In Vivo Transcription of the Bovine Leukemia Virus *tax/rex* Region in Normal and Neoplastic Lymphocytes of Cattle and Sheep

WAYNE A. JENSEN,* JOEL ROVNAK, AND GARY L. COCKERELL

Department of Pathology, Colorado State University, Fort Collins, Colorado 80523

Received 6 August 1990/Accepted 7 February 1991

Expression of bovine leukemia virus (BLV) has been considered to be blocked at the transcriptional level in vivo, since viral RNA species are not readily detected in freshly isolated leukocytes from BLV-infected animals. However, the presence of a persistent antiviral antibody response in infected animals suggests that some degree of virus expression must occur in vivo. The purpose of this study was to determine whether BLV RNA species could be detected by using the polymerase chain reaction in normal or neoplastic lymphoid cells freshly isolated from naturally or experimentally BLV-infected cattle and sheep, respectively. Primers designed to detect a 2.1-kb doubly spliced BLV *tax/rex*-specific mRNA were used to amplify cDNA copies of RNA derived from infected animals. The amplified viral product was then detected with a radiolabeled BLV *tax/rex*-specific probe. BLV-specific RNA was detected readily in freshly isolated peripheral blood leukocytes derived from BLV-seropositive cattle or sheep with persistent lymphocytosis and less readily in peripheral blood leukocytes from BLV-seropositive but hematologically normal animals. BLV-specific RNA was also detected in fresh samples of BLV-induced lymphosarcomas. Normal and neoplastic lymphoid cells from BLV-seronegative animals were uniformly negative under similar conditions. These primers also amplified the same viral product from genomic DNA derived from BLV-seropositive animals, providing further evidence for in vivo transcription and suggesting that BLV RNA-dependent DNA polymerase is capable of reverse transcribing the 2.1-kb mRNA in vivo. The demonstration of transcriptional products of BLV in vivo proves that viral latency in BLV infection is incomplete.

Bovine leukemia virus (BLV) is a naturally occurring, exogenous, B-lymphocytotropic retrovirus of cattle and is the etiologic agent of enzootic bovine leukosis (EBL), a neoplastic proliferation of B lymphocytes. BLV infection in cattle also induces persistent lymphocytosis (PL), a nonneoplastic proliferation of B lymphocytes (5). Sheep are susceptible to experimental infection with BLV and develop lymphosarcoma at an increased rate and frequency compared with those of the naturally occurring infection in cattle (5, 15). BLV is structurally and biologically related to the human T-cell leukemia virus types I and II (HTLV-I and HTLV-II). These viruses lack *onc* genes, integrate randomly into the host cell genome (4, 11, 20), and contain several unique open reading frames at the 3' end of their genome which encode the *trans*-acting regulatory proteins Tax and Rex (9, 10, 25, 28, 38). These regulatory proteins are translated from a 2.1-kb mRNA which is derived from doubly spliced full-length mRNA (31, 34-36, 42).

BLV was identified after short-term in vitro incubation of B lymphocytes from cattle with PL (33). Similarly, HTLV-I was identified after in vitro incubation of leukocytes from patients with a rare form of cutaneous T-cell lymphoma now known as adult T-cell leukemia (14, 44). BLV expression is most readily detected in cultured peripheral blood leukocytes (PBL) from cattle with PL (7) or sheep with elevated blood lymphocyte numbers and an inverted B/T-lymphocyte ratio (8), but only infrequently in cultured PBL from hematologically normal BLV-infected animals (7). Expression of BLV in vivo is thought to be blocked at the transcriptional level since neither viral particles, proteins, nor RNA has been readily detected in freshly isolated PBL or tumor cells (1, 16, 21, 23, 40). By using in situ hybridization, the presence of

viral RNA has been detected in <1/2,000 to 1/500,000 freshly isolated PBL from clinically normal BLV-infected sheep (26). Paradoxically, BLV-infected animals develop a marked and persistent humoral immune response against all viral proteins (12). In contrast to BLV, HTLV-I viral proteins have been detected in serum and spinal fluid from HTLV-I-infected patients affected with adult T-cell leukemia or chronic progressive myelopathy, respectively (3, 17), and HTLV-I RNA was recently detected, by using the polymerase chain reaction (PCR), in freshly isolated PBL from adult T-cell leukemia patients and viral carriers (24).

The current study was undertaken to determine whether BLV transcriptional activity could be detected in freshly isolated cells from BLV-infected cattle and sheep by using the PCR. Animals in all recognized stages of BLV infection were studied, including hematologically normal animals and those with PL or lymphosarcoma-leukemia.

