 5′ end of the LTR, and the remainder was reinserted into the CAT plasmid. The plasmids containing the shortened LTRs were cotransfected with either pMB-RS or pUC19 (carrier DNA) onto RD-4 cells to assess their transcriptional activities (Fig. 7A). Deletion of sequences 5′ of the *Pvu*II site located 143 bp upstream of the RNA start site reduced CAT activity by 64% compared with pBLS1cat. Deletion of the region 5′ of the *Pvu*II site located 118 bp before the RNA start site nearly abolished promoter activity. The deletion analyses thus defined a region of the LTR, between 100 and 180 bp upstream of the cap site, which was required for promoter activity in response to p38(XBL) expression.

The definitive test of whether this region contains a response element sensitive to the effects of p38(XBL) is to show that it can confer p38(XBL) responsiveness to a heterologous promoter. To this end, a chimeric promoter was constructed and coupled to *cat*-coding sequences (Fig. 7B). The plasmid pSVΔEcat contains the SV40 early promoter unit lacking sequences upstream of the promoter-proximal *Sph*I site that form an essential part of the SV40 enhancer (5). This deletion renders the promoter essentially inactive; however, it can be reactivated by the insertion of heterologous enhancer units (14, 23). A BLV LTR fragment containing sequences located 105 to 179 bp upstream of the RNA start site was inserted, in either orientation, upstream of the SV40 core promoter of pSVΔEcat to produce pΔE75Bg-1 and pΔE75Bg-2. In RD-4 cells, transfection of either pΔE75Bg-1 or pΔE75Bg-2 alone yielded a negligible level of CAT activity (Fig. 7B). This level of activity was increased approximately 150 times when either pΔE75Bg

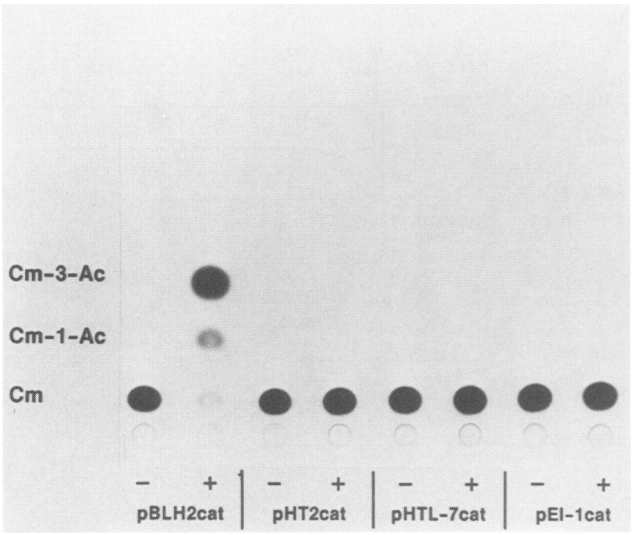

FIG. 6. Levels of CAT activity directed by various retroviral promoters in response to p38(XBL) expression. CAT activities were monitored after cotransfection of RD-4 cells with 5 μg of CAT plasmids containing the BLV LTR (pBLH2cat), the HTLV-II LTR (pHT2cat), the HTLV-I LTR (pHTLV-7cat), or the equine infectious anemia virus LTR (pEI-1 cat), and 5 μg of either pMB-RS (+) or pUC19 (−). Reaction products were resolved and visualized as described in the legend to Fig. 5C.

plasmid was cotransfected with pMB-RS. Thus, this fragment, previously identified as a cell-specific "enhancer" (5), contains one or more response elements sensitive to p38(XBL) expression.

DISCUSSION

The experiments presented here revealed that BLV transcription is controlled by an interaction of the virus-coded p38(XBL) protein and target sequences upstream of the viral promoter. Whether this is a direct protein-DNA interaction awaits further examination. The expression of p38(XBL), directed by the transcriptional promoters derived from SV40 or RSV, produced a strong *trans*-activation of the BLV LTR in both transient and long-term cotransfection experiments. The level of activity of the BLV promoter in the transient assays was dependent on the concentration of the pX-containing plasmid introduced into the cells, as well as on the strength of the promoter controlling its expression. Interestingly, cotransfection of the cloned provirus, pBLV913, produced a negligible *trans*-activation effect in both transient and long-term expression experiments. A similar pattern of inactivity was observed by using pXB-BL, in which pX expression is controlled by the natural promoter. However, *trans*-activation by pXB-BL was observed in transient cotransfections of certain cell lines. Self-activation leading to very high levels of *trans*-activation was not observed in either case, suggesting that additional events or factors may be involved in the normal control of BLV gene expression. One testable interpretation of these results is that p38(XBL) has a short functional half-life. If this was the case, p38(XBL) would be unable to accumulate in the cell nucleus and consequently, a self-activation cycle dependent on a critical amount of p38(XBL) would be interrupted. Moreover, *trans*-activation would be observed only if p38(XBL) was continuously overproduced, as when its expression is directed by strong promoters. It has been reported that the half-life of p38(XBL) is relatively short (5 h) in the productively infected cell line FLK-BLV (31). However, the functional half-life in the nucleus may even be shorter. Furthermore, the stability of p38(XBL) may vary among cell lines and could play a role in the ability of a cell to actively produce virus. For example, the switch from a nonproductive to a productive infection in cells harboring BLV might be related to the ability of the cell to transiently stabilize p38(XBL).

