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Identification of Alternatively Spliced mRNAs Encoding Potential New Regulatory Proteins in Cattle Infected with Bovine Leukemia Virus

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The polymerase chain reaction was used to detect and characterize low-abundance bovine leukemia virus (BLV) mRNAs. In infected cattle we could detect spliced mRNA with a splice pattern consistent with a Tax/Rex mRNA, as well as at least four alternatively spliced RNAs. Two of the alternatively spliced mRNAs encoded hitherto unrecognized BLV proteins, designated RIII and GIV. The Tax/Rex and alternatively spliced mRNAs could be detected at their highest levels in BLV-infected cell cultures; the next highest levels were found in samples from calves experimentally infected at 6 weeks postinoculation. Alternatively spliced mRNAs were also expressed, albeit at lower levels, in naturally infected animals; they were detected by a nested polymerase chain reaction. Interestingly, the GIV mRNA was specifically detected in naturally infected cows with persistent lymphocytosis and in two of five calves at 6 months after experimental infection with BLV. Furthermore, the calf with the strongest signal for GIV had the highest lymphocyte counts. These data may suggest a correlation between expression of the GIV product and development of persistent lymphocytosis. Some of the donor and acceptor sites in the alternatively spliced mRNAs were highly unusual. The biological mechanisms and significance of such a choice of unexpected splice sites are currently unknown.

Bovine leukemia and lymphosarcoma, an economically important disease of cattle (103), attracted attention early in this century. In several European countries, the finding of clusters of herds with a high incidence of leukemia and lymphosarcoma suggested an infectious etiology. Nevertheless, bovine leukemia virus (BLV) was not isolated until 1969 (70), and it was shown to be the infectious agent of persistent lymphocytosis and chronic leukemia and lymphoma in cows. BLV belongs to the oncovirus group of the family *Retroviridae* and is structurally related to human T-cell lymphotropic virus types I and II (HTLV-I and HTLV-II), agents associated with adult T-cell leukemia and perhaps with hairy T-cell leukemia in humans (43, 46, 96, 99, 110, 111). However, BLV causes B-cell proliferation and neoplasia whereas HTLV causes T-cell neoplasia. An exact characterization of the BLV-induced tumor cells has not yet been established. They seem to belong to the B-lymphocyte lineage, and their blastlike morphology and reactivity to heavy-chain immunoglobulin M suggest that they most probably are pre-B cells (21, 35, 40).

BLV and HTLV have no known oncogene incorporated in their genomes and apparently have no preferred integration sites in tumor cells, suggesting that their mechanisms for tumor initiation are different from those of other oncogenic retroviruses. BLV and HTLV genomes have a special X region located between the *env* region and the 3′ long terminal repeat (LTR) (81, 89, 96, 99), and it has been speculated that expression of this X region is somehow involved in the early events of tumorigenesis (21, 58). The X region contains several open reading frames (ORFs), one of which encodes a *trans*-activating protein, Tax (36, 46, 47, 84, 85, 87, 88), and another encodes the Rex protein, involved in promoting the expression of viral structural proteins (1, 37, 51, 79, 88). The mRNAs and protein products of these two genes have been characterized for both HTLV and BLV (1, 36–38, 46, 47, 51, 79, 84, 85, 88), and the functional roles of these proteins in gene regulation of the two viruses have been established.

Transcription of the BLV genome in animals with chronic infection or with tumors is nearly undetectable by conventional techniques (59, 60, 65), although the persistent presence of antibodies to viral proteins suggests a continuous, albeit low-level, production of viral proteins (21, 56, 75, 87). Transcription of the Tax/Rex mRNA has recently been shown in HTLV-infected individuals and in BLV-infected cattle by using the sensitive technique of reverse transcriptase-polymerase chain reaction (PCR) (44, 55, 62, 98).

It was originally proposed that, in addition to the Tax and Rex ORFs, both HTLV and BLV may potentially express several other small ORFs in the X region (80, 81, 89, 96, 111). These ORFs have been termed XBL-III and XBL-IV for BLV and pXI and pXII for HTLV. Proteins encoded by these genes would have to be translated from mRNAs that are spliced differently from the mRNA for the Tax and Rex proteins (88, 108, 109). Alternative splicing of the small multiple-spliced mRNAs encoding regulatory proteins of the lentiviruses, especially human immunodeficiency virus (HIV), is well established (8, 14, 41, 48, 83, 93–95), and complex splicing has also been shown for the spumaretrovi-

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ruses (73). Recently, complex splicing was shown for HTLV, together with preliminary data on BLV (15, 27, 42, 76). These data indicated that complex splicing is a feature of the BLV/HTLV group of viruses.

In the present study we have characterized the pattern of mRNA splicing in BLV-infected animals and have attempted to correlate the findings with the development of clinical disease. In infected cattle we could detect spliced mRNA with a splice pattern consistent with a Tax/Rex mRNA, as well as at least four alternatively spliced RNAs. Two of the alternatively spliced mRNAs encoded hitherto unrecognized BLV proteins, designated RIII and GIV, from the XBL-III and XBL-IV ORFs, respectively. The GIV mRNA was specifically detected in naturally infected cows with persistent lymphocytosis and in two of five calves at 6 months after experimental infection with BLV. Interestingly, the calf with the strongest signal for GIV had the highest lymphocyte counts. Taken together, the data may suggest a correlation between expression of the GIV product and development of persistent lymphocytosis.

MATERIALS AND METHODS

Cells and virus. Bovine kidney (MDBK) cells (ATCC CCL 22), primary fetal bovine lung (FBL) cells (107), and feline kidney (CRFK) cells (31) were used for the in vitro experiments. A chronically BLV-infected continuous fetal lamb kidney cell line (FLK-BLV) was kindly provided by Janice Miller, National Animal Disease Center, Ames, Iowa. Cells were grown in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum, antibiotics, and glutamine.

Animals. Eight colostrum-deprived Holstein calves, 4 to 6 months of age, were repeatedly tested and found to be free of bovine retroviruses, including bovine immunodeficiency-like virus, bovine spumaretrovirus, and BLV. Five of the eight calves were inoculated intravenously with 10^7 peripheral blood mononuclear cells (PBMC) from a BLV-infected cow with persistent lymphocytosis (cow 9321), while three age-matched control calves were inoculated with 10^7 PBMC from a retrovirus-free calf. Additional in vivo samples were obtained from an age-matched Holstein calf naturally infected with BLV (calf 357) and from two cows naturally infected with BLV (cows 9321 and 9324). Both cows had exhibited persistent lymphocytosis for more than 2 years.

Virus isolation. Blood samples were collected three times per week during the first 3 months postinoculation (p.i.) and at 4- to 6-week intervals thereafter. At 2, 6, 19, and 28 weeks p.i., whole blood was collected into acid citrate dextrose and PBMC were isolated as described previously (25, 86). For isolation of BLV, 10^7 PBMC were cocultivated with 5×10^5 FBL cells in the presence of Polybrene (25, 107). Cells were subcultured once, fixed in 70% acetone–30% methanol, and tested for the presence of BLV by using an indirect immunofluorescence assay similar to that described earlier (25). For these studies, we used a 1:100 dilution of polyclonal BLV antiserum obtained from a sheep experimentally infected with BLV as the primary antibody and a 1:30 dilution of fluoresceinated rabbit anti-sheep immunoglobulin G (Jackson Immunoresearch Labs, West Grove, Pa.) as the secondary antibody.

In situ hybridization. Fragments spanning the full length of the BLV913 infectious clone (kindly provided by David Derse, Frederick Cancer Research Facility, Frederick, Md.) were subcloned into a transcription vector based on the SP6 and T7 phage promoters by using previously described techniques (2, 5, 105). The BLV DNA and RNA contents in infected cells were measured by using ^{35}S-labeled RNA probes and in situ hybridization as described previously (4, 5, 7, 105). Preliminary studies have shown that this strategy, paired with image analysis, gives excellent quantitative results on BLV-infected cells (106a).

Samples for RNA analysis. RNA samples analyzed consisted of total RNA extracted from FLK-BLV cells, from uninfected MDBK cells, and from bovine PBMC. Samples were collected from experimentally infected animals at 2, 6, and 28 weeks after infection.

PBMC were isolated as described above. To avoid in vitro activation of BLV transcription, the PBMC were not in contact with heterologous serum and were processed as fast as possible. Total RNA was extracted by the guanidine thiocyanate method followed by centrifugation through a cesium chloride cushion as described previously (3). Total cellular DNA was extracted from the interphase of the cesium chloride cushion.

