JOURNAL OF VIROLOGY, Feb. 1997, p. 1514–1520 Vol. 71, No. 2
0022-538X/97/$04.00+0

In Vivo Study of Genetically Simplified Bovine Leukemia Virus Derivatives That Lack *tax* and *rex*†

KATHLEEN BORIS-LAWRIE,[1,2]* VERONIKA ALTANEROVA,[3] CESTMIR ALTANER,[3] LUCIA KUCEROVA,[3] AND HOWARD M. TEMIN[2]‡

Department of Veterinary Biosciences and Center for Retrovirus Research, Ohio State University, Columbus, Ohio[1]; McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin[2]; and Cancer Research Institute, Slovak Academy of Sciences, SK-812 32 Bratislava, Slovakia[3]

Received 17 May 1996/Accepted 16 October 1996

Genetically simplified derivatives of complex retroviruses that replicate in animal models are useful tools to study the role of the complex regulatory genes in virus infection and pathogenesis and were proposed as a novel approach toward the development of vaccines against complex retroviruses. Previously we developed genetically simple derivatives of bovine leukemia virus (BLV) that can replicate in tissue culture independently of the BLV regulatory proteins, Tax and Rex, and the RIII and GIV open reading frames (K. Boris-Lawrie and H. M. Temin, J. Virol. 69:1920–1924, 1995). These derivatives are encoded on novel, hybrid retrovirus genomes that contain transcriptional control sequences of a simple retrovirus and *gag-pol* or *env* genes of the complex BLV. The first-generation simple BLV derivatives replicate as complementary viruses (coviruses) by using separate *gag-pol* or *env* genomes, and therefore virus spread is limited to cells that are infected with both covirus genomes. Here we describe a second-generation simple BLV derivative that is encoded on a single hybrid genome. We show the virus to be replication competent by successive passage on D17 target cells and by analysis of viral RNA and proteins in the infected cells. Furthermore, we evaluate the immunogenicity and infectivity of the simple BLV derivatives in a BLV animal model. Small groups of rats were injected either with virus-producing cells or with proviral DNA. Western immunoblot analysis revealed that antibodies against the major viral antigenic determinants are induced in response to either method of introduction and that seroconversion is sustained in most of the rats for at least 6 months (the duration of the study). The magnitudes of the antiviral responses were similar in rats infected with the first-generation simple BLV coviruses, the second-generation replication-competent derivative, or wild-type BLV. Wild-type BLV typically infects peripheral blood mononuclear cells (PBMC), and the simple BLV derivatives were also found to infect PBMC as demonstrated by PCR amplification of proviral sequences and reverse transcriptase PCR amplification of viral RNA in treated rats. These results establish that simple BLV derivatives lacking *tax* and *rex* are infectious and immunogenic in rats. These viruses will be useful tools in comparative studies with BLV to evaluate the role of *tax* and *rex* in maintenance of virus load and in disease outcome.

All retroviruses share a conserved modular genomic structure and replicate by the same basic series of steps. Genetically simple retroviruses encode *gag*, *pol*, and *env* genes that are common to all retroviruses. Complex retroviruses encode additional, regulatory genes that dramatically affect the replication cycle by regulating specific steps in replication. How these modifications influence virus persistence and pathogenicity remains to be defined. Previously we developed genetically simplified derivatives of bovine leukemia virus (BLV) that can replicate in tissue culture independently of the regulatory proteins, Tax and Rex, and the RIII and GIV open reading frames (5). Genetically simplified derivatives of BLV that can replicate in animals would be important tools for studying the role of the complex regulatory genes in maintenance of virus load, virus persistence, and disease progression. Furthermore, simplified derivatives were also proposed as a novel approach to the development of vaccines against diseases caused by complex retroviruses (20). The previously described simple BLV derivatives were produced from novel, hybrid retrovirus genomes that contain the BLV *gag*, *pol*, and *env* genes and BLV *cis*-acting sequences important for replication, including the encapsidation signal (E), primer binding site (PBS), polypurine tract (PPT), and att sites. The genomes lack the major BLV splice donor and splice acceptors, *tax*, *rex*, the RIII and GIV open reading frames, and most of the BLV long terminal repeat (LTR) sequences, including the Tax and Rex response elements. The LTRs are hybrid molecules composed primarily of transcriptional control sequences from a simple retrovirus (spleen necrosis virus [SNV]). To facilitate gene expression in the absence of the BLV splice sites, the *gag-pol* open reading frame and the *env* open reading frame were expressed from separate hybrid genomes, and the progeny virions replicate as complementary viruses (coviruses). In this configuration, successive production of infectious virus was limited to cells infected with both coviruses. This is similar to rescue of the Bryan high-titer strain of Rous sarcoma virus, which lacks *env*, by Rous-associated helper virus-1 and -2 (7).