MATERIALS AND METHODS

Animals studied and cell isolations. BLV serological status was determined by using a commercial agar gel immunodiffusion assay (Leukassay B; Pitman-Moore Inc., Washington Crossing, N.J.). Twenty-three clinically normal BLV-seropositive adult female Holstein cattle were studied, 11 of which had PL and the remaining 12 of which were hematologically normal. Eleven BLV-seronegative cattle were used as controls. Eight cattle with EBL were studied; abdominal tumor nodules or neoplastic lymph nodes were collected from six animals, and PBL from leukemic cattle (112,000 and 190,000 PBL/ μ l of venous blood) were collected from two. One cow with non-BLV-associated thymic lymphosarcoma was also studied.

Eight experimentally BLV-infected sheep were studied. Four of these sheep had been inoculated intraperitoneally

* Corresponding author.

within 3 months of birth with 10^7 PBL from a BLV-seropositive cow with PL. Two of these four sheep developed lymphosarcoma and lymphocytic leukemia (190,000 and 388,000 PBL/ μ l of venous blood) within 9 months postinoculation, a third developed PL at 16 months postinoculation, with an elevated lymphocyte count (6,600 to 9,300/ μ l) and an inverted B/T-lymphocyte ratio (65% B cells, 35% T cells), and the fourth sheep remained hematologically normal. Two of the remaining four sheep were inoculated intraperitoneally at 3 months of age with 10^7 bovine embryonic spleen (BESP) cells infected with an infectious clone of BLV, pBLV913 (9), derived from the persistently BLV-infected fetal lamb kidney (FLK) cell line (41). FLK cells contain four proviral copies, all of which have been cloned; pBLV913 is a construct of the 5' *Eco*RI fragment of proviral clone 9 and the 3' *Eco*RI fragment of proviral clone 13 (5a). pBLV913 is infectious in vitro following microinjection into BESP cells, as evidenced by induction of syncytia, expression of BLV RNA and proteins, and reverse transcriptase activity in culture supernatants (5b). Both sheep inoculated with pBLV913-infected BESP cells seroconverted to BLV by 4 weeks postinoculation. At 15 months postinoculation, 10^8 PBL from one of the BLV913-infected sheep were inoculated intravenously into the remaining two sheep at 3 months of age. These two sheep seroconverted to BLV at 1 and 3 months postinoculation (35a). Two BLV-seronegative sheep served as controls.

PBL were collected in buffy coats isolated by centrifugation of 60 ml of venous blood followed by removal of residual erythrocytes by hypotonic lysis. Samples were maintained on ice, except during centrifugation, until lysis for RNA isolation within 60 to 90 min of blood collection. PBL from leukemic animals were frozen in liquid nitrogen until lysed for RNA isolation. Single-cell suspensions of lymphosarcoma tissue were isolated by manual disruption of tumor nodules in cold Hanks balanced salt solution and frozen in liquid nitrogen until lysed for RNA isolation. RNA was also isolated from the persistently BLV-infected FLK cell line to serve as a positive control.

RNA isolation and cDNA production. Total cellular RNA was isolated by lysis of PBL or 3×10^7 lymphosarcoma cells in 4 M guanidine isothiocyanate solution and pelleting through cesium chloride as previously described (6) with minor modifications. Cells were lysed in 2.5 ml of 4 M guanidine isothiocyanate solution containing 4 M guanidine isothiocyanate, 20 mM sodium acetate, 0.1 mM dithiothreitol, and 0.5% sodium *N*-lauroylsarcosine. Genomic DNA was sheared by passage four times through a 20-gauge needle, and then 1 g of CsCl was added to each sample. The lysates were layered over 0.5 ml of 5.7 M CsCl–0.1 M EDTA (pH 7.5) in 3.5-ml ultracentrifuge tubes (*g*-Max konical Quick-seal tubes; Beckman Instruments, Inc., Palo Alto, Calif.) and centrifuged in a Beckman SW41 rotor for 14 h at $280,000 \times g$ and 20°C. The RNA pellets were subsequently dissolved in H_2O , precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol, and pelleted by centrifugation for 10 min at $16,000 \times g$. Pellets were again dissolved in H_2O and then quantitated by a colorimetric assay (DNA Dipstick; Invitrogen, San Diego, Calif.). Samples containing at least 2 μ g of RNA were divided equally, and half of each sample was digested for 1 h at 37°C with 60 U of DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 50 μ l of 50 mM Tris (pH 7.6)–10 mM $MgCl_2$ –1 mM dithiothreitol–200 U of placental RNase inhibitor per ml (Boehringer Mannheim Biochemicals). The remaining half of each sample was simultaneously

TABLE 1. Sequences and positions of BLV primers

Primer designation	Sequence (5'-3')	BLV nucleotide position ^a
5' primer	AGGCGCTCTCCTGGCTACTG	4758–4776
3' primer	GGCACCAGGCATCGATGGTG	7333–7314
5' probe primer (splice junction)	CATCAGATGGCAAGTGTGT	4862–4871 7247–7256

^a Nucleotide position in the proviral sequence of Sagata et al. (37).

digested with 1 μ g of RNase T₁ (Sigma Chemical Co. St. Louis, Mo.) as well as with DNase I under the same conditions. Samples containing less than 2 μ g RNA were digested only with DNase I. Digested samples were sequentially extracted with phenol-chloroform-isoamyl alcohol (24:24:1) and chloroform–1-butanol (4:1). RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. RNA (200 ng to 2.5 μ g) was reverse transcribed at 37°C for 1 h in 25 μ l of buffer containing 100 mM NaCl, 50 mM Tris hydrochloride (pH 8.2), 6 mM $MgCl_2$, 1 mM dithiothreitol, 1,000 U of placental RNase inhibitor (Boehringer Mannheim Biochemicals), per ml, 50 μ g of oligo(dT)₁₅ per ml, and 1,000 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) per ml.

