

Unintegrated Bovine Leukemia Virus DNA: Association with Viral Expression and Disease

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The correlation between bovine leukemia virus (BLV) unintegrated DNA, viral expression, and stage of disease was determined in cattle naturally infected with BLV. The concomitant presence of unintegrated BLV DNA with viral transcriptional activity was observed in 53% (18 of 34) of hematologically normal, BLV-seropositive cattle and in 100% (10 of 10) of BLV-seropositive cattle with the preneoplastic syndrome persistent lymphocytosis. In vitro studies suggested that accumulation of unintegrated BLV DNA resulted from a process of reinfection rather than intracellular reverse transcription of newly synthesized BLV RNA. Interestingly, unintegrated BLV DNA was not detected in tumor cells from cattle with BLV-associated lymphocytic leukemia/malignant lymphoma despite viral transcriptional activity in 100% (eight of eight) of these cattle. Thus, the presence of unintegrated BLV DNA differentiated nonneoplastic from neoplastic conditions in BLV-infected cattle. These results demonstrate that unintegrated viral DNA serves as a marker of disease progression in BLV-infected cattle but is not necessarily associated with induction or maintenance of the neoplastic state.

Bovine leukemia virus (BLV) is a B-lymphocytotropic retrovirus closely related by genomic organization and disease progression to human T-cell leukemia virus type 1 (HTLV-1) (for reviews, see references 7 and 12). Infection by BLV is characterized by a long clinical latency and a persistent immune response associated with low-level or transient viral expression (9, 11). Approximately 65% of infected cattle remain clinically and hematologically normal, whereas ~30% develop a persistent nonneoplastic proliferation of B lymphocytes (persistent lymphocytosis [PL]). In less than 5% of infections, a fatal, monoclonal, neoplastic transformation of B lymphocytes appears, resulting in lymphocytic leukemia (LL)/malignant lymphoma (ML). Because the risk of developing LL/ML is greater in cattle with PL than in BLV-seropositive cattle with normal hematological parameters, PL should be considered a preneoplastic condition (15).

The chronic nature of BLV infections reflects the capacity of BLV to integrate within host chromosomal DNA. As is typical of most retroviruses, integrated proviral DNA serves as a template for the synthesis of viral gene products. In addition to integrating within the host genome, the provirus can exist in both unintegrated linear and circular forms (4). Although limited gene expression from unintegrated retroviral DNA has been reported (29–31), these proviral species are generally believed to be transcriptionally silent remnants of the viral replication cycle (3, 18, 22).

In several retroviral systems, high levels of unintegrated viral DNA have been correlated with superinfection, the onset of disease, and cytopathicity (19, 20, 24). Although unintegrated viral DNA has not been reported in cattle naturally infected with BLV, these obligate intermediates of retroviral reverse transcription (4, 8) could serve as markers for BLV infection and disease progression.

The purpose of this study was to determine if unintegrated viral DNA was present in lymphocytes of BLV-infected cattle and, if so, to correlate its presence with viral expression and

stage of disease. The circular form of the BLV provirus was chosen as a marker for unintegrated viral DNA on the basis of our ability to specifically detect this form in the presence of both integrated and linear unintegrated viral DNA. The use of this animal model, with cattle in well-defined stages of infection and disease, may provide insight into the progression of analogous retroviral infections in humans.

MATERIALS AND METHODS

Animals. Seventy-four adult Holstein female cattle from a commercial dairy were screened for antibodies against BLV gp51 by agar gel immunodiffusion (Leukassay B; Rhone Merieux, Inc., Athens, Ga.). Cattle were categorized as having PL if their lymphocyte counts exceeded $9.5 \times 10^3/\mu\text{l}$ on consecutive evaluations at ≥ 3 -month intervals. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from all seronegative (BLV⁻), seropositive (BLV⁺), and BLV⁺/PL cattle on three independent occasions and analyzed for unintegrated BLV DNA and viral transcriptional activity. Fresh tumor tissues, either PBMC from cattle with LL or solid tissue masses from cattle with ML, were collected at necropsy and analyzed in duplicate. Following collection, all tissues were either processed immediately or maintained at -70°C until use.