PCR amplification. Data on BLV infection have suggested that ordinary Northern (RNA) blotting or cDNA mapping can be difficult or impossible because the specific mRNAs in question are of very low abundance. Therefore, single-stranded cDNA synthesis and amplification by PCR followed by cloning and sequencing of the products were used.

PCR amplifications were done on single-stranded cDNA from control and BLV-infected cells by using selected pairs of oligonucleotide primers, a commercially available temperature cycler, and a standard protocol supplied by the manufacturer (Perkin Elmer Cetus, Norwalk, Conn.) or a protocol with minor modifications (6, 23, 45). Samples (1 μg) of total RNA were converted into cDNA by using oligo(dT) as the primer and avian myeloblastosis virus reverse transcriptase in a volume of 20 μl under the conditions described by the manufacturer (Promega Corp., Madison, Wis.). A 10-μl sample of this single-strand cDNA was then used directly for PCR in a 100-μl total volume under standard conditions (Perkin Elmer Cetus) involving 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. Nested PCR was done for another 35 cycles on 1-μl samples from the first PCR amplifications by using internal primers and standard conditions. For PCR analysis of cDNA from BLV-infected cells, we constructed nine oligonucleotide primers each 20 to 22 nucleotides (nt) in length. The sequences, locations in the BLV genome (89), and designations of the oligonucleotide primers are as follows: BLV221, 5′-TTCTCCTGAGACCCT CGTGC-3′ (nt 221 to 240); BLV251, 5′-GTCCTGAGCTCTC TTGCTCC-3′ (nt 251 to 270); BLV530C′, 5′-TGTTTGCCG GTCTCTCCTGG-3′ (nt 530 to 511); BLV4761, 5′-CGCTCT CCTGGCTACTGACC-3′ (nt 4761 to 4780); BLV6831C′, 5′-A GGTATCTCTAGAAAGGGTG-3′ (nt 6831 to 6812); BLV 6900C′, 5′-GGAGGTTGCGGCTCGAGCTA-3′ (nt 6900 to 6881); BLV7140C′, 5′-GCAGCCGTTGTGGAAACGGA-3′ (nt 7140 to 7121); BLV7221C′, 5′-CGTTATCAGGTAATG GATCCCG-3′ (nt 7221 to 7200); BLV7557C′, 5′-AAGTGA AACCGGGCCGGGCT-3′ (nt 7557 to 7538). The locations of these primers are shown in Fig. 1.

Samples of amplified DNA were run on 3 to 4% agarose gels, and the identity of the bands was confirmed by hybridization to specific probes radiolabeled as described previously (3, 6, 17, 23, 45). For use as probes, we subcloned several segments of the BLV913 full-length clone of BLV (Fig. 1).

Cloning and nucleotide sequence analysis. Samples of amplified DNA were extracted with phenol and chloroform,

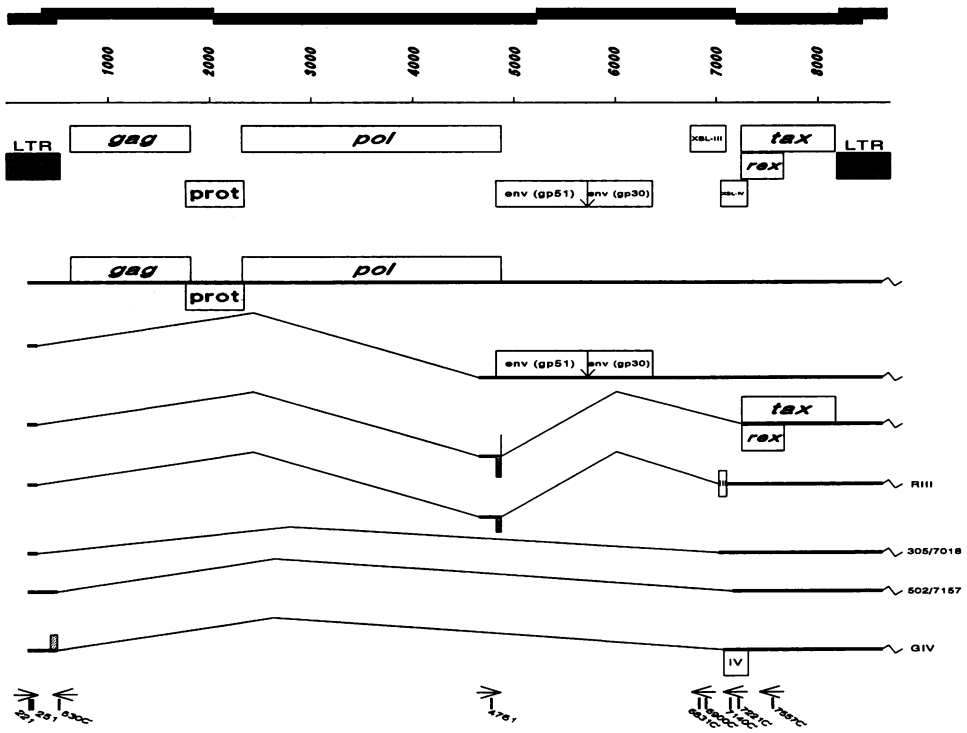

FIG. 1. Schematic representation of the BLV provirus. All numbering of nucleotides in the present report is based on the available GenBank sequence of BLV published by Sagata et al. (89). The total proviral genome, including both LTRs, is thus 8,714 nt. The locations of the two complete LTRs (shaded boxes), the ORFs *gag*, prot, *pol*, *env*, *tax*, *rex*, XBL-III, and XBL-IV are indicated. The putative cleavage site in the *env* gene generating gp51 and gp30 is indicated as a vertical arrow. Regions of BLV which we have subcloned for use as probes are indicated by thick, horizontal bars at the top of the figure. The proposed structure for the mRNA encoding Gag, Prot, and Pol is shown as an unspliced, full-length RNA; the mRNA encoding Env is shown as a singly spliced transcript; and the mRNA encoding Tax and Rex is shown as a doubly spliced transcript. The initiating methionines on this mRNA are shown as a vertical line for the Tax methionine and a hatched box for the Rex methionine. The alternatively spliced mRNAs described in this paper are shown below the Tax/Rex mRNA. The designations of the alternatively spliced mRNAs are indicated to the right, and the potential new translation products of these mRNAs are indicated with boxes. The designation, position, and direction of the primers used for splice-specific cDNA-PCR are shown at the bottom.

digested with proteinase K, phenol-chloroform extracted again, and precipitated with ethanol. Amplified DNAs were then restricted with *Sac*I, which cuts at nt 256, 353, and 7192 in the BLV genome (89). Such restricted DNA samples were then ligated into *Sac*I-digested pGEM3Z (Promega) by using standard techniques. Ligated DNA was transformed into *Escherichia coli* JM109, and the colonies were screened by using subgenomic fragments of BLV radiolabeled with ^{32}P. Clones that reacted with the BLV probes were digested with selected restriction enzymes and nucleotide sequenced by the dideoxy-chain termination method (91) with [^{35}S]dATP as the label as previously described (3, 6, 16, 17, 23, 45). Templates for sequencing were double-stranded plasmid DNAs prepared as described previously (26). The primers used were either commercially available or BLV-specific oligonucleotides. All regions were sequenced at least twice in both directions. DNA sequences were analyzed by using the Microgenie (Beckman Instruments) and the PC/GENE (IntelliGenetics) sequence analysis programs. The nucleotide numbering for BLV described by Sagata et al. (89) is used in the present paper.

Construction of expression plasmids. Functional cDNAs were generated by ligation of the PCR-generated clones into the *Sac*I site of a pGEM4Z plasmid containing BLV sequences from the *Sac*I site at nt 7192 to the *Eco*RI site at nt 7924. Functional inserts were cut out of these plasmids by *Sma*I, which cuts in the multiple cloning site 5′ to the BLV sequences, in the middle exon at nt 4699 (for clones having the middle exon), and at nt 7895 for subsequent subcloning into the eukaryotic expression plasmids pRSPA-S and pSVL (Pharmacia, Uppsala, Sweden). The pRSPA-S vector contains the strong Rous sarcoma virus promoter and the splice and polyadenylation sequences from simian virus 40 and was kindly provided by David Derse, who also provided a Tax expression construct in this vector (36, 37). A construct expressing primarily Rex was constructed by PCR-generated mutation of the Rex ATG into the sequence AAAATGG (nt 4818 to 4824) and cloning of a construct from nt 4810 to 4871 spliced to the Rex/Tax acceptor at nt 7247 and ending at nt 7924.