The aims of the current study were to derive a second-generation simple BLV derivative that is replication competent and to introduce the simple BLV derivatives into a BLV animal model to test their in vivo infectivity and immunogenicity. Here we describe a single-genome simple BLV derivative that is replication competent in successive replication cycles on D17 cells. The experiments establish that the simple BLV derivatives are infectious in vivo and induce antiviral

* Corresponding author. Mailing address: Department of Veterinary Biosciences, Ohio State University, 1925 Coffey Rd., Columbus, OH 43210. Phone: (614) 292-1392. Fax: (614) 292-6473. E-mail: boris-lawrie.1@osu.edu.

† This article is dedicated to the memory of Howard M. Temin.

‡ Deceased.

antibodies. These data validate simple BLV derivatives as useful reagents to study the roles of *tax*, *rex*, and the RIII and GIV open reading frames in BLV infection and pathogenesis. These results constitute an important step in the evaluation of the simple BLV derivatives as prototypic retrovirus vaccines.

MATERIALS AND METHODS

Nomenclature. Plasmid vectors are designated with names that start with p; names without p denote viruses or proviruses derived from the vector.

Vector construction. The poliovirus internal ribosome entry site (IRES) from pP2A-5′ (16) was amplified by PCR and cloned into pCRII (Invitrogen) to make pKB451. The sequences of the PCR primers were GTC GAC CAG CTC TGG GGT TGT TCC C and CCC GGG CAT TGA GTG GTG ATG ATA TCA AC, and they contain *Sal*I and *Sma*I termini, respectively. BLV *env* was amplified with Vent polymerase with DNA of fetal lamb kidney cells chronically infected with BLV (FLK/BLV cells) and primers KB566 (TGG CCA AAA GAA CGA CGG TCC CG) and KB567 (GTC GAC TCA AGG GCA GGG TCG) and cloned into pCRII to make pKB450. The IRES was excised from pKB451 with *Sma*I and ligated into the *Msc*I site of pKB450 to make an in-frame fusion between the IRES ATG and the *env* open reading frame and create pKB453. pKB453 was digested with *Sph*I, and the IRES-*env* cassette was subcloned into the *Sph*I site of pU5*gag-pol* to yield pU5*gag-pol-env*. pU5Hyg was constructed from the precursor of p*hyg*, pKB406 (5). This plasmid contains the SNV LTRs and BLV 3′ untranslated region (UTR) sequences. BLV 5′ UTR sequences beginning at U5 (nucleotide [nt] 440 as numbered in reference 19) and extending to nt 1147 were amplified by PCR with primers containing *Eco*RI and *Kpn*I termini and cloned into the homologous sites in pKB406 between the 5′ LTR and 3′ UTR to construct pKB430. The BLV UTR contains sequences important for integration (att), reverse transcription (PBS and PPT), and encapsidation (E). The BLV E signal is discontinuous and includes *gag* sequences to nt 1147 (12) and U5 sequences (5a). The *Sph*I fragment from p*gag-pol*IH (5) that contains the encephalomyocarditis virus IRES joined to *hyg* was inserted between the 5′ UTR and 3′ UTR in pKB430 to yield pU5*hyg*.