Detection of unintegrated BLV DNA. (i) **Qualitative PCR.** Cattle were screened for the presence of unintegrated BLV DNA by using qualitative PCR. Total cell lysates were prepared from PBMC and tumor tissues as described by Higuchi (10). Lysates were subjected to hot-start PCR (2), using oligonucleotides U5 and U3 (Table 1). These primers, derived from sequences within *pX* and adjacent to *gag*, respectively, selectively amplify discrete products from circular BLV DNA having one or two long terminal repeats (LTRs) (Fig. 1). Each 100- μl reaction mixture contained 40 μl of lysate (2.4×10^5 mononuclear cells), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4.5 mM MgCl₂, 0.2 mM deoxyribonucleoside triphosphates (dNTPs), 0.2 μM oligonucleotide primers, and 2.5 U of *Taq* polymerase (Boehringer Mannheim Corp., Indianapolis, Ind.). The PCR temperature profile consisted of 94°C for 3 min, 2 cycles of 94°C for 60 s, 64°C for 60 s, and 72°C for 35 s, and 37 cycles of 94°C for 60 s, 63°C for 60 s, and 72°C for 35 s. Amplified products were identified by Southern blot hybridization using an alkaline phosphatase-labeled oligonucleotide (UP [Table 1]) (23). To evaluate the sensitivity of this assay, serial dilutions of the linearized plasmid pR5 (Fig. 1; also see below) were added to BLV⁻ PBMC lysates and analyzed as described above.

(ii) **CC-PCR.** The abundance of unintegrated BLV DNA was evaluated by competitive comparative PCR (CC-PCR). Total cell lysates of PBMC ($20 \mu\text{l}$, 8×10^4 cells) were added to serial dilutions of the linearized competitor plasmid pR5 and prepared for hot-start PCR as described above. The PCR temperature profile consisted of 94°C for 3 min, 2 cycles of 94°C for 60 s, 64°C for 30 s, 72°C for 35 s, and 26 cycles of 94°C for 60 s, 63°C for 30 s, and 72°C for 35 s. Amplified products were identified by Southern blot hybridization and analyzed by densitometry using an IS-1000 Digital Imaging system (Alpha Innotech, San Leandro,

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TABLE 1. Sequences and positions of oligonucleotide primers and probes

Primer or probe	Sequence (5'→3')	Nucleotide position
A5	GCAAGCAGGAGTATGACGAG	41–60 ^a
A3	GAATCAAAGATATGCACAGGG	340–320 ^a
P5	GGTCCGAATTGGTTGCTAGCAGA	8081–8103 ^b
P3	GCAGAAGGTTGAGCCAGTCTGA	694–673 ^b
TR5	AGGCGCTCTCTGGTACTG	4758–4776 ^b
TR3	GGCACCAGGCATCGATGGTG	7333–7314 ^b
TRP	CATCAGATGGCAAGTGTGT	4862–4871:7247–7256 ^b
U5	TGCTAGAAAATGAATGGCTCT	8138–8158 ^b
U3	CCAGAGTTGTTAGGGTCC	581–561 ^b
UP	AACCGTGCTTGCTTACCTGAC	322–301 ^b

^a Nucleotide position in the bovine actin sequence of Degen et al. (5).

^b Nucleotide position in the BLV sequence of Sagata et al. (26).

Calif.) as previously described (6). Linear regression was used to predict the copy numbers of unintegrated BLV DNA on the basis of comparisons of competitor and virus-specific band intensities.

Detection of BLV transcriptional activity. (i) **Qualitative RT-PCR.** BLV transcriptional activity was evaluated by reverse transcription-PCR (RT-PCR) using oligonucleotides TR5 and TR3 (Table 1), which selectively amplify a 201-bp doubly spliced BLV *tax/rex* mRNA (9, 11). Total RNA, isolated from 2.5×10^5 mononuclear cells by using RNeasy (Qiagen, Inc., Chatsworth, Calif.), was in-