Transfections and CAT assays. The above-mentioned expression constructs were transfected into CRFK cells by using standard calcium phosphate precipitation (9) procedures essentially as described previously (16). Briefly, nearly confluent monolayers of cells in 60-mm tissue culture dishes were cotransfected with the expression plasmids and a BLV-LTR-CAT construct kindly provided by David Derse. In some experiments the BLV-LTR-CAT plasmid was cotransfected with a BLV-Tax expression plasmid (36–38) or with the parental pRSPA-S plasmid as a negative control. The pCATCONTROL plasmid (Promega), which contains the simian virus 40 promoter and enhancer and the bacterial

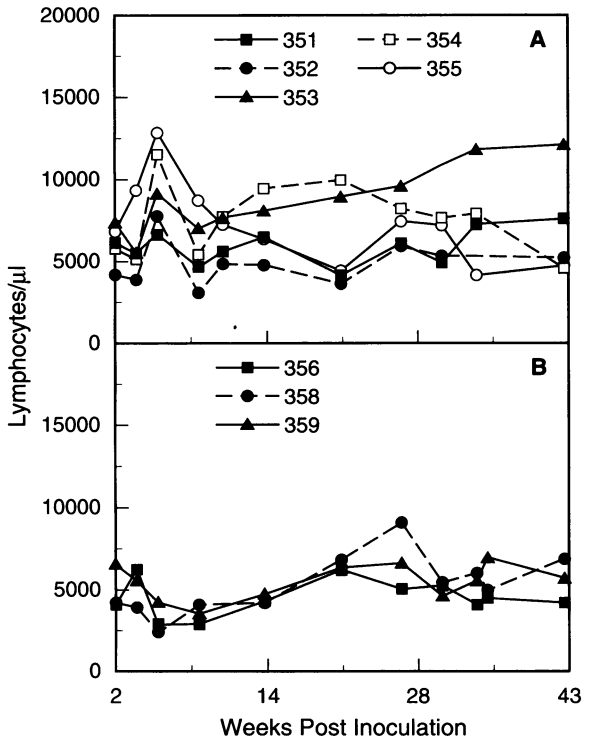

FIG. 2. Numbers of peripheral blood lymphocytes following experimental inoculation. (A) Five calves inoculated with 10^7 PBMC from a BLV-infected cow with persistent lymphocytosis. (B) Three control calves inoculated with 10^7 PBMC from a retrovirus-free calf. Numbers represent individual animals.

chloramphenicol acetyltransferase (CAT) gene, served as a useful monitor of transfection efficiency. Lysates were analyzed for CAT activity by a liquid scintillation assay essentially as described previously (9).

Experiments were done in duplicate by using reaction times in the linear range of the assay and equivalent amounts of protein.

In vitro transcription and translation. The functional cDNA clones in pGEM4Z described above were linearized with *Eco*RI, which cuts at nt 7924, or *Cla*I, which cuts at nt 7318 immediately downstream of the XBL-III and XBL-IV ORFs (Fig. 1). Capped runoff transcripts were synthesized by using T7 RNA polymerase as specified by the manufacturer (Stratagene, La Jolla, Calif.). In vitro translations were done in a nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [^{3}H]leucine or [^{35}S]cysteine. The resulting proteins were analyzed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Rabbit antipeptide antisera. A 14-amino-acid peptide (AL RDPLPDNDKIIS) from the XBL-IV ORF, with a high probability of containing an antigenic site, was synthesized at the Iowa State University Peptide Synthesis Facility. A cysteine was added to the N terminus to facilitate cross-linking to keyhole limpet hemocyanin (Sigma). Cross-linking was kindly performed by Arne Holm, Department of Chemistry, Royal Veterinary University, Denmark. Antipeptide antibodies were produced in rabbits by subcutaneous injection of 500 μg of keyhole limpet hemocyanin-peptide conjugate in Freund's complete adjuvant followed by two booster injections of the conjugate in incomplete adjuvant. An anti-C-terminal BLV-Rex antibody (77) was kindly provided by Kathryn Radke, Department of Avian Sciences, University of California, Davis.

Immunoprecipitation. For immunoprecipitation, 10 μl of in vitro-translated products was mixed with 190 μl of RIPA buffer (20 mM Tris [pH 7.3], 150 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 U of aprotinin per ml, and 5 μg of leupeptin per ml). Serum (1 or 10 μl) was added, and the mixture was incubated at 4°C for 16 h with agitation before addition of 50 μl of 50% protein A-Sepharose Fast Flow (Pharmacia) and another incubation for 3 h. After extensive washing of the precipitates, these were analyzed by SDS-PAGE. Gels were fixed, soaked in Amplify (Amersham, Aylesbury, Buckinghamshire, England), dried, and exposed to X-ray film as described previously (24).

RESULTS

Clinical disease and virus replication. All five BLV-infected calves experienced a mild, transient increase in lymphocyte numbers at 2 to 4 weeks p.i. (Fig. 2A). A similar increase was not observed in the control animals (Fig. 2B). A second phase of increased lymphocyte numbers (10,000 to 12,000 lymphocytes per μl of whole blood) was observed in calf 353 at 30 weeks p.i. and persisted through 43 weeks p.i. (Fig. 2A). Virus was recovered from all experimentally infected calves through 28 weeks p.i., although the levels of virus replication appeared to be variable (Table 1). Larger numbers of BLV-infected cells were consistently observed in calf 353, whereas smaller numbers of infected cells were characteristic of calf 351. Temporal changes in the level of virus replication were observed for the other calves. Over time, there was a decrease in the number of infected cells in calf 355, whereas increased numbers of BLV-infected cells were observed in calf 352 (Table 1).

Levels of virus replication and expression in PBMC were

TABLE 1. Quantitation of BLV in infected cattle

Animal	No. of BLV syncytia/10^5 PBMC[a]		No. of hybridization-positive cells/10^5 PBMC[b] (19 wk p.i.)
	8 wk p.i.[c]	19 wk p.i.[d]	
351[e]	32	11	0
352[e]	26	>500	50–100
353[e]	740	>500	50–100
354[e]	720	ND[f]	ND
355[e]	560	54	1
9321[g]	ND	>500	20,000

[a] The number of BLV-induced syncytia in FBL cells following cocultivation with PBMC isolated from BLV-infected calves at 8 and 19 weeks p.i.

[b] Quantitation of BLV-positive cells by in situ hybridization was done on serum-treated PBMC hybridized with an antisense BLV probe as described in the text.

[c] FBL cells were cocultured with 10-fold serial dilutions of PBMC. After 4 days, the cells were fixed and stained for the presence of BLV-induced syncytia by immunofluorescence as described in the text.

[d] FBL cells were cultured with PBMC, subcultured once, and stained for the presence of BLV-induced syncytia by immunofluorescence as described in the text.

[e] Calves inoculated with 10^7 PBMC from a BLV-infected cow with persistent lymphocytosis.

[f] ND, not done.

[g] Cow naturally infected with BLV that had been persistently lymphocytotic for more than 2 years. Samples from this cow were analyzed at the same time as the 19-week p.i. samples from the experimentally infected calves.

also analyzed by in situ hybridization, using full-length RNA antisense BLV probes to detect BLV RNA. A few positive cells could be detected in all five calves at 2 weeks p.i., but at 6 weeks p.i. calf 351 was negative and the other four calves had only 1 positive cell per 10^5 PBMC (data not shown). PBMC from four of these calves were isolated at 19 weeks p.i. and incubated at room temperature for 4 to 6 h in phosphate-buffered saline (PBS) alone or in PBS with 10% fetal calf serum. PBMC from cow 9321 with persistent lymphocytosis were incubated similarly. Cytospins were then made and processed for in situ hybridization. Analysis of these cytospins showed no positive cells in any of the calf PBMC or in the cow PBMC incubated in PBS. After incubation in 10% serum, no positive cells were detectable in calf 351, but calf 355 had a single positive cell and calves 352 and 353 had 50 to 100 positive cells per 10^5 PBMC (0.05 to 0.1% positive cells) (Table 1). Unfortunately, PBMC from calf 354 were not available for analysis. Analysis of PBMC from cow 9321 that were incubated in PBS showed no positive cells, whereas incubation in PBS with 10% fetal calf serum induced BLV expression in approximately 20% of the cells (Table 1). Moreover, a significant portion of the PBMC had more than 100 grains per positive cell, in contrast to the PBMC from experimentally infected calves, of which only a few cells contained more than 30 grains (data not shown). These findings indicated that the cow with persistent lymphocytosis had a much higher percentage of BLV-infected cells than did the experimentally infected calves at 19 weeks p.i. and, moreover, that these cells could be induced to transcribe BLV at a high level. Hybridization with a BLV sense probe showed no positive cells, indicating that the signal detected with the antisense probe represented hybridization to BLV RNA and not to BLV proviral DNA. Thus, the in situ hybridization analysis indicated that our procedure for isolating PBMC, as expected, did not induce BLV expression but that subsequent stimulation with serum activated expression.