The BLV pX mRNA is bicistronic, encoding two overlapping open reading frames joined to different translation initiation codons. That the two reading frames are used has been verified by the immunological detection of both 38- and 18-kilodalton proteins in BLV-infected cells. Both proteins appear to have a nuclear localization, and the smaller is phosphorylated (31; Nancy Rice, personal communication). The translation of such an unusual mRNA is probably aided by the location of the two, staggered initiation codons within sequences having suboptimal efficiencies (22). For example, the upstream ATG used for envelope and p18(XBL) synthesis is in the sequence TCAAATGC; the downstream ATG used for p38(XBL) is in the sequence TCAGATGG. Thus, the upstream ATG is likely to be bypassed a certain fraction of the time, allowing synthesis of p38(XBL). It will be of interest in the future to determine the relative amounts of p18(XBL) and p38(XBL) in cells and to examine whether their expression is differentially regulated. The results presented here show that p38(XBL) is responsible for the *trans*-activation of the BLV LTR, but no function for p18(XBL) has yet been identified.

The structural and functional aspects of transcriptional control in BLV are paralleled in HTLV-I and HTLV-II. These viruses all contain a pX region containing several open reading frames (16, 35, 38). The pX mRNAs of HTLV-I and HTLV-II are, like BLV, generated by a double splicing mechanism and encode two alternative, overlapping reading frames (17, 32, 50). In HTLV-I and HTLV-II, the products of the larger open reading frames are proteins of 40 and 38 kilodaltons, respectively (21, 24, 39, 42). These proteins, termed x-lor or tat, were localized to the nucleus of infected cells (13, 41) and transcriptionally activated their LTRs (8, 10, 37, 43). The smaller pX open reading frame of HTLV-I yields nuclear phosphoproteins of 27 and 21 kilodaltons analogous to p18(XBL) of BLV (21, 32). Although the functions of these smaller pX gene products are currently unknown, their biochemical and cellular properties suggest a possible role in regulating virus or cellular gene expression.

The ability of p38(XBL) to exclusively activate the BLV LTR parallels the previous observations that the HTLV-I and HTLV-II LTRs were not active in BLV-infected cell lines (30). Conversely, the BLV LTR was inactive in HTLV-infected cells and in cells expressing the HTLV pX genes (30, 43). Thus, the transcriptional effects produced by the BLV and HTLV pX proteins, which share little sequence similarity, are apparently mediated by unique promoter control elements (5, 11). A region of the BLV LTR, which contains sequences required for the p38(XBL)-induced activation of transcription, was located by deletion analyses. Chimeric promoter units, containing an LTR fragment coupled to the core promoter sequences of SV40, were activated in response to p38(XBL) expression. The active BLV LTR fragment is normally located between 100 and 180 bp upstream of the RNA start site, indicating that *trans*-activation is at the level of transcription initiation. This same region was previously identified as a cell-specific enhancer element