Cells, transfections, and infections. D17 dog osteosarcoma cells were cultured in Temin's modified Eagle medium and transfected by the dimethyl sulfoxide-Polybrene method (9). For infections, producer-cell medium was changed 16 h in advance. After harvest, the medium was clarified by centrifugation for 10 min at $3,000 \times g$ in a clinical centrifuge, mixed with Polybrene (final concentration, 30 μg/ml), and incubated on 2×10^5 D17 cells in a 60-mm-diameter dish for 4 to 6 h. Medium containing hygromycin B (80 U/ml; Calbiochem) was added 24 h after infection.

PCR amplification. For analysis of proviral DNA, D17 cells (10^7/ml) were treated with lysis buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg of gelatin per ml, 0.45% Nonidet P-40, 0.45% Tween 20, and 60 μg of proteinase K per ml), and the lysate was incubated for 2 to 16 h at 55°C and then for 10 min at 94°C. Five microliters of lysate was used in a 50-μl PCR mixture with primer pairs complementary to *hyg* and the PPT or to BLV *pol*. The sequences of the *hyg* and PPT primers were GGA AGT GCT TGA CAT TGG GGA (*hyg*) and G GTC GGC ATG ATC TTT CAT ACA AAT GAC TCC CCC TCA GCA TG (PPT), which yield a PCR product 800 bp in length. The BLV *pol* primers were KB560 (GGA GGT TTG TGC ATG ACC TAC) and KB576 (GTT AGG TGT ACG TAG GCT AG), and they yield a PCR product of 793 bp. Typically, the PCR mixtures were incubated for 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, cycled for 30 cycles, and analyzed on agarose gels. To prepare peripheral blood mononuclear cell (PBMC) samples for PCR, PBMCs were isolated by a Ficoll-Hypaque gradient centrifugation and washed three times with phosphate-buffered saline. Lymphocytes from 1 ml of blood were resuspended in 10 μl of 10× lysis buffer and diluted 10-fold with sterile water containing 200 μg of proteinase K per ml, followed by incubation at 55°C overnight and treatment at 100°C for 10 min to inactivate proteinase K. Five microliters of lysate was combined in a 50-μl PCR mixture with BLV *pol* primers KB560 and KB576 or with primers complementary to pUC19 sequences, KB595 (CAG GGG ATA ACG CAG GAA AG) and KB596 (CCA CTG GCA GCA GCC ACT G), which yield a 440-bp PCR product. The reaction mixtures were incubated as described above for 40 cycles. The PCR products were detected by Southern blot hybridization with the homologous PCR product that had been ^{32}P labeled by the random-primer method with the Rediprime reagent (Amersham). Hybridization was performed in Rapid Hyb solution (Amersham) under stringent conditions.

RNA and protein analysis. Total cellular RNA was prepared in Tri-Reagent (Molecular Research Center, Inc.) according to the manufacturer's instruction. To prepare viral RNA, virus particles were sedimented from clarified medium by ultracentrifugation at 25,000 rpm for 2 h in an SW28 rotor, and RNA was extracted with Tri-Reagent. RNA was applied to a Hybond N membrane (Amersham) in a slot blot apparatus (Millipore) and probed with ^{32}P-labeled BLV *pol* DNA probe (nt 2459 to 3252; amplified by PCR with primers KB560 and KB576) or *env* DNA probe (nt 4827 to 6369; amplified by PCR with primers KB566 and KB567), which were prepared with Rediprime reagent (Amersham). Hybridization was conducted in QuikHyb buffer (Stratagene) at 68°C for 1 h, and the blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature, washed once in 0.1× SSC–0.1% SDS at 65°C, and exposed by PhosphorImager technology (Molecular Dynamics, Sunnyvale, Calif.). RNA for reverse transcriptase-PCR (RT-PCR) analysis was isolated with Tri-Reagent, DNase treated, and incubated with avian myeloblastosis virus RT by the manufacturer's protocol (Access RT-PCR System; Promega). RNA samples without RT were used as controls to detect DNA contamination. BLV-specific cDNAs were amplified by PCR with thermostable Tf1 DNA polymerase and BLV *pol* primers KB560 and KB561 (CAT TGG AGG TCT CCT AAG ACC), which yield a 591-bp product.