Taken together, the data indicated that the largest number of infected cells able to express BLV were present in cow 9321 followed by calves 352 and 353. Moreover, of the experimentally infected calves, calf 353 also had the highest lymphocyte counts.

Amplification of Tax/Rex mRNA. In initial experiments we looked for Tax/Rex mRNA in the samples by using cDNA-PCR amplification of sequences containing the R region (primer BLV251) and sequences in the Tax/Rex region (primer BLV7557C′) (Fig. 1). Amplification of RNA from the BLV-infected cell culture, FLK/BLV, resulted in strong bands at approximately 590 and 550 bases and a weak band at ca. 800 bases (Fig. 3A). Amplification of RNA extracted from leukocytes from a pool of five BLV-infected calves at 6 weeks p.i. resulted in two weak bands at 590 and 550 bases. These two bands had approximately the same intensity (Fig. 3A). The size of the bands (550 and 590 bases) is consistent with the presence of a Tax/Rex mRNA in the cell culture and in the infected calves. The presence of two bands may indicate that one of the predicted splice sites is heterogeneous. Probing with BLV probes spanning the Tax/Rex region or the middle exon was positive on both bands, whereas probing with a probe specific for the XBL-III and XBL-IV regions (Fig. 1) was negative, indicating that the products were amplified from genuine Tax/Rex mRNA. The band at 800 bases seen in the FLK/BLV sample reacted with the XBL-III/IV probe and may represent alternatively spliced mRNAs (see below). Amplification of RNA isolated from a cow with persistent lymphocytosis (cow 9321) resulted in bands of approximately 590 and 400 nt (data not shown). Probing of these bands indicated that the 590-nt band represented genuine Tax/Rex mRNA while the 400-nt band lacked the middle exon, comparable to a truncated Rex mRNA described for HTLV (15, 42, 76). Amplification by using the same primers on RNA isolated from PBMC from a naturally BLV-infected calf, another naturally BLV infected cow (cow 9324) with persistent lymphocytosis, a group of noninfected calves, and control cell cultures was consistently negative (Table 2).

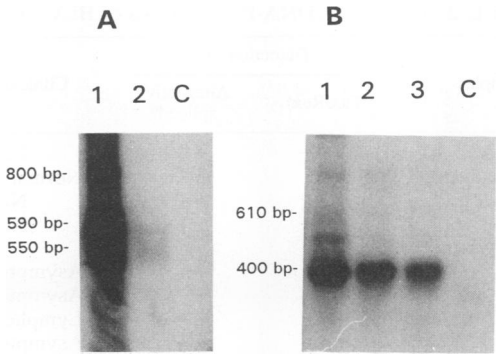

FIG. 3. Southern blot analysis of PCR-amplified cDNAs. Samples were electrophoresed into agarose gels, Southern blotted onto nylon filters, and hybridized with a full-length BLV probe as described in the text. The sizes of amplified products are indicated in base pairs. (A) PCR with primers BLV251 and BLV7557C′. (B) Nested PCR with primers BLV251 and BLV7557C′ followed by primers BLV4761 and BLV7557C′. Lanes: 1, FLK/BLV; 2, calves experimentally infected with BLV at 6 weeks p.i.; 3, calf 357 naturally infected with BLV; C, control calves.

Nested PCR (nested in only one side) was then performed on the previous PCR products by using primer BLV4761 (located in the middle exon) and primer BLV7557C′. This resulted in a strong band around 400 bases and a very weak band at 610 bases in the samples from the BLV-infected cell cultures, the experimentally infected calves at 6 weeks p.i., the naturally BLV-infected calf (Fig. 3B), the two naturally infected cows with persistent lymphocytosis, and four of five experimentally infected calves (Table 2). The strong bands were consistent with abundant amplification of a Tax/Rex mRNA with a theoretical size of ca. 410 bases. This band was mapped to the middle exon region and the Tax/Rex region by using subgenomic BLV probes (data not shown). The fact that we detected only one strong Tax/Rex amplified band by using these primers may indicate that the heterogeneity we observed when using primers BLV251 and BLV7557C′ is due to a heterogeneity in the first splice (Fig. 1). The weak band at 610 bases could be from amplification of an XBL-IV mRNA with a theoretical product around 600 to 650 bases (see below). The intensity of this band (Fig. 3B) was, however, too weak to be convincingly mapped with subgenomic probes. The control calves gave consistently negative results.

PCR samples from the FLK/BLV cell culture and cow 9321 with persistent lymphocytosis were reamplified with primers located at nt 4802 to 4834 (this primer adds a *Sma*I site at nt 4810) and nt 7324 to 7350 (this primer adds a *Sma*I site at nt 7341). The amplified products were cut with *Sma*I and cloned into pGEM3Z. Two clones from the FLK/BLV cell culture and six from cow 9321 were sequenced, and all eight clones had nt 4871 spliced to nt 7247 and thus represented the BLV Tax/Rex splice described previously (108).

TABLE 2. Results of cDNA-PCR analysis of BLV samples

Sample	Detection of[a]: Tax/Rex[c]	Detection of[a]: Alternative spliced[d]	Clinical stage[b]
FLK-BLV[e]	+++	+++	NA[f]
Control calves[g]	−	−	Normal
BLV 2 wk p.i.[h]	++	++	NA
BLV 6 wk p.i.[h]	+++	+++	NA
BLV 28 wk p.i.[h]			
351	−	−	Asymptomatic
352	+	+	Asymptomatic
353	++	++	Lymphocytosis
354	++	++	Asymptomatic
355	+	+	Asymptomatic
Cow 9321[i]			
Sample 1	+++	++	Lymphocytosis
Sample 2	+++	++	Lymphocytosis
Cow 9324[i]	++	++	Lymphocytosis
Calf 357[j]			
Sample 1	++	++	Asymptomatic
Sample 2	++	++	Asymptomatic

[a] The different samples and the detection of Tax/Rex or alternative spliced mRNA are indicated. Symbols: +++, samples are positive by direct PCR; ++, strong positive reaction by nested PCR; +, weak but consistent positive reaction in nested PCR; −, negative reaction by Southern blot of both direct and nested cDNA-PCR reactions.

[b] In vivo samples were collected from control or BLV-infected animals. The clinical outcome of BLV infection is listed as either asymptomatic or lymphocytosis (lymphocytes consistently above 10,000/μl of blood).

[c] Tax/Rex mRNA was amplified by using primers in the LTR or middle exon paired with primer BLV7557C′ in the Tax/Rex ORF.

[d] Alternatively spliced mRNA containing sequences in the XBL-IV or XBL-III ORF was detected by cDNA-PCR with specific primers as described in the text. Southern blots were probed with a subgenomic fragment of BLV containing sequences in the XBL-IV and XBL-III ORFs.

[e] A chronically BLV-infected cell line.

[f] NA, not applicable.

[g] Control calves were inoculated with PBMC from a clinically normal, retrovirus-free calf. PBMC from the three animals were pooled, and RNA was extracted and analyzed at 11, 16, and 20 weeks p.i.

[h] Five calves experimentally infected with PBMC from cow 9321. PBMC collected at 2 and 6 weeks p.i. were pooled for RNA isolation. At 28 weeks p.i., RNA was isolated from individual animals as indicated.

[i] Cows naturally infected with BLV and persistently lymphocytotic for more than 2 years. RNA was extracted from PBMC from cow 9324 once and from cow 9321 twice with a 3-month interval (samples 1 and 2).

[j] Calf 357 was naturally infected with BLV. PBMC were sampled at approximately 4 months (sample 1) and 5 months (sample 2) of age.