DNA isolation. Genomic DNA was isolated from PBL and tumor cells by lysis in sodium dodecyl sulfate (SDS) and digestion with proteinase K. Cells were suspended in 100 mM sodium chloride–10 mM Tris (pH 7.5)–2 mM EDTA. Proteinase K (Sigma Chemical Co.) and SDS were added to final concentrations of 0.2 mg/ml and 1.2% (wt/vol), respectively. The lysates were incubated overnight at 37°C and then extracted twice with Tris-buffered phenol (pH 8.0), twice with phenol-chloroform-isoamyl alcohol (24:24:2), and twice with chloroform-isoamyl alcohol (49:1). Genomic DNA was then precipitated with 0.1 volume of 3 M sodium acetate and 2.0 volumes of absolute ethanol, spooled on a glass rod, dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA, and quantitated by measuring the A_{260} .

PCR. The sequence and nucleotide position of the primers used in the PCR are shown in Table 1. The 5' primer was located upstream of the *env-tax* splice junction site, and the 3' primer was located downstream of this splice junction site so that the amplified product crossed the second splice junction (Fig. 1). This set of primers yielded a 201-bp product from cDNA of the doubly spliced 2.1-kb BLV mRNA. The 201-bp product was amplified by adding 75 μ l of buffer containing 50 mM KCl, 10 mM Tris hydrochloride (pH 8.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM deoxynucleoside triphosphates, 0.5 μ M each primer, and 2 U of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, Conn.) to each cDNA sample. Genomic DNA samples were amplified by adding 1 to 2 μ g of DNA to 100 μ l of the same buffer. The amplification sequence consisted of a 1-min denaturation at 95°C, a 1-min annealing at 62°C, and a 20-s polymerization at 72°C with a 5-s extension of polymerization per cycle. This sequence was repeated for 35 cycles (DNA Thermocycler; Perkin-Elmer/Cetus).

Southern blotting and hybridization. A 5- to 25- μ l sample of the amplified products was electrophoresed either through 1% agarose GTG–3% Nusieve or 1% agarose GTG–1% Nusieve (Research Organics Inc., Cleveland, Ohio). After being stained with ethidium bromide, amplified products were blotted onto nylon membrane (Biotrace RP; Gelman

FIG. 1. Genomic organization of BLV provirus and generation of the doubly spliced 2.1-kb mRNA. Abbreviations: LTR, long terminal repeat; gag, group-specific antigen; prt, protease; pol, reverse transcriptase; env, envelope glycoprotein; pX, X region; Sd, splice donor; Sa, splice acceptor; Sj, splice junction; S, *SacI* site; E, *EcoRI* site. The locations of the 5' and 3' primers used for amplification are shown as short horizontal bars. Note the difference in the relative size of the two maps as shown by the scales at the right.

Sciences, Ann Arbor, Mich.) and hybridized overnight at 73°C in buffer (rapid hybridization buffer; Amersham Corp., Arlington Heights, Ill.) containing 5×10^6 cpm of α - 32 P-labeled BLV *tax/rex*-specific probe per ml of hybridization mix. After hybridization, the filters were washed twice in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature, twice in $0.1 \times$ SSC–0.1% SDS at 73°C, and once in $0.1 \times$ SSC–0.1% SDS at 78°C and then autoradiographed for 8 to 20 h at -70°C with an intensifying screen. The probe used to detect the PCR-amplified product was a 97-bp fragment produced by PCR from the full-length BLV proviral clone, pBLV913, by using a 5' probe primer (Table 1) that covered the *env-tax* spliced junction and the same 3' primer described above. The probe was formed by annealing of the 3' 10 bases of the 5' probe primer to unspliced pBLV913. Conditions of amplification were as described above, except that deoxynucleoside triphosphates were decreased to 5 μM , 100 μCi of [α - 32 P]dCTP was included, and the polymerization time was increased to 1 min with a 5-s extension of polymerization per cycle. The specific activity of the probe was approximately 5×10^7 cpm/ μg of DNA.