To analyze BLV proteins in infected cells, subconfluent cultures were incubated in methionine- and cysteine-deficient medium for 30 min, followed by the addition of ^{35}S-labeled methionine and cysteine (18.5 MBq/ml) (Pro-mix; Amersham) and fetal bovine serum (10% final concentration) for 5 h. The cells were rinsed with phosphate-buffered saline and lysed in 150 mM NaCl–10 mM Tris HCl (pH 8.0)–1% Triton–0.5% sodium deoxycholate–0.1% SDS. Clarified lysates were immunoprecipitated with either polyclonal anti-BLV antibody or preimmune serum for 5 h at 4°C. The immune complexes were collected by adding 30 μl of 50% protein A-Sepharose suspension (Pharmacia) and incubating 1 h at 4°C, followed by extensive washing and resuspension in sample buffer (40 mM Tris-HCl [pH 6.8], 1% SDS, 50 mM dithiothreitol, 5% sucrose, 0.002% bromophenol blue). The proteins were resolved on SDS–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) gels and visualized by exposure to X-ray film.

Animals. Adult male Wistar rats 200 to 300 g in weight were used. Blood samples for Western blotting and PCR analysis were collected from the tail veins of the experimental rats after various periods of time. Blood samples for RT-PCR were collected by cardiac venipuncture upon killing of the animals.

Western immunoblot analysis. BLV Gag proteins were prepared by sucrose gradient centrifugation of supernatant from a chronically infected rat cell line (2). Since BLV Env protein is not efficiently harvested by this procedure, $gp51^{Env}$ was prepared by immunoaffinity column chromatography. Virus-containing medium was treated with Tween 20 and exposed to an immunoaffinity column containing anti-$gp51^{Env}$ antibodies, and the $gp51^{Env}$ was eluted. The BLV proteins were then subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electrophoretic transfer. The membranes were cut in strips and incubated in Tris-buffered saline–0.05% Tween 20 with 1% of blot-qualified bovine serum albumin (Promega) for 3 h at room temperature, followed by incubation overnight at 4°C with the test rat sera, known positive anti-BLV control sera, or preimmune rabbit sera at various dilutions. After the membranes were washed with Tris-buffered saline–0.05% Tween 20 three times for 15 min each, anti-rabbit or anti-rat immunoglobulin G conjugated to alkaline phosphatase (dilution, 1:5,000) was added. Western blue stabilizer substrate (Promega) for alkaline phosphatase was used for color development.

RESULTS

The single-genome simple BLV derivative is replication competent. We sought to produce a replication-competent simple BLV derivative by expressing BLV *gag*, *pol*, and *env* from a single hybrid genome, rather than from separate complementary virus genomes. The previously described complementary virus genome that encodes BLV *gag-pol* (pU5*gag-pol*) was modified by inserting BLV *env* (from pU5*env*) directly downstream of *pol* (Fig. 1B) (5). The resultant genome lacks the BLV major splice donor and *env* splice acceptor (pU5*gag-pol-env*) (Fig. 1C). Instead, a poliovirus IRES (16) was fused at the *env* AUG to promote cap-independent translation of BLV *env* from the pU5*gag-pol-env* RNA.

To determine if virus expressed from pU5*gag-pol-env* is replication competent, D17 (dog osteosarcoma) cells were transfected with pU5*gag-pol-env*, progeny virus was harvested and passaged on D17 target cells, and infected cells were analyzed. An initial approach was to passage virus on D17 cells, allow 7 days for virus replication and spread, and then screen the infected-cell pools for replicated provirus sequences by PCR. BLV *pol* sequences were detected in cells infected with the single-genome simple BLV derivative or the complementary virus derivatives but not in mock-treated D17 cells (data not shown). These results are consistent with production of replication-competent virus from the pU5*gag-pol-env* hybrid genome.

A second approach to examine if the single-genome simple BLV is replication competent was to evaluate the ability of the virus to replicate a selectable vector (pU5Hyg) (Fig. 1D) through successive replication cycles. pHyg contains a hygromycin B resistance gene, *hyg*, between SNV transcriptional