Taken together, these results demonstrated the presence of low levels of Tax/Rex mRNA in the in vivo samples, similar to recently published results (55), and established the validity of our techniques.

mRNA containing sequences in the XBL-IV or XBL-III ORF. To establish whether BLV-encoded mRNAs alternatively spliced to contain XBL-IV or XBL-III coding sequences (Fig. 1), we designed a series of oligonucleotide primers to detect alternatively spliced mRNAs by using PCR. First, we attempted specific amplification of an mRNA potentially containing the XBL-IV ORF (nt 7044 to 7307). Direct cDNA-PCR with primers BLV251 and BLV7140C′ resulted in amplification of one strong band and several weak bands in amplified RNA from FLK/BLV cell cultures (Table 2). A weaker band could be detected in amplified RNA isolated 6 weeks after experimental BLV infection of calves. Importantly, bands were positive with probes spanning the LTR region, the middle exon, and the XBL-IV region but negative with a probe in the Tax/Rex region (because the primer ends at nt 7140). Amplifications of RNA from the

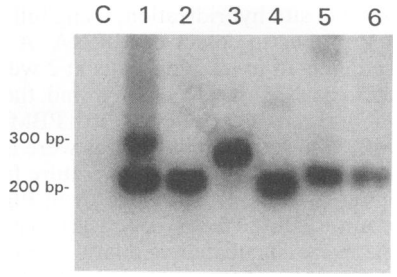

FIG. 4. Southern blot analysis of PCR-amplified cDNAs. Total RNA was converted to cDNA and subjected to nested PCR by using primers BLV251 and BLV7557C′ followed by primers BLV4761 and BLV7140C′. Samples of the PCR products were electrophoresed into agarose gels, Southern blotted onto nylon filters, and hybridized with a full-length BLV probe as described in the text. Approximate sizes are indicated in base pairs. Lanes: C, control calves; 1, FLK/BLV; 2, calves experimentally infected with BLV at 6 weeks p.i.; 3, calf 357 naturally infected with BLV; 4, calf 353 experimentally infected with BLV at 28 weeks p.i.; 5, calf 354 experimentally infected with BLV at 28 weeks p.i.; 6, cow 9321 with BLV-induced persistent lymphocytosis.

naturally infected animals, from the control animals, and from control cell cultures were negative by direct PCR (Table 2).

Nested PCR was then done on samples already amplified with the BLV251 and BLV7557C′ primer sets. For these amplifications we used primer BLV4761 paired with BLV7140C′ or BLV7221C′. Both set of primers resulted in amplified products in the samples from FLK/BLV, in the pool of experimentally infected calves at 2 and 6 weeks p.i., in four of five calves at 28 weeks p.i., in the naturally infected calf, and in the two cows with persistent lymphocytosis (Table 2). No bands were detected in amplified RNA from noninfected calves or from control cell cultures (Table 2). The number and size of bands varied among the positive samples (Fig. 4) but were consistent in repeated experiments. Furthermore, amplified bands consistently reacted with a probe containing the XBL-III/IV ORFs. These results indicated that alternatively spliced mRNAs that potentially could encode proteins were present at low levels in BLV-infected cell cultures and cattle.

To examine whether we could detect alternatively spliced mRNA specifically containing sequences in the XBL-III ORF (nt 6742 to 7098), we performed cDNA-PCR with primers located in this region. Direct PCR with primer BLV251 paired with either BLV6831C′ or BLV6900C′ (Fig. 1) detected no specific signals. Nested PCR with primer BLV4761 paired with BLV6831C′ or BLV6900C′ resulted in extremely weak signals, which probably represent a spurious reaction. Our primer BLV6831C′, in combination with BLV251, could amplify an abundant product of more than 2 kb from cDNA (representing unspliced genomic or envelope single-spliced RNA) or from DNA extracted from FLK/BLV cells, indicating that this primer works well. Thus, our results indicated that mRNAs with a specific acceptor in the 5′ end of the XBL-III ORF are either not expressed or expressed at extremely low levels during BLV infection in vivo.

As controls for the specificity of the PCR amplifications, we performed PCR on mRNA, omitting the reverse transcriptase step. No amplified products could be detected in these samples (data not shown). Amplification of purified genomic DNA from the same samples usually resulted in no

amplified products. In certain samples, especially from FLK/BLV cells, very large or very small bands could be detected. These bands did not line up with any of the bands having the predicted splice patterns for a Tax/Rex, XBL-IV, or XBL-III mRNA, indicating that our bands amplified from converted RNA are from spliced mRNA and not spurious RNA transcribed from deleted proviruses. It has recently been suggested that DNA representing spliced Tax/Rex sequences could be detected in genomic DNA from BLV-infected cattle (55). In our amplifications we did detect certain bands in amplified DNA, but these bands had a different size from those detected in the RNA samples. Moreover, these bands did not react with the BLV probe spanning the middle exon (data not shown). This suggests that the sequences amplified from genomic DNA most probably represent proviruses having most of the 3′ end and central region deleted. This would be consistent with the structure of deleted proviruses reported previously (21). Amplification of genomic DNA with primers BLV251 (located in the R region) and BLV530C′ (located in the U5 region) resulted in a positive band at 280 bases in all BLV-infected samples examined and no reaction in control samples, indicating that all infected samples contained BLV proviral DNA (data not shown).

In summary, by using region-specific PCR we could detect spliced mRNA having a splice pattern consistent with a Tax/Rex mRNA and with alternatively spliced mRNAs containing sequences in the XBL-IV ORF. An mRNA containing sequences in the 5′ end of the XBL-III ORF could not be convincingly amplified and may not be expressed in the samples we tested. The Tax/Rex and alternatively spliced mRNAs could be detected at the highest levels in BLV-infected cell cultures and at the next highest levels in the 6-week p.i. samples from experimentally infected calves. These mRNAs are also expressed, albeit at lower levels, in naturally infected animals, where they could be detected by nested PCR (Table 2).

Cloning and sequencing of alternatively spliced cDNAs. To characterize specific mRNAs containing sequences in the XBL-IV or XBL-III ORF, we performed nested PCR on selected samples by using primers BLV221 or BLV251 together with BLV7557C′ followed by nested PCR with primers BLV251 and BLV7221C′. Probing of the amplifications with a full-length BLV probe or a probe spanning the XBL-IV and XBL-III ORF resulted in several bands between 250 and 500 bp in the 6-week p.i. sample and in samples from four of five experimentally infected calves at 28 weeks p.i. but in only a single band of approximately 410 bp in the cows with persistent lymphocytosis (Fig. 5; Table 2). The strongest signal was detected in the cows with persistent lymphocytosis and in calf 353 at 28 weeks p.i. Amplified products were cut with *Sac*I and cloned into pGEM3Z. Colonies were screened with a ^{32}P-labeled full-length BLV probe. From the pool of five calves infected for 6 weeks, a total of 32 clones were sequenced. Of these, 17 had a single splice joining nt 305 to nt 7018 (designated splice pattern 305/7018), 10 had a single splice joining nt 502 to nt 7157 (splice pattern 502/7157), and 5 had two splices, one joining nt 305 to nt 4649 and the other joining nt 4871 to nt 7018 (designated splice pattern RIII) (Fig. 1; Table 3). From cow 9321 (with persistent lymphocytosis), 10 clones were sequenced, and all had a single splice joining nt 502 to nt 7066 (designated splice pattern GIV) (Fig. 1; Table 3). To confirm this unusual splice, amplifications were done on RNA from another cow with persistent lymphocytosis (cow 9324) and on another RNA sample isolated from cow 9321 3 months after the first sample. Sequence analysis of three clones from each of these samples revealed the same splice pattern (GIV), joining nt 502 to nt 7066, confirming this as a genuine BLV splice pattern. Sequencing of clones derived from two of the experimentally infected calves at 28 weeks p.i. showed that two of three clones from calf 353 had the GIV splice (nt 502 to nt 7066) while a single clone had the double splice described above (RIII). One of three clones from calf 354 had the nt 502-to-nt 7066 (GIV) splice, whereas two clones had nt 305 joined to nt 7018 (305/7018) (Fig. 1).