For Southern blot hybridization of genomic DNA, 10 μg of DNA was digested with either *SacI* (Boehringer Mannheim Biochemicals) or *SacI* and *EcoRI* (Boehringer Mannheim Biochemicals), electrophoresed through 1% agarose GTG–1% Nusieve, blotted onto nylon membrane (Biotrace RP), and hybridized overnight at 68°C in buffer (rapid hybridization buffer) containing 2×10^6 to 5×10^6 cpm of α - 32 P-labeled BLV *tax/rex*-specific probe per ml of hybridization mix. After hybridization, the filters were washed twice in $2 \times$ SSC–0.1% SDS at room temperature, twice in $0.1 \times$ SSC–0.1% SDS at room temperature, twice in $0.1 \times$ SSC–0.1% SDS at 68°C, and once in $0.1 \times$ SSC–0.1% SDS at 73°C and then autoradiographed for 24 to 72 h at -70°C with an intensifying screen. The probe used for Southern blot hybridization of genomic DNA was a 921-bp fragment derived from a subclone of pBLV913 containing the *Clal-NarI* fragment (nucleotides 7113 to 8004 [43]) of the *tax/rex* region.

RESULTS

Detection of BLV-specific mRNA in PBL from BLV-seropositive cattle and sheep. A representative autoradiograph of amplified cDNA derived from PBL of a cow and a sheep with PL and from persistently BLV-infected FLK cells is

shown in Fig. 2. DNase-digested samples yielded a 201-bp product which hybridized with the 97-bp probe derived from the BLV *tax/rex* region. RNase digestion prior to cDNA production eliminated the signal in identical samples. FLK RNA which was not reverse transcribed also failed to generate the 201-bp product (data not shown). The 201-bp amplified product was detected in five of five BLV-seropositive cattle affected with PL and one of seven hematologically normal, BLV-seropositive cattle (Table 2). Similarly, the 201-bp amplified product was detected in two of five hematologically normal, BLV-seropositive sheep and in the single sheep affected with PL (Table 2). The two hematologically normal sheep in which the 201-bp amplified product was detected were animals inoculated intravenously with PBL from one of the sheep inoculated with pBLV913-infected BESP cells. The 201-bp product was not detected in amplified cDNA from PBL of BLV-seronegative cattle or sheep.

Detection of BLV-specific mRNA in tumor cells from BLV-seropositive cattle and sheep. A representative autoradiograph of amplified cDNA from a cow and a sheep affected with lymphosarcoma is shown in Fig. 3, and the data are

FIG. 2. Southern blot analysis of amplified cDNA derived from freshly isolated PBL from a BLV-seronegative (lanes BLV Neg) cow and sheep, a BLV-seropositive (lanes BLV Pos) cow and sheep affected with persistent lymphocytosis, and the persistently BLV-infected FLK cell line (lanes FLK). Samples marked with an asterisk are identical to the adjacent sample except that they were digested with RNase prior to cDNA production. Primers used in the PCR were located on each side of the second splice junction to produce a 201-bp amplified product from cDNA of doubly spliced BLV mRNA. The blots were hybridized with a 97-bp probe derived from the BLV *tax/rex* region.

TABLE 2. Detection of in vivo BLV RNA in cattle and sheep

Animal	No. of animals positive for BLV RNA/no. tested:				
	BLV seronegative		BLV seropositive		
	PBL	Tumor	PBL (PL ⁻) ^a	PBL (PL ⁺)	Tumor
Cattle	0/7	0/1	1/7	5/5	4/6
Sheep	0/2	0/0	2/5	1/1	1/2

^a PL⁻, Animals without PL; PL⁺ animals with PL.

summarized in Table 2. A 201-bp amplified product was detected in four of six samples derived from BLV-seropositive cattle affected with EBL. Three of the four positive samples were derived from tumor nodules or neoplastic lymph nodes, and one positive sample was PBL isolated from a leukemic animal. RNA isolated from tumor cells from a BLV-seronegative cow with thymic lymphosarcoma was negative for the 201-bp amplified product. A 201-bp amplified product was also detected in one of two samples derived from BLV-seropositive sheep with lymphosarcoma. Both samples were PBL isolated from leukemic sheep (190,000 and 388,000 PBL/ μ l).

Detection of 2.1-kb cDNA in genomic DNA derived from BLV-seropositive cattle and sheep. The same PCR primers used to amplify cDNA samples were used to amplify genomic DNA from PBL of BLV-seropositive cattle, BLV-infected FLK cells, and pBLV913 plasmid DNA (Fig. 4). All three DNA preparations yielded an approximately 2.6-kbp amplified product derived from unspliced BLV provirus. Surprisingly, the primers also amplified a product of the same size (201 bp) as that amplified from cDNA samples derived from BLV-seropositive cattle and FLK cells. This product was not amplified from pBLV913 DNA (Fig. 4). The 201-bp product was amplified from PBL genomic DNA from six of six BLV-seropositive cattle affected with PL and two of five hematologically normal BLV-seropositive cattle. This amplified product was also detected in genomic DNA from three of four tumors derived from cattle and two of two tumors derived from sheep affected with BLV-induced lymphosarcoma which contained clonally integrated provirus

FIG. 3. Southern blot analysis of amplified cDNA from freshly isolated tumor cells from a BLV-seronegative cow with thymic lymphosarcoma, a BLV-seropositive cow with EBL, and a BLV-seropositive sheep with lymphocytic leukemia. Primers used in the PCR were located on each side of the second splice junction to produce a 201-bp amplified product from cDNA of doubly spliced BLV mRNA. The blots were hybridized with a 97-bp probe derived from the BLV *tax/rax* region.