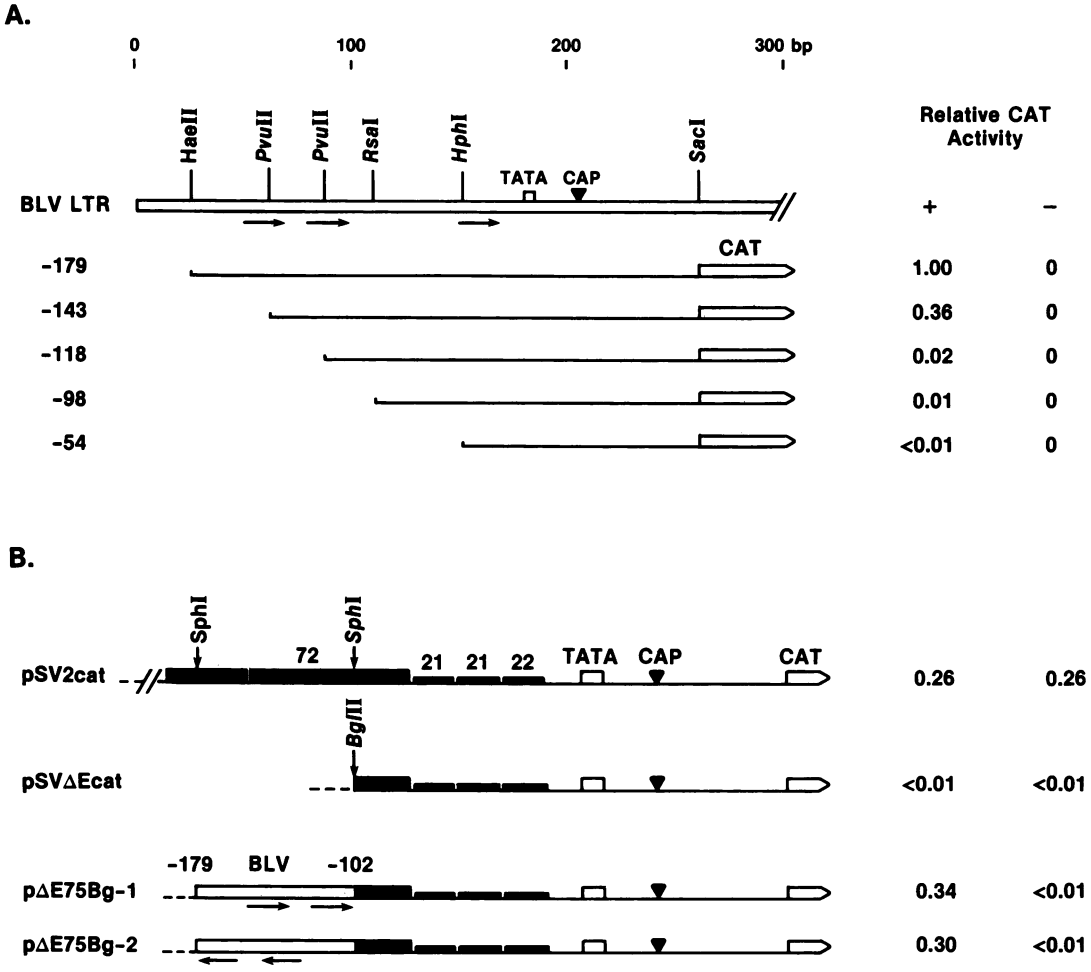

FIG. 7. (A) Deletion analysis of 5′ LTR sequences. Progressively shortened LTR fragments, bounded at the 3′ ends by the *Sac*I site and at the 5′ ends by the indicated restriction sites, were inserted into the *Hin*dIII site of pSV0cat. The numbers at the left indicate the position of the 5′ deletion with respect to the RNA start site (CAP). Each of these plasmids (5 μg) was cotransfected onto RD-4 cells with 5 μg of either pMB-RS (+) or pUC19 (−). Levels of CAT activity are expressed relative to the activity obtained after cotransfection of pMB-RS and pBL-S1cat (containing intact LTR sequences 5′ of the cap site and 3′ sequences to the *Sac*I site). (B) Response of chimeric promoter units to p38(XBL) expression. A portion of the SV40 early promoter, contained in the plasmid pSV2cat, is shown. The 72- and 21-bp direct repeats are indicated by the black boxes. Sequences upstream of the promoter-proximal *Sph*I site, which form an essential part of the SV40 enhancer, were deleted and replaced with a *Bgl*II linker to form pSVΔEcat (5). A BLV LTR fragment spanning the region between 102 and 179 bp upstream from the RNA start site was inserted, in either orientation, into the *Bgl*II site of pSVΔEcat. The plasmids pΔE75Bg-1 and pΔE75Bg-2 contain the chimeric promoters in which the BLV fragment (open box) is fused upstream of the SV40 core promoter elements. The arrows below the BLV regions indicate short repeat elements (also shown under the BLV LTR in panel A). Each of these CAT plasmids (5 μg) was cotransfected onto RD-4 cells with 5 μg of either pMB-RS (+) or pUC19 (−). CAT activities, shown at far right, are expressed as in panel A.

because its activity was restricted to cells productively infected with BLV (5). Clearly, this restricted enhancer activity, observed in the BLV-infected cells, was due to an interaction of p38(XBL) (either directly or indirectly) with response elements which constitute, or lie within the enhancer region.