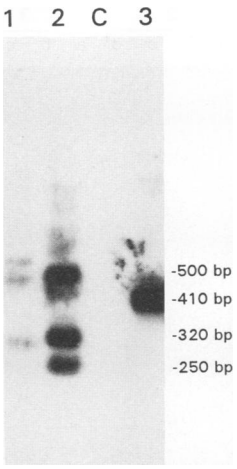

FIG. 5. Southern blot analysis of PCR-amplified cDNAs. Total RNA was converted to cDNA and subjected to nested PCR by using primers BLV251 and BLV7557C′ followed by primers BLV251 and BLV7221C′. Samples of the PCR products were electrophoresed into agarose gels, Southern blotted onto nylon filters, and hybridized with a full-length BLV probe as described in the text. Sizes of amplified products are indicated in base pairs. Lanes: 1, calf 357 naturally infected with BLV; 2, calves experimentally infected with BLV at 6 weeks p.i.; 3, cow 9321 with BLV-induced persistent lymphocytosis; C, control calves.

In vitro translation and immunoprecipitation. To determine whether the alternatively spliced mRNAs would encode protein products, we performed in vitro transcription of

TABLE 3. Splice sites used in BLV[a]

Splice site	Sequence	Found in mRNA
	305 ↓	
5′ donor	GCGGUCAG**guaaggcaag**	*env*, Tax/Rex, 305/7018
	502 ↓	
	CGGCGCCC**ucuagcggcc**	GIV, 502/7157
	4871 ↓	
	UCAGAUGG**guaag**ucuca	Tax/Rex, RIII
	↓ 4649	
3′ acceptor	ca**uuuucag**AGGGCGGAGA	*env*, Tax/Rex, RIII
	↓ 7018	
	ucca**aaaag**GUCCUGAUGA	RIII, 305/7018
	↓ 7066	
	gaca**uucc**AGCCACAUCC	GIV
	↓ 7157	
	cuucu**auu**UCCACCUCGG	502/7157
	↓ 7247	
	cuuuu**aag**CAAGUGUUGU	Tax/Rex

[a] Splice donor and acceptor sites used in the different BLV mRNAs are listed. The sites used in the alternatively spliced mRNAs and the Tax/Rex mRNA were determined in the present study, whereas sites used in the envelope (*env*) mRNA were based on data in the literature (21, 80, 81, 108).

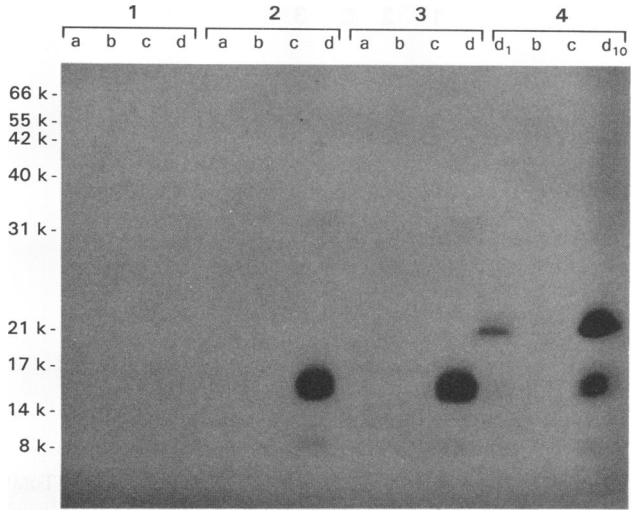

FIG. 6. In vitro translation and immunoprecipitation of truncated Rex encoded by the cDNAs. cDNAs were linearized and transcribed in vitro by using T7 polymerase. Transcribed RNA was translated in the presence of [^{35}S]cysteine and subjected to immunoprecipitation as described in the text. In vitro translation reaction products (10 μl) were used for the immunoprecipitations except for no. 4, in which 1 μl (lane d_1) and 10 μl (lane d_{10}) were used. Immunoprecipitated products were electrophoresed into an SDS–15% polyacrylamide gel followed by fluorography. The positions of molecular mass markers (in kilodaltons) are indicated. Truncated Rex runs around 16 kDa, whereas full-length Rex runs around 22 kDa. Samples: 1, GIV plasmid cut with *Cla*I; 2, GIV plasmid cut with *Eco*RI; 3, RIII plasmid cut with *Eco*RI; 4, BLV Rex plasmid cut with *Eco*RI. Antibodies for precipitations: a, preimmune rabbit 1 serum; b, preimmune rabbit 2 serum; c, rabbit 1 immunized with the GIV peptide as described in Materials and Methods; d, rabbit anti-Rex serum.

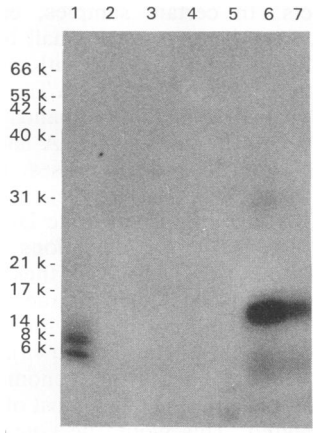

FIG. 7. In vitro transcription and translation of cDNA clones. cDNAs were linearized and transcribed in vitro by using T7 polymerase. Transcribed RNA was translated in the presence of [^{3}H]leucine, and samples were electrophoresed into an SDS–15% polyacrylamide gel followed by fluorography. The positions of molecular mass markers (in kilodaltons) are indicated. Samples: 1, RIII plasmid cut with *Cla*I; 2, no RNA added; 3, GIV plasmid cut with *Cla*I; 4, splice 305/7018 plasmid cut with *Cla*I; 5, splice 502/7157 plasmid cut with *Cla*I; 6, splice 305/7018 plasmid cut with *Eco*RI; 7, splice 502/7157 plasmid cut with *Eco*RI.

reconstructed cDNAs in pGEM4Z plasmids linearized with *Cla*I or *Eco*RI followed by in vitro translation in the presence of [^{3}H]leucine or [^{35}S]cysteine. Translation of full-length constructs (cut with *Eco*RI at nt 7924) resulted in an abundant protein product from all the alternatively spliced mRNAs. This product had a molecular mass of ca. 16 kDa, comigrated with truncated Rex from a Rex-splice construct, and could be specifically immunoprecipitated by antibodies to the C-terminal part of Rex (Fig. 6). This indicated that the alternatively spliced mRNAs all expressed truncated Rex initiating at one of the internal AUGs in the Rex ORF. Analysis of the coding regions of the RIII and GIV constructs indicated that these mRNAs could potentially express additional proteins (Fig. 1). Therefore, these constructs were cut by *Cla*I (which cuts at nt 7318) to avoid expression of the truncated Rex proteins that obscured the detection of additional small proteins. Translation of these constructs resulted in a fairly abundant product from the RIII mRNA construct. This product migrated as a doublet with a molecular mass of around 6 to 8 kDa (Fig. 7). This is in fairly good agreement with an estimated molecular mass of 5.5 kDa. No additional products could be directly observed in translated RNA from the GIV construct (Fig. 7). However, immunoprecipitation with an antibody directed against a synthetic peptide in the XBL-IV ORF resulted in a very weak product migrating at around 17 kDa. By increasing the amount of translation reaction 10-fold, the immunoprecipitation of this 17-kDa GIV product could be convincingly demonstrated (Fig. 8, lane 3b). The immunoprecipitation was specific because no products were detected with preimmune sera or with the anti-Rex antibody (Fig. 8). The apparent molecular mass of 17 kDa of the GIV protein is in good agreement with the estimated mass (11.6 kDa), considering the apparent 16-kDa mobility of the truncated Rex protein (estimated size, 13.1 kDa).

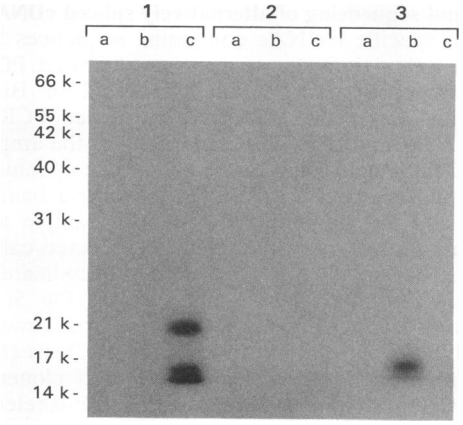

FIG. 8. In vitro translation and immunoprecipitation of proteins encoded by the cDNAs. cDNAs were linearized and transcribed in vitro by using T7 polymerase. Transcribed RNA was translated in the presence of [^{35}S]cysteine and subjected to immunoprecipitation as described in the text. In vitro translation reaction products (100 μl) were used for the immunoprecipitations, except for no. 1, for which 10 μl was used. Immunoprecipitated products were electrophoresed into an SDS–12% polyacrylamide gel followed by fluorography. The positions of molecular mass markers (in kilodaltons) are indicated. Samples: 1, BLV Rex plasmid cut with *Eco*RI; 2, RIII plasmid cut with *Cla*I; 3, GIV plasmid cut with *Cla*I. Antibodies for precipitations: a, preimmune rabbit 1 serum; b, rabbit 1 immunized with the GIV peptide as described in Materials and Methods; c, rabbit anti-Rex serum.