FIG. 4. Southern blot analysis of DNA products amplified from genomic DNA isolated from the persistently BLV-infected FLK cell line (1 μ g), PBL from a BLV-seropositive cow with persistent lymphocytosis (1 μ g), and pBLV913 (0.1 μ g), a full-length proviral clone of BLV. Primers used in the PCR were located on each side of the second splice junction. A 201-bp amplified product is derived from spliced BLV cDNA, whereas a 2.6-kbp amplified product containing the intervening intron is derived from BLV provirus. The blots were hybridized with a 97-bp probe derived from the BLV *tax/rax* region.

(data not shown). The 201-bp product was not amplified from PBL genomic DNA derived from five BLV-seronegative cattle.

To determine whether the 2.1-kb cDNA could be detected by Southern blot hybridization of genomic DNA, restriction endonucleases were chosen which would differentiate full-length provirus from the 2.1-kb cDNA as follows. Digestion with *Sac*I would be expected to yield a 1,248-bp *tax/rax* fragment from proviral DNA and a 1,714-bp *tax/rax* fragment from 2.1-kb cDNA as a result of deletion of the *Sac*I site located 5' to the second splice acceptor (Fig. 1). Similarly, double digestion with *Sac*I and *Eco*RI would be expected to yield 728- and 520-bp *tax/rax* fragments from full-length BLV provirus and 1,194- and 520-bp *tax/rax* fragments from 2.1-kb cDNA (Fig. 1). DNA derived from FLK cells or PBL DNA from a cow affected with PL was digested with *Sac*I or *Sac*I plus *Eco*RI, electrophoresed through 1% agarose GTG-1% Nusieve, and then assayed by Southern blot hybridization with a BLV *tax/rax*-specific probe. The 2.1-kb cDNA was not detected by Southern blot hybridization of FLK DNA (Fig. 5) or DNA derived from a BLV-seropositive cow affected with PL. To investigate whether the 2.1-kb cDNA was detectable in a clonal tumor cell population, genomic DNA derived from four bovine and two ovine lymphosarcomas containing clonally integrated BLV provirus (data not shown) were assayed by Southern blot hybridization as described above. The 2.1-kb cDNA was not detected in genomic DNA from any of the tumors (data not shown).

DISCUSSION

Using PCR amplification, we demonstrated the expression of the BLV 2.1-kb mRNA in freshly isolated PBL and

FIG. 5. Southern blot analysis of genomic DNA isolated from the persistently BLV-infected FLK cell line. Genomic DNA (10 μ g) was digested with *SacI* or *SacI* plus *EcoRI* and electrophoresed through 1% agarose GTG-1% Nusieve. The blots were hybridized with a 921-bp probe derived from the BLV *tax/rex* region. Digestion with *SacI* would be expected to yield a 1,248-bp *tax/rex* fragment from proviral DNA and a 1,714-bp *tax/rex* fragment from 2.1-kb cDNA. Double digestion with *SacI* and *EcoRI* would be expected to yield 728- and 520-bp *tax/rex* fragments from full-length BLV provirus and 1,194- and 520-bp *tax/rex* fragments from 2.1-kb cDNA.

lymphosarcoma cells. The 2.1-kb species of RNA was chosen for assay because previous reports have shown it to be necessary for translation of the 38-kDa transcriptional activator, Tax, and the 18-kDa mRNA processing regulator, Rex (9, 10). Therefore, it seemed likely that the 2.1-kb mRNA species would predominate when expression was minimal. The PCR primers used in this study will not detect mRNA species from which structural proteins are translated. However, since expression of *tax* and *rex* activates transcription and selects for unspliced or singly spliced mRNA species, respectively, it can be hypothesized that structural protein mRNA species would also be expressed. This would explain the persistent anti-BLV humoral immune response in infected animals.

The ready detection of BLV 2.1-kb mRNA in BLV-infected animals affected with PL may reflect the greater number of infected cells in these animals (approximately 30%) as compared with hematologically normal BLV-infected animals (less than 5%) (22, 23), or possibly differential expression of BLV in animals with and without PL. The increased expression of the 2.1-kb mRNA in animals with PL may also account for the higher antiviral antibody titers seen in these animals (29). This increased expression could also be important in the leukemogenicity of BLV, since the risk of developing EBL is higher in animals with PL than in hematologically normal animals (13, 23). In HTLV-I-infected cells, *tax* activates the expression of various cellular genes such as the receptor for interleukin-2, which has been suggested to play a role in leukemogenesis through an autocrine or paracrine mechanism (27, 32).