cubated with 100 pmol of each primer at 75°C for 10 min. The reaction mixture was subsequently cooled at 0°C for 10 min and combined with 1 U of Prime RNase inhibitor (5 Prime→3 Prime, Inc., Boulder, Colo.), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4.5 mM MgCl₂, 0.2 mM dNTPs, 2.7 U of Rous-associated virus-2 reverse transcriptase (Amersham Corp., Arlington Heights, Ill.), and 2.5 U of *Taq* polymerase in a total volume of 100 μl. The RT-PCR temperature profile consisted of 48°C for 30 min, 94°C for 3 min, 2 cycles of 94°C for 60 s, 62°C for 60 s, and 72°C for 35 s, and 37 cycles of 94°C for 60 s, 61°C for 60 s, and 72°C for 35 s. Amplified products were identified by Southern blot hybridization using an alkaline phosphatase-labeled oligonucleotide (TRP [Table 1]) complementary to the *tax/rex* splice junction site. To evaluate the sensitivity of this assay, the 201-bp doubly spliced BLV *tax/rex* mRNA was cloned into the pCRII vector (Invitrogen, San Diego, Calif.) and transcribed as sense RNA, using commercial reagents (Ampliscribe System T7; Epicenter Technologies, Madison, Wis.). Serial dilutions of this transcript were subsequently added to BLV⁻ total RNA and analyzed as described above. All RNAs were pretreated with RNase-free DNase prior to RT-PCR.

(ii) **CC-RT-PCR.** The relative level of BLV transcriptional activity was evaluated by CC-RT-PCR using oligonucleotide pairs A5-A3 and TR5-TR3 (Table 1) to coamplify β-actin and *tax/rex* mRNAs, respectively. RNA isolation and reaction conditions were as described for qualitative RT-PCR except that the following temperature profile was used: 48°C for 30 min, 94°C for 3 min, 2 cycles of 94°C for 60 s, 62°C for 60 s, and 72°C for 35 s, and 25 cycles of 94°C for 60 s, 61°C for 60 s, and 72°C for 35 s. The products were visualized on ethidium bromide-stained agarose gels and analyzed by densitometry.

BLV activation. To stimulate BLV transcription in vitro, PBMC were incubated (37°C, 5% CO₂) for 8 h with 5 μg of lipopolysaccharide-L4561 (LPS; Sigma Chemical Co., St. Louis, Mo.) per ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Construction of PCR control plasmids. The BLV molecular clone pBLV913, which contains the entire BLV sequence with 400 bp of 5' flanking irrelevant ovine genomic DNA (25), was used to construct control plasmids pBLV913-2 and pR5. A 9.1-kbp fragment of clone pBLV913, free of vector sequence, was isolated by complete digestion with *Kpn*I. Following ligation, this fragment was cleaved with *Hind*III and inserted into the unique *Hind*III site of pUC19. This construct, designated pBLV913-2, contains two LTRs in tandem with 400 bp of intervening irrelevant ovine genomic DNA as described above. To construct plasmid pR5, pBLV913 was partially digested with *Aat*II, and a 8,184-bp BLV fragment (nucleotides 60 to 8244 [26]) isolated. Following ligation, oligonucleotides P5 and P3 (Table 1) were used to amplify a 697-bp fragment, using reaction conditions as described for qualitative PCR. This fragment, containing a single LTR and 104 bp of 5' and 149 bp of 3' flanking BLV sequences, was ligated into the *Eco*RV site of the pT7Blue(R) vector (Novagen, Madison, Wis.). To differentiate pR5 from other PCR templates in the CC-PCR assay, a 322-bp cassette of irrelevant DNA was ligated into the BLV LTR *Sty*I site of this plasmid (Fig. 1).

RESULTS

Comparison of BLV unintegrated DNA and BLV transcriptional activity. The specificity of the qualitative PCR protocol for the detection of unintegrated BLV DNA was first evaluated by using two control plasmids, pR5 (Fig. 1) and pBLV913-2 (not shown). Interestingly, pBLV913-2, which contains two tandem LTRs analogous to the two-LTR circular species of BLV DNA, served as a template to generate both single- and double-LTR-size products (data not shown). This was likely a result of self or product priming of the target template in addition to specific oligonucleotide PCR priming. Amplification of plasmid pR5 yielded a single product of the predicted size (950 bp), and the sensitivity was determined to be two copies of pR5 target in a background of 2.4×10^5 PBMC (data not shown).

A total of 74 adult Holstein female cattle (22 BLV⁻, 34 BLV⁺, 10 BLV⁺/PL, and 8 BLV⁺/LL/ML) were then screened for the presence of unintegrated BLV DNA (Fig. 2). Both one-LTR circular and two-LTR circular forms of BLV DNA were identified. However, the two-LTR circular form was never detected in the absence of the one-LTR circular species. Unintegrated viral DNA was identified in 53% (18 of 34) of BLV⁺, 100% (10 of 10) of BLV⁺/PL, and 0% (0 of 8) of BLV⁺/LL/ML cattle. No unintegrated BLV DNA was detected in any of the 22 BLV⁻ cattle.