In summary, the in vitro data indicated that the alternative spliced mRNAs, as described for the Tax/Rex mRNA (42, 76, 77), can express truncated Rex protein originating at one of the multiple AUGs in the Rex ORF. In addition, the RIII mRNA expresses a protein of 6 to 8 kDa and the GIV mRNA can express, albeit at low levels, a protein of 17 kDa.

CAT assays. A potential effect on LTR-driven transcription of the RIII and GIV proteins was examined by transient cotransfection with a BLV-LTR-CAT reporter construct into CRFK cells. These studies indicated that the GIV protein transactivated expression from the BLV-LTR by two- to threefold while the RIII protein had no effect. Under the same conditions, the BLV-Tax expression construct transactivated LTR-driven transcription by 200-fold (data not shown).

DISCUSSION

Using region-specific PCR, we have shown alternative and complex splicing of BLV mRNA. In infected cattle we could detect spliced mRNA with a splice pattern consistent with a Tax/Rex mRNA and at least four alternatively spliced RNAs, of which two encoded hitherto unrecognized BLV proteins, designated RIII and GIV. The Tax/Rex and alternatively spliced mRNAs could be detected at the highest levels in BLV-infected cell cultures and at the next highest levels in the 6-week p.i. samples from experimentally infected calves. These mRNAs are also expressed, albeit at lower levels, in naturally infected animals, in which they could be detected by using nested PCR. Interestingly, the GIV mRNA was specifically detected in naturally infected cows with persistent lymphocytosis and in two of five calves at 28 weeks after experimental infection with BLV. Furthermore, the calf with the strongest signal for GIV (calf 353) also had the highest lymphocyte counts (Fig. 2A). These data may suggest a correlation between expression of the GIV product and development of persistent lymphocytosis.

In situ hybridization analysis of PBMC showed the presence of a very few positive cells in the experimentally infected calves early after infection (2 and 6 weeks p.i.). No positive PBMC could be directly detected in the experimentally infected calves at 19 weeks p.i. or in a naturally infected cow with persistent lymphocytosis. Interestingly, incubation of the PBMC from these animals in PBS with 10% fetal calf serum for a few hours resulted in up to 0.1% positive cells in the experimentally infected calves and in approximately 20% positive PBMC from the cow with persistent lymphocytosis. Also, the level of serum-induced BLV transcription in the individual cells was highest in the cow with persistent lymphocytosis. These results are in good agreement with in situ hybridization data from BLV infection in sheep, published by Lagarias and Radke (65). Whether this restriction of BLV expression in vivo is mediated by antibodies against the viral surface glycoprotein, as described for HTLV (104), or by a plasma repressing factor (30, 49, 50) is currently unknown. Nevertheless, our in situ hybridization data indicated that our normal procedure for isolating PBMC did not induce BLV expression and hence that our PCR experiments detected low levels of BLV RNA present in vivo in PBMC with severely restricted BLV expression.

In vitro translation experiments and examination of the coding potential of the alternatively spliced mRNAs indicated that all four could encode an N-terminally truncated form of Rex starting at one of the multiple internal AUGs in the Rex ORF (first possible AUG at nt 7280). Such a truncated form of Rex could also be encoded by the Tax/Rex mRNA. The size of the truncated Rex product(s) estimated on gels was around 16 kDa, and the theoretical size would be 13.1 kDa or less depending on the AUG used. This difference in apparent mobility correlates well with results published by others (77). The 305/7018 and 502/7157 RNAs (Fig. 1) apparently encode only the truncated Rex protein. mRNAs encoding only truncated Rex have been shown for HTLV (15, 42, 76), although no specific function has been shown for such a truncated Rex protein that lacks the nucleolus-targeting and RNA-binding motif.

In addition to truncated Rex, the GIV mRNA could encode a very interesting protein. The use of the alternative donor site at nt 502 incorporates an mRNA leader that potentially could initiate protein translation at one of the multiple CUGs in frame 1. The CUG at nucleotide 430 (CGUCUGG) is in a reasonable context, and we speculated that the GIV mRNA could encode a protein starting at this CUG. The murine leukemia retroviruses initiate glycosylated Gag protein (78), equine infectious anemia retrovirus initiates translation of Tat protein from CUGs in this region of their genomes (39, 92), and initiation at non-AUGs has also been described for other viral (11, 12, 18, 68) and cellular (52) proteins. Initiation at the CUG at nt 430 could give a hypothetical protein of 24 amino acids in frame 1 linked to 81 amino acids in the XBL-IV ORF (frame 3) at nt 7066 until terminated at nt 7307 (Fig. 9). This gives a total of 105 amino acids with a molecular mass of 11.6 kDa. Furthermore, this hypothetical protein has a Rex-like, potential nucleolus-targeting and RNA-binding motif consisting of 6 arginines in a stretch of 13 amino acids (Fig. 9). Translation of the GIV RNA followed by immunoprecipitation with an antibody directed against a peptide in the XBL-IV ORF resulted in a weak band migrating at 17 kDa. This result suggests that the GIV mRNA does express a GIV protein, and, moreover, the low level of translation is in agreement with a potential suboptimal CUG initiation codon. Computer analysis of the potential GIV protein initiating at the CUG at nt 430 indicated that the first 24 amino acids encoded in reading frame 1 had the characteristics of a hydrophobic leader peptide (Fig. 9). Furthermore, the analysis indicated potential proteolytic cleavage sites between amino acids 21 and 22 or 23 and 24. If this is the case, the final GIV protein would consist of the 81 amino acids from the XBL-IV ORF.

GIV

```
                              ↓ ↓
  1  LACTRVCFLS YFLFLAARAL SFGAQPHPAA FGPPFLTVPI KSLPFPQRLP

 51  LHLLFPPRHR LPRRALRALR DPLPDNDKII SCLLSKCCWL GAPLSTCLPG

101  PGFVQ
```

RIII

```
  1  MPKERRSRRR PQPIIRWVLM NVFPCNKPQQ RHSSHIQQHL GRLF
```

FIG. 9. Amino acid sequences of the potential GIV and RIII proteins. The hydrophobic leader from exon 1 in the GIV protein has a double underline, and the potential proteolytic cleavage sites are indicated by arrows. The 7-amino-acid stretch in GIV with homology to members of the Myb family of proteins has a single underline. The arginine-rich potential targeting signal in the GIV protein is indicated in boldface, as are the 17 amino acids of exon 1 in the RIII protein identical to the nucleolus-targeting signal and RNA-binding motif in exon 1 of Rex.

This ORF does not contain any potential N-linked glycosylation sites, indicating that the functional significance of the potential leader peptide is not glycosylation. This is in contrast to the glycosylated Gag protein of the murine leukemia viruses, which uses a suboptimal CUG codon to add a leader peptide transporting the protein to the endoplasmic reticulum for glycosylation (32, 78).

Our theory of the GIV protein being expressed in vivo is supported by the fact that the nucleotide sequence of the XBL-IV ORF is highly conserved (more than 99%) between the two published sequences of BLV (80, 81, 89). Considering the highly variable nature of retroviruses, it seems unlikely that this region would be so highly conserved unless it encoded a functional protein. To get a possible indication of potential function of the GIV protein, we searched the NBRF and Swiss-Prot (release 20) protein data bases for amino acid sequence homologies. Only limited, but interesting, areas of homology between the BLV GIV amino acid sequence and proteins in the data base could be demonstrated. The potential role of GIV in tumorigenesis is strengthened by the observed identity of the 7-amino-acid stretch ProIleLysSerLeuProPhe to members of the Myb protein family of proto-oncogenes (57, 66, 97) (Fig. 9). Multiple Myb-binding motifs can be found in the BLV enhancer region, and it has been shown that Myb can transactivate the HTLV and HIV LTRs (20, 33, 34). Thus, it is possible that BLV transcription is regulated by Myb or, alternatively, that cellular genes are regulated by the potential GIV protein. In any case, the homology is interesting considering the role of Myb in myeloid and lymphoid regulation and in leukemia. The GIV protein was also found to be 55% homologous, over an 18-amino-acid stretch, to the measles virus phosphoprotein P. This protein binds to RNA and is probably part of the transcription complex initiating measles virus transcription (13). Taken together, the amino acid homologies of the GIV protein suggest a potential role of this hypothetical protein in viral or cellular transcriptional regulation and perhaps in tumorigenesis.