Approximately 60% (five of eight) of the tumors in the current study maintained the ability to express the 2.1-kb mRNA species. The fact that some of the BLV-induced tumors were negative for the 2.1-kb mRNA species suggests that, as previously proposed (19, 21), expression of *tax* is not necessary to maintain the neoplastic state. The lack of 2.1-kb mRNA expression in some tumors may be due to deletion of

the 5' portion of the viral genome, as has been reported for some BLV-induced lymphosarcomas (21); however, the presence of deleted provirus in the lymphosarcoma samples examined was not determined in this study. We cannot exclude the possibility that the 201-bp product generated from tumor cells was due to contaminating BLV-infected, nonneoplastic PBL. However, we consider this unlikely since the 201-bp product was amplified not only from leukemic PBL but also from solid tumor nodules, which represent an essentially pure neoplastic cell population. Additionally, in naturally BLV-infected cattle and experimentally BLV-infected sheep, antibody titers reach maximal values with the development of lymphosarcoma, which suggests that viral expression is increased at that time (2, 12). Failure to detect BLV 2.1-kb mRNA in tumor cells from the cow with thymic lymphosarcoma further substantiates the conclusion that this condition is not associated with BLV infection (18).

Our results do not completely exclude the possibility that the 2.1-kb mRNA in freshly isolated PBL and tumor cell populations was generated during the short time interval between sample collection and cell lysis. However, amplification of the 201-bp amplified product from BLV-induced tumor or PBL genomic DNA is evidence that the 2.1-kb mRNA is transcribed *in vivo*. Only cDNA of RNA which has been spliced between the splice donor located in the 5' region of the *env* gene and the splice acceptor located just 5' to the pX region can generate the appropriate 201-bp product. The BLV primer-binding site used for initiation of reverse transcription is located 3' to the splice donor in the 5' long terminal repeat and is not present in the 2.1-kb mRNA (37). Therefore, the cDNA species detected by PCR must have originated from aberrantly spliced genomic RNA in which only the *env*-to-*tax/rex* splice occurred, or from doubly spliced 2.1-kb mRNA in which reverse transcription was initiated through an unidentified mechanism. In either case, detection of the 201-bp amplified product in genomic DNA is evidence of previous BLV transcription with subsequent reverse transcription by BLV RNA-dependent DNA polymerase. It is not known whether this cDNA species integrates into the host cell genome or remains unintegrated. If this cDNA species integrates, the lack of its detection by Southern blot hybridization of DNA from the six BLV-induced tumors suggests that this is a relatively rare event. Additionally, detection of this cDNA species by PCR, but not in Southern blot hybridization assays, suggests that it is present in low copy numbers. Pseudogenes derived from reverse transcription of cellular mRNA with subsequent integration have been described for numerous genes (39). Additionally, Rous sarcoma virus reverse transcriptase has been shown to reverse transcribe mRNA specific for the plasmid neomycin gene with subsequent cDNA integration into the genome of infected cells (30).

The results of this study indicate that viral latency in BLV infection is incomplete and provide evidence for a previously undescribed cDNA species derived from spliced BLV mRNA. If cDNA of the BLV *tax* gene integrates into the genome of the infected cell, it then becomes possible that the *tax* gene will come under the control of a host cell promoter, leading to inappropriate expression of *tax*. The expression of BLV *tax*, whether from the BLV provirus or integrated cDNA copies, may play a role in leukemogenesis by BLV.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-00924 from the National Institute of Allergy and Infectious Diseases and CA-43728 from the National Cancer Institute.