To determine if the presence of unintegrated BLV DNA

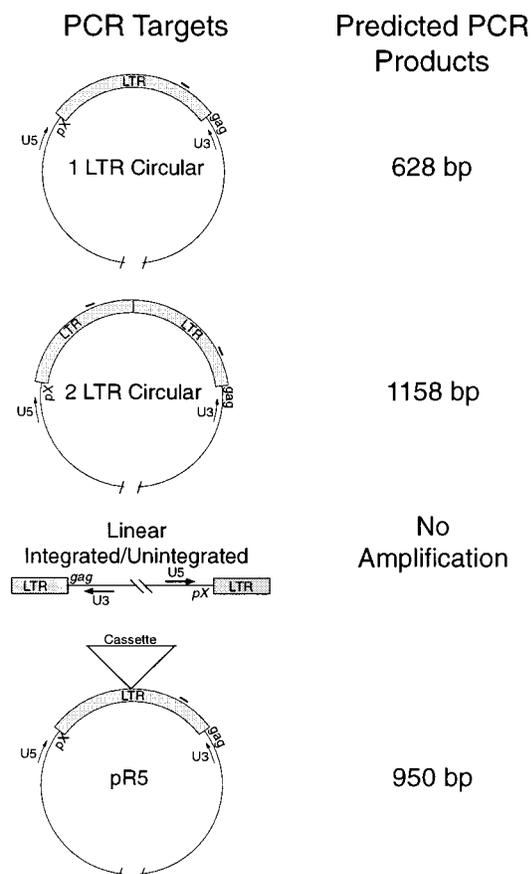


FIG. 1. BLV unintegrated DNA-specific PCR. The competitor plasmid pR5 and potential forms of BLV proviral DNA are illustrated with the corresponding sizes of their predicted PCR products. All DNA targets utilize the same BLV-specific primers, which are shown by the arrows. Solid bars indicate binding locations of the alkaline phosphatase-labeled probe UP.

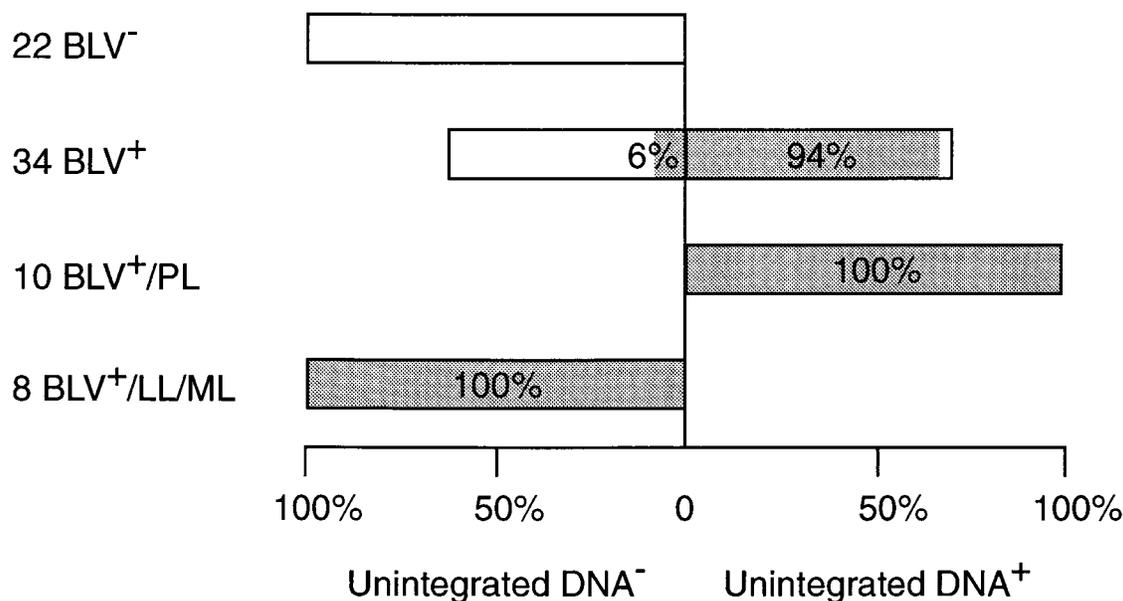


FIG. 2. Relationship between unintegrated BLV DNA, viral transcription, and disease. The number of cattle in each category is given on the left. Lengths of the horizontal bars represent the unintegrated BLV DNA status of cattle within each group as a percentage. Transcriptional activity (solid fills) is presented as a percentage of BLV unintegrated DNA⁺ or BLV unintegrated DNA⁻ cattle.