The minimal nucleotide sequence which constitutes an active p38(XBL) response element is currently being examined. Nucleotide sequence data and the functional deletion analyses of the BLV LTR strongly suggest a role for two short repeats within the active 75-bp region. The two repeats centered around 148 and 123 bp upstream of the RNA start site are separated by 15 bp and have the sequence CACGTCAGCTG. Each repeat thus contains a *Pvu*II site (CAGCTG) near its 3′ end. Removal of LTR sequences 5′ of the *Pvu*II site in the upstream repeat reduced promoter activity, in response to p38(XBL), by 60 to 70%. Deletion of an additional 26 bp, which includes all sequences 5′ of the *Pvu*II site in the downstream repeat, nearly abolished promoter activity, indicating an important role for these sequences. A third, related element is found centered around 48 bp upstream of the RNA cap site (Fig. 7 and 8) and is apparently inactive in the absence of upstream sequences. Each of these 11-bp repeats is part of a longer 18- to 21-bp repeat element; together they are very similar with respect to nucleotide sequence and spatial array to elements found in the LTRs of HTLV-I and HTLV-II (Fig. 8). All of these elements have a common, 7-bp core sequence TGACGTC. The HTLV-I and HTLV-II sequences surrounding this core are very similar to each other but differ from those of BLV.

BLV	-148	CAGACAG	AGACGTC	AGCTGCC
BLV	-123	AAGCTGG	TGACGTC	AGCTGGT
BLV	-48	GAGCTGC	TGACCTC	ACCTGCT
HTLV-I	-243	AAGGCCC	TGACGTG	TCCCCCT
HTLV-I	-195	TAGGCTC	TGACGTC	TCCCCCC
HTLV-I	-95	CAGGCGT	TGACGAC	AACCCTC
HTLV-II	-214	AAGGCTC	TGACGTC	TCCCCCT
HTLV-II	-174	ACGGCCC	TGACGTC	CCTCCCG
HTLV-II	-80	AAGGCTC	TGACGAT	TACCCCC
	BLV	NAGNQPG		AGCTGNQ
Consensus			TGACGTC	
	HTLV	AAGGCQC		TCCCCCQ

FIG. 8. Comparisons of the nucleotide sequences of repeat elements found in the BLV, HTLV-I, and HTLV-II LTRs. The three 18- to 21-bp repeat elements observed in each virus have been aligned around a common 7-bp core sequence. The position of each repeat within its LTR is given as the distance from the RNA start site to the A residue near the center of each repeat. A consensus sequence based on these data is shown at the bottom. N, Any nucleotide; P, purine; Q, pyrimidine. Sequence data were obtained from reference 6 for BLV, reference 36 for HTLV-I, and reference 40 for HTLV-II.

The mutually exclusive *trans*-activation of the BLV and HTLV promoters by their cognate factors would suggest that the shared 7-bp core represents a functional center of a larger response element whose specificity is determined by the flanking nucleotides. It will be of interest to determine the relative importance of specific nucleotides within these elements and the significance of the number and position of the elements with respect to promoter activation. In several respects, BLV is similar to mouse mammary tumor virus; their expression is highly regulated by an interaction of well-defined promoter control sequences with specific *trans*-acting factors. In mouse mammary tumor virus, the control elements are responsive to the activated glucocorticoid receptor; a response apparently resulting from direct receptor-DNA binding (2, 28, 34). Interestingly, there are multiple glucocorticoid receptor elements arrayed in the mouse mammary tumor virus LTR, somewhat like the arrangement of repeats in the BLV and HTLV LTRs. DNA-binding and footprinting studies should soon reveal whether the actions of the pX proteins are mediated by binding to the *cis*-acting sequences.

The existence of a positive feedback loop, in which the p38(XBL) protein activates the transcriptional promoter controlling its own expression, raises some questions with respect to the restricted expression of BLV observed in nature. The absence of viremia in BLV-infected animals (12) suggests that other regulatory mechanisms are superimposed on this activation cycle. Although this may be attributable, in part, to some biochemical property of p38(XBL), it is also possible that the virus contains negative regulatory sequences or encodes other gene products which regulate virus expression. The latter could perhaps be effected by the products of pX open reading frames X-II and X-III. The greatest obstacle in approaching an understanding of the molecular genetics of BLV has been the difficulty in reproducibly obtaining virus production in cell cultures infected with virus or transfected with cloned provirus. It is likely that this barrier can now be overcome as a result of our understanding the strategies of virus transcriptional control.

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