REFERENCES

1. Baliga, V., and J. F. Ferrer. 1977. Expression of the bovine leukemia virus and its internal antigen in blood lymphocytes. *Proc. Soc. Exp. Biol. Med.* **156**:388-391.
2. Bex, F., C. Bruck, M. Mammerickx, D. Portetelle, J. Ghysdael, Y. Cleuter, M. Leclercq, D. Dekegel, and A. Burny. 1979. Humoral antibody response to bovine leukemia virus infection in cattle and sheep. *Cancer Res.* **39**:1118-1123.
3. Bhagavati, S., G. Ehrlich, R. W. Kula, S. Kwok, J. Sninsky, V. Udani, and B. J. Poiesz. 1988. Detection of human T-cell lymphoma/leukemia virus type I DNA and antigen in spinal fluid and blood of patients with chronic progressive myelopathy. *N. Engl. J. Med.* **318**:1141-1147.
4. Broder, S., and R. C. Gallo. 1985. Human T-cell leukemia viruses (HTLV): a unique family of pathogenic retroviruses. *Annu. Rev. Immunol.* **3**:321-336.
5. Burny, A., C. Bruck, Y. Cleuter, D. Couez, J. Deschamps, J. Ghysdael, D. Gregoire, R. Kettmann, M. Mammerickx, G. Marbaix, and D. Portetelle. 1985. Bovine leukemia virus: a new mode of leukemogenesis, p. 35-55. *In* G. Klein (ed.), *Advances in viral oncology*. Raven Press, New York.
- 5a. Casey, J. W. Unpublished data.
- 5b. Casey, J. W., A. L. Boyd, M. A. Gonda, J. Rovnak, and G. L. Cockerell. Unpublished data.
6. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
7. Cockerell, G. L., and J. Rovnak. 1988. The correlation between the direct and indirect detection of bovine leukemia virus infection in cattle. *Leuk. Res.* **12**:465-469.
8. Cornil, I., P. Delon, A. Parodi, and D. Levy. 1988. T-B cell cooperation for bovine leukemia virus expression in ovine lymphocytes. *Leukemia* **2**:313-317.
9. Derse, D. 1987. Bovine leukemia virus transcription is controlled by a virus-encoded *trans*-acting factor and by *cis*-acting response elements. *J. Virol.* **61**:2462-2471.
10. Derse, D. 1988. *trans*-Acting regulation of bovine leukemia virus mRNA processing. *J. Virol.* **62**:1115-1119.
11. Deschamps, J., R. Kettmann, and A. Burny. 1981. Experiments with cloned complete tumor-derived bovine leukemia virus information prove that the virus is totally exogenous to its target animal species. *J. Virol.* **40**:605-609.
12. Deshayes, L., D. Levy, A. L. Parodi, and J. Levy. 1980. Spontaneous immune response of bovine leukemia-virus-infected cattle against five different viral proteins. *Int. J. Cancer* **25**:503-508.
13. Ferrer, J. F. 1980. Bovine lymphosarcoma. *Adv. Vet. Sci. Comp. Med.* **24**:1-68.
14. Gallo, R. C. 1981. Growth of human normal and leukemic T cells: T-cell growth factor (TCGF) and the isolation of a new class of RNA tumor viruses (HTLV). *Blood Cells* **7**:313-329.
15. Ghysdael, J., C. Bruck, R. Kettmann, and A. Burny. 1984. Bovine leukemia virus. *Curr. Top. Microbiol. Immunol.* **112**:1-19.
16. Gupta, P., S. V. S. Kashmiri, and J. F. Ferrer. 1984. Transcriptional control of the bovine leukemia virus genome: role and characterization of a nonimmunoglobulin plasma protein from bovine leukemia virus-infected cattle. *J. Virol.* **50**:267-270.
17. Ishibashi, K., S. Hanada, and S. Hashimoto. 1987. Expression of HTLV-I in serum of HTLV-I-related subjects and the early detection of overt ATL in HTLV-I carriers. *J. Immunol.* **139**:1509-1513.
18. Jacobs, R. M. 1985. Bovine lymphoma, p. 21-51. *In* R. G. Olsen, S. Krakowka, and J. R. Blakeslee (ed.), *Comparative pathobiology of viral diseases*. CRC Press, Inc., Boca Raton, Fla.
19. Kettmann, R., Y. Cleuter, D. Gregoire, and A. Burny. 1985. Role of the 3' long open reading frame region of bovine leukemia virus in the maintenance of cell transformation. *J. Virol.* **54**:899-901.
20. Kettmann, R., Y. Cleuter, M. Mammerickx, M. Meunier-Rotival, G. Bernardi, A. Burny, and H. Chantrenne. 1980. Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis. *Proc. Natl. Acad. Sci. USA* **77**:2577-2581.
21. Kettmann, R., J. Deschamps, Y. Cleuter, D. Couez, A. Burny, and G. Marbaix. 1982. Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3' proximate cellular sequences. *Proc. Natl. Acad. Sci. USA* **79**:2465-2469.
22. Kettmann, R., G. Marbaix, A. Burny, M. Meunier-Rotival, J. Cortadas, G. Bernardi, and M. Mammerickx. 1980. Genomic integration of bovine leukemia provirus. *Cold Spring Harbor Conf. Cell Proliferation* **7**:927-941.
23. Kettmann, R., G. Marbaix, Y. Cleuter, D. Portetelle, M. Mammerickx, and A. Burny. 1980. Genomic integration of bovine leukemia provirus and lack of viral RNA expression in the target cells of cattle with different responses to BLV infection. *Leuk. Res.* **4**:509-519.
24. Kinoshita, T., M. Shimoyama, K. Tobinai, M. Ito, S. Ito, S. Ikeda, K. Tajima, K. Shimotohno, and T. Sugimura. 1989. Detection of mRNA for the *tax1/rex1* gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86**:5620-5624.
25. Kiyokawa, T., M. Seiki, S. Iwashita, K. Imagawa, F. Shimizu, and M. Yoshida. 1985. p27x-III and p21x-III, proteins encoded by the pX sequence of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* **82**:8359-8363.
26. Lagarias, D. M., and K. Radke. 1989. Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections. *J. Virol.* **63**:2099-2107.
27. Lando, Z., P. Sarin, M. Megson, W. C. Greene, T. A. Waldman, R. C. Gallo, and S. Broder. 1983. Association of human T-cell leukaemia/lymphoma virus with the Tac antigen marker for the human T-cell growth factor receptor. *Nature (London)* **305**:733-736.
28. Lee, T. H., J. E. Coligan, J. G. Sodroski, W. A. Haseltine, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, and M. Essex. 1984. Antigens encoded by the 3'-terminal region of human T-cell leukemia virus: evidence for a functional gene. *Science* **226**:57-61.
29. Levy, D., L. Deshayes, A. L. Parodi, J. P. Levy, J. R. Stephenson, S. G. Devare, and R. V. Gilden. 1977. Bovine leukemia virus specific antibodies among French cattle. Radioimmunoassay with the major structural protein (BLV p24). *Int. J. Cancer* **20**:543-550.
30. Linial, M. 1987. Creation of a processed pseudogene by retroviral infection. *Cell* **49**:93-102.
31. Mamoun, R. Z., T. Astier-Gin, R. Kettmann, J. Deschamps, N. Rebeyrotte, and B. J. Builemain. 1985. The pX region of the bovine leukemia virus is transcribed as a 2.1-kilobase mRNA. *J. Virol.* **54**:625-629.
32. Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J. Inoue, M. Yoshida, and T. Taniguchi. 1987. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-I-encoded p40x and T3/Ti complex triggering. *Cell* **48**:343-350.
33. Miller, J. M., L. D. Miller, C. Olson, and K. G. Gillette. 1969. Virus-like particles in phytohemagglutinin-stimulated lymphocyte cultures with reference to bovine lymphosarcoma. *J. Natl. Cancer Inst.* **43**:1297-1305.
34. Nagashima, K., M. Yoshida, and M. Seiki. 1986. A single species of pX mRNA of human T-cell leukemia virus type I encodes *trans*-activator p40x and two other phosphoproteins. *J. Virol.* **60**:394-399.
35. Rice, N. R., S. L. Simek, G. C. Dubois, S. D. Showalter, R. V. Gilden, and R. M. Stephens. 1987. Expression of the bovine leukemia virus X region in virus-infected cells. *J. Virol.* **61**:1577-1585.
- 35a. Rovnak, J., J. W. Casey, W. A. Jensen, A. L. Boyd, M. G. Weiser, and G. L. Cockerell. 1990. Abstr. 19th Annu. UCLA Symp. *J. Cell. Biochem. Suppl.* **14D**:71.