correlated with viral transcriptional activity, RT-PCR was used to detect BLV *tax/rex* mRNA in the same tissues from the 74 cattle described above. Using serial dilutions of in vitro-transcribed *tax/rex* RNA, the sensitivity of this assay was determined to be 10 copies of target in a background of BLV⁻ RNA (data not shown). BLV transcriptional activity correlated with the presence of unintegrated viral DNA in PBMC from 94% (17 of 18) of BLV⁺ and 100% (10 of 10) of BLV⁺/PL cattle (Fig. 2). In contrast, BLV transcriptional activity was identified in tumor cells from 100% (eight of eight) of BLV⁺/LL/ML cattle despite the absence of detectable unintegrated viral DNA (Fig. 2). BLV transcriptional activity was not detected in any of the 22 BLV⁻ cattle.

Comparison of BLV unintegrated DNA accumulation and BLV transcription in vitro. To examine the possibility that the accumulation of unintegrated BLV DNA was due to intracellular reverse transcription of newly synthesized viral RNA, PBMC were cultured with LPS under conditions which have been shown to induce BLV transcriptional activity in vitro (11). Although BLV can undergo cell-to-cell transmission, infection of susceptible cells is inefficient and slow. Therefore, a change in the level of unintegrated viral DNA following short-term in vitro stimulation of BLV-infected or -susceptible PBMC should not reflect the process of reinfection by progeny virus.

Figure 3 illustrates the results of a typical CC-PCR experiment used to evaluate the relative abundance of unintegrated viral DNA in LPS-stimulated PBMC. In contrast to the qualitative PCR protocol for the detection of unintegrated BLV DNA, amplification of the control plasmids pR5 and pBLV913-2 by the CC-PCR procedure yielded only products of the expect sizes. This increased specificity was achieved through modification of the temperature profile while maintaining a reduced but acceptable reaction sensitivity. In each experiment, negative and positive control reactions (Fig. 3, lanes 2, 3, and 13) were performed to monitor sensitivity, specificity, and contamination. Because the two-LTR circular form of BLV DNA was infrequently identified, only the one-LTR circular species was considered in the CC-PCR analysis of

unintegrated BLV DNA. Amplification of increasing numbers of the competitor plasmid pR5 resulted in progressive competition between a fixed number of PBMC containing predominantly one-LTR circular BLV DNA.

PBMC from three cattle in each of three different stages of infection (unintegrated DNA⁻/BLV⁺, unintegrated DNA⁺/BLV⁺, and unintegrated DNA⁺/BLV⁺/PL) were studied before and after 8 h of LPS stimulation in vitro. The results, correlating the accumulation of unintegrated BLV DNA with viral transcription before and after LPS stimulation, are shown in Fig. 4. In unintegrated DNA⁻/BLV⁺ cattle, no increase in unintegrated DNA or transcriptional activity was observed after 8 h of stimulation (Fig. 4A or B, respectively). The absence of detectable unintegrated DNA and viral expression in these cattle was confirmed by using the more sensitive qualitative PCR and RT-PCR assays described above (data not shown).

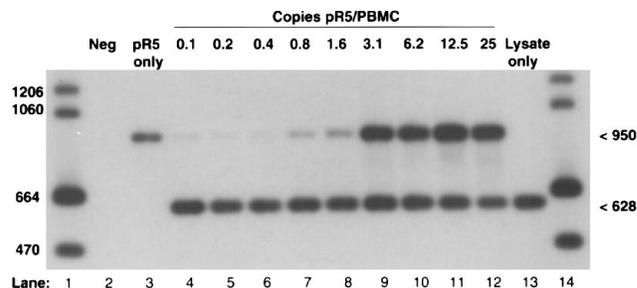


FIG. 3. CC-PCR analysis of BLV unintegrated DNA in fresh PBMC from an unintegrated DNA⁺/BLV⁺/PL cow. The number of copies of the competitor plasmid pR5/PBMC added to each reaction is indicated at the top (lanes 4 to 12). Sizes and locations of the one-LTR circular (628-bp) and pR5 (950-bp) products are indicated on the right. Lanes 3 and 13 contain PCR products amplified from 3.1×10^4 copies of pR5 DNA and 8×10^4 PBMC, respectively. Size of the molecular weight markers (lanes 1 and 14) are indicated at the left. The equivalence point is between lanes 8 and 9, representing a one-LTR circular BLV relative abundance of ~ 2.9 copies per PBMC. This image was digitized and imported into Aldus PageMaker.