The RIII mRNA could potentially encode a protein starting at the Rex/envelope AUG at nt 4821, continuing for 17 amino acids in frame 3 spliced to the 3′ end of the XBL-III ORF (frame 1) at nt 7018, and continuing in this frame for 27 amino acids until terminated at nt 7098. The protein would consist of 44 amino acids (Fig. 9) and have a calculated molecular mass of 5.5 kDa. A protein doublet of 6 to 8 kDa could be shown by in vitro translation of the RIII RNA. The RIII protein contains a potential phosphorylation site and the two bands could correspond to phosphorylated and unphosphorylated forms of RIII. Alternatively, the smaller form could be a proteolytic cleavage product.

Comparison of the C-terminal 27-amino-acid sequence of the RIII protein with the data bases did not show any significant homologies. The amino-terminal part of the RIII protein has the same nucleolus-targeting and RNA-binding motif as Rex (Fig. 9). Thus, it is likely that the RIII protein can bind to the Rex-responsive element RexRE (10, 37, 101). This interaction could potentially act as a transdominant repressor of Rex function as described for C-terminally truncated forms of Rex (10, 19, 53, 82). Alternatively, the function of RIII could be significantly different depending on a specific functional role of the 27 amino acids in the XBL-III ORF. We are currently designing assay systems to test for a possible role of the RIII protein on Rex regulation of BLV expression.

Some characteristics of the potential products of GIV and RIII are shown in Table 4, together with similar potential products of HTLV (27), the Rex and Tax proteins of BLV and HTLV, and Rev and Vpr of HIV for comparison. The characteristics summarized in Table 4 indicate that the potential new proteins of BLV and HTLV, together with the Rex proteins of BLV and HTLV and Rev and Vpr of HIV, have several similar features. This may suggest that these proteins belong to a common family of complex retrovirus regulatory proteins. The features linking these proteins are as follows: relatively small size (i.e., less than 30 kDa), highly basic, presence of an arginine-rich positively charged stretch characteristic of a nucleus- or nucleolus-targeting signal (shown to be necessary for Rex/Rev function [54, 64, 67, 82]), stretches of repeated leucine or isoleucine residues resembling leucine zipper motifs to some degree (63, 74, 90, 106, 112), and, in the Rex, GIV, and HTLV Tof proteins, the presence of multiple cysteines. These features suggest that the putative new proteins of BLV and HTLV may play a role in nucleic acid binding and in viral and cellular regulation. Alignment of the BLV and HTLV proteins did not show a high degree of homology among the proteins (less than 30% homology). However, this is also true for the Rex and Tax proteins of BLV and HTLV, although these proteins have been shown to have similar functions in the two viruses. Thus, functional motifs may be more important than amino acid sequence similarity in assessment of the relatedness of this group of proteins.

TABLE 4. Characteristics of known and potential BLV and HTLV regulatory proteins[a]

Protein	Mol mass (kDa)	pI	Potential nucleolus-targeting signal	Repeated leucines	No. of cysteines/ total no. of amino acids
BLV Rex	16.5	11.2	Yes	Yes	4/156
BLV RIII	5.5	12.7	Yes	Yes	1/44
BLV GIV	11.6	10.6	Yes	Yes	6/105
HTLV Rex	20.5	9.6	Yes	Yes	6/189
HTLV Rof	17.4	13.0	Yes	Yes	1/152
HTLV Tof	26.7	12.9	Yes	Yes	9/241
HIV Rev	13.3	9.5	Yes	Yes	3/118
HIV Vpr	11.3	8.7	Yes	Yes	1/96
BLV Tax	34.3	6.5	No	Yes	12/309
HTLV Tax	39.5	6.5	No	Yes	8/353

[a] The primary amino acid sequences of the BLV, HTLV, and HIV proteins shown were extracted from sequences in GenBank or from published data (27). The molecular mass and isoelectric point of the proteins were calculated by using the Microgenie or PC/Gene programs. A potential nucleolus-targeting signal was determined by comparison with the known nucleolus-targeting signals present in Rex or Tof (27, 54, 64, 67, 82). The Tax proteins do not have any obvious nucleolus-targeting signals, but regions with nuclear localization signals have been described (102). The number of cysteines and total number of amino acids in the proteins are indicated.

Transient-expression experiments indicated that the RIII protein had no effect on the LTR-driven expression of BLV whereas the GIV protein could transactivate the LTR by two- to threefold. This low level of transactivation resembles the degree of transactivation observed with Vpr activation of the HIV LTR (28) and with Myb activation of the HTLV or HIV LTR (20, 33, 34) or Myb activation of cellular genes like CD4 and Myc (100, 113). This may indicate that the function of the GIV protein may be as a transactivator of certain genes, although the observed level on the BLV LTR seems extremely low compared with Tax transactivation. Since expression of the GIV mRNA also correlated with levels of virus-infected cells, the possible effect of GIV in tumorigenesis may be indirect. That is, GIV may increase virus

expression of other viral proteins, which may have a more direct effect on cell proliferation. We tested the activating potential of the RIII and GIV proteins in a feline kidney cell line that has shown great versatility for testing a diverse range of transactivating proteins (our unpublished observations). It is possible that this particular cell line does not support full functional activity of the RIII and GIV proteins or that the main functional capabilities of these proteins are not directed at regulation of the BLV LTR.

Analysis of splice donor and acceptor sites of the alternatively spliced mRNAs revealed several interesting findings. The donor at nt 305 used in the 305/7018 mRNA (Fig. 1) and as the first donor in the RIII mRNA has the canonical GU as the 5′ nucleotides in the spliced-out intron and is identical to the donor used in the single spliced envelope mRNA and the first donor in the Tax/Rex mRNA (108) (Table 3). The second donor in the RIII mRNA at nt 4871 is also identical to the second donor of the Tax/Rex mRNA and fits well with established consensus (71). The donor at nt 502 in the 502/7157 and the GIV mRNA is quite unusual in having UC as the first two bases in the intron (Table 3). This donor was used in combination with two different acceptors at nt 7066 or 7157 (Fig. 1; Table 3). These two acceptors are also quite unusual in having a CC (nt 7066) or a UU (nt 7157) dinucleotide as the 3′ bases in the intron. However, these donors and acceptors were used in RNA samples from several individual animals, indicating that such mRNAs are examples of bona fide BLV transcripts in vivo. The acceptors at nt 4649 (RIII, Env, and Tax/Rex) and 7018 (RIII and 305/7018) both fit well with established consensus having an AG as the 3′ dinucleotide in the intron. Surprisingly, the RIII splice pattern uses only a small fraction of the XBL-III ORF. However, the use of an RIII acceptor at nt 7018 would be likely if there is a stop signal in the XBL-III ORF at nt 6973 as described by Rice et al. (80, 81). Using our techniques, we could not find any evidence for use of the theoretical splice acceptor at the beginning of the XBL-III ORF at nt 6742 or at the acceptor at nt 6824 described by Ciminale et al. (27).

The mechanisms involved in the use of alternative, and unusual, splice sites are currently obscure. The use of splice sites could be controlled by virus-encoded factors, as suggested for HIV Rev (69), or could be determined by cellular factors as described for several cellular genes (22, 29, 61, 72). Regardless of the mechanisms involved, it seems as if at certain stages during BLV infection, the use of several, suboptimal, splice donors and acceptors results in joining of specific RNA sequences and generation of additional functional mRNAs. In this context, generation of the RIII and GIV mRNAs in infected cells may be important in the pathogenesis of persistent lymphocytosis and eventually leukemia in BLV-infected animals.

In conclusion, we identified alternatively spliced mRNAs encoding potential new regulatory proteins of BLV. Computer analysis revealed that the hypothetical new BLV proteins share interesting features and homologies with proteins involved in viral and cellular regulation. Furthermore, our studies may suggest that the GIV protein is expressed in vivo and is involved in BLV-induced persistent lymphocytosis. On the basis of the potential properties discussed above, the GIV protein is a promising candidate for a virally encoded protein involved in the BLV-induced proliferation of B cells and, perhaps, in development of leukemia.

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