36. Sagata, N., J. Tsuzuku-Kawamura, M. Nagayoshi-Aida, F. Shimizu, K. Imagawa, and Y. Ikawa. 1985. Identification and some biochemical properties of the major XBL gene product of bovine leukemia virus. *Proc. Natl. Acad. Sci. USA* **82**:7879–7883.
37. Sagata, N., T. Yasunaga, J. Tsuzuku-Kawamura, K. Ohishi, Y. Ogawa, and Y. Ikawa. 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. *Proc. Natl. Acad. Sci. USA* **82**:667–681.
38. Slamon, D. J., K. Shimotohno, M. J. Cline, D. W. Golde, and I. S. Y. Chen. 1984. Identification of the putative transforming protein of the human T-cell leukemia viruses HTLV-I and HTLV-II. *Science* **226**:61–65.
39. Temin, H. M. 1985. Reverse transcription in the eukaryotic genome: retroviruses, pararetroviruses, retrotransposons, and retrotranscripts. *Mol. Biol. Evol.* **2**:455–468.
40. Van Der Broeke, A., Y. Cleuter, G. Chen, D. Portetelle, M. Mammerickx, D. Zagury, M. Fouchard, L. Coulombel, R. Kettmann, and A. Burny. 1988. Even transcriptionally competent proviruses are silent in bovine leukemia virus-induced sheep tumor cells. *Proc. Natl. Acad. Sci. USA* **85**:9263–9267.
41. Van der Maaten, M. J., and J. M. Miller. 1976. Replication of bovine leukemia virus in monolayer cell cultures. *Bibl. Haematol.* **43**:360–362.
42. Wachsman, W., K. Shimotohno, S. C. Clark, D. W. Golde, and I. S. Y. Chen. 1984. Expression of the 3' terminal region of human T-cell leukemia viruses. *Science* **226**:177–179.
43. Weiss, R., N. Teich, H. Varmus, and J. Coffin. 1985. RNA tumor viruses. *Molecular biology of tumor viruses*, vol. 2, p. 1086–1101. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. Wong-Staal, F., and R. C. Gallo. 1985. The family of human T-lymphotropic leukemia viruses: HTLV-I as the cause of adult T cell leukemia and HTLV-III as the cause of acquired immunodeficiency syndrome. *Blood* **65**:253–263.