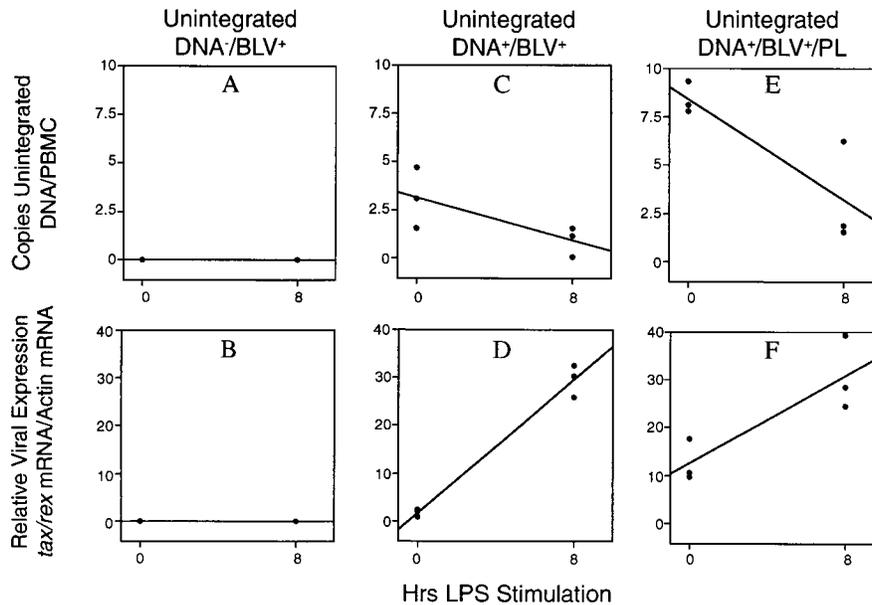


FIG. 4. Comparison of unintegrated BLV DNA and transcriptional activity following LPS stimulation. The unintegrated BLV DNA and infection status of each group of three cattle prior to stimulation is shown at the top. Copies of unintegrated viral DNA per PBMC represent a comparison of the amplification products from the competitor plasmid pR5 and native viral DNA. Viral expression is presented as a ratio of *tax/rex* mRNA to actin mRNA after CC-RT-PCR. Lines connecting datum points were determined by linear regression.

Unintegrated DNA⁺/BLV⁺/PL cattle consistently showed higher levels of unintegrated provirus and transcriptional activity prior to stimulation (Fig. 4E and F, respectively) compared with unintegrated DNA⁺/BLV⁺ cattle (Fig. 4C and D, respectively). Both groups demonstrated a decrease in unintegrated viral DNA 8 h poststimulation, in spite of a readily detectable up-regulation in BLV *tax/rex* expression.

DISCUSSION

Retroviral reverse transcription typically leads to the formation of three species of double-stranded DNA: linear molecules flanked by LTRs, and covalently closed circular forms containing one or two LTRs. The immediate precursor to the integrated provirus is the linear species. Reports of low-level protein expression from the circular forms of viral DNA (29–31) are typically overshadowed by evidence that these unique molecules are formed by processes of homologous recombination (one-LTR circular), self-ligation, or autointegration (two-LTR circular) and represent dead-end products of replication (4).

In this study, we used the unique structure of circular retroviral DNA and PCR to identify unintegrated BLV provirus in cattle naturally infected with BLV. Previous studies on the presence of unintegrated viral DNA in experimentally BLV-infected sheep reported conflicting results (14, 17), while results of similar studies of naturally BLV-infected cattle were negative (14). It is interesting that these unique molecules have eluded detection in spite of their abundant accumulation in specific groups of BLV⁺ cattle in the current study. In BLV⁺ and BLV⁺/PL cattle containing transcriptionally active virus, we found ~3 and ~8 copies of circular BLV DNA per cell, respectively, in freshly isolated PBMC. In human immunodeficiency virus type 1 (HIV-1)-infected patients, a similar pattern of unintegrated viral DNA accumulation with disease progression has been reported, albeit with a maximum unintegrated proviral load of <0.2 copies per cell (20, 21). This

variance in unintegrated viral DNA abundance is likely due to differences in the biological activities of HIV-1 and BLV. The validity of our data is supported in part by the observation that the one-LTR circular form of BLV DNA is more abundant than the two-LTR circular form, reflecting previous reports that circular molecules formed by homologous recombination are more abundant than products of autointegration (22, 28).

The analysis of unintegrated viral DNA in patients infected with HTLV-1 has produced conflicting results. Kitamura et al. (16) reported the detection of unintegrated provirus in a single patient of seven with adult T-cell leukemia but not in either of two asymptomatic HTLV-1 carriers or any of five patients with HTLV-1-associated myelopathy/tropical spastic paraparesis. In contrast, Bazarbachi et al. (1) reported unintegrated provirus in PBMC isolated from patients with adult T-cell leukemia (two of two) or HTLV-1-associated myelopathy/tropical spastic paraparesis (one of one) despite the absence of detectable viral expression. These studies do not provide evidence for a direct association between the presence of unintegrated viral DNA, viral expression, or disease progression in HTLV-1-infected patients.

The correlation of unintegrated BLV DNA with transcriptional activity in BLV⁺ and BLV⁺/PL cattle and the absence of detectable unintegrated provirus in BLV⁺/LL/ML cattle demonstrate that unintegrated viral DNA serves as a marker for disease progression. Without evidence for gene expression from unintegrated BLV DNA, it is difficult to suggest any direct role of the unintegrated provirus in BLV-induced transformation in cattle. Although studies with HIV-1 have demonstrated that integration is essential for efficient gene expression of retroviral proteins (27), it is possible that transient or low-level expression from unintegrated provirus is involved in retrovirus-induced disease. Previous attempts to demonstrate protein expression from unintegrated retroviral DNA have used integration-incompetent viruses and *in vitro* systems (29–31). Similar experiments were considered inappropriate in the current study because of the unavailability of integration-de-

fective BLV and because the in vitro behavior of BLV would undoubtedly differ from that which occurs in vivo.

Kettmann et al. (13) reported that 2 of 17 BLV-induced tumors contained only defective proviral DNA with 5' deletions, suggesting that expression of the 3' region of the BLV genome is required for maintenance of the neoplastic state. In two subsequent studies using RT-PCR for the analysis of BLV transcriptional activity, *tax/rex* mRNA was detected in four of six and three of three BLV-induced neoplasms (9, 11). These reports reflect the need for sensitive techniques to analyze viral activity in order to better characterize BLV-induced disease. Viral expression in all BLV⁺/LL/ML cattle in the current study may represent transcriptional activity from nonneoplastic infected cells or an as yet uncharacterized process to maintain the neoplastic state, which is indistinguishable from earlier stages of disease. In contrast, the existence of unintegrated BLV DNA appears to differentiate nonneoplastic and neoplastic conditions. It would be interesting to monitor cattle over an extended period to confirm our hypothesized sequence of BLV disease progression: unintegrated DNA⁻/BLV⁺ → unintegrated DNA⁺/BLV⁺ → unintegrated DNA⁺/BLV⁺/PL → unintegrated DNA⁻/BLV⁺/LL/ML. If this hypothesis is correct, disease progression could be monitored by the accumulation of unintegrated BLV DNA in the early stages of infection, prior to development of the typical hematological abnormalities associated with PL.

In HIV-1-infected cells, the in vitro accumulation of unintegrated viral DNA results from a process of reinfection rather than intracellular reverse transcription of newly synthesized HIV-1 RNA (24). Decrease in the levels of unintegrated BLV DNA, concomitant with rising viral expression in LPS-stimulated PBMC in vitro, suggests a similar process is operative in BLV. This view is supported by the inherently inefficient and slow rate of BLV infection and the reported in vitro instability of unintegrated retroviral DNA (21). If the intracellular reverse transcription of newly synthesized BLV RNA was the source of unintegrated viral DNA, increased transcription would be reflected in an accumulation of unintegrated DNA.

Our findings of the absence of both unintegrated provirus and *tax/rex* expression in freshly isolated PBMC from ~50% of BLV⁺ cattle may represent a strategy of BLV to evade immunosurveillance. The persistent immune response in BLV-infected cattle suggests that a small population of infected cells are permissive to viral expression. By analogy to the behavior of HIV-1 in humans, a quiescent B-cell reservoir where BLV integration is restricted may result in the long latency period typical of BLV infections in cattle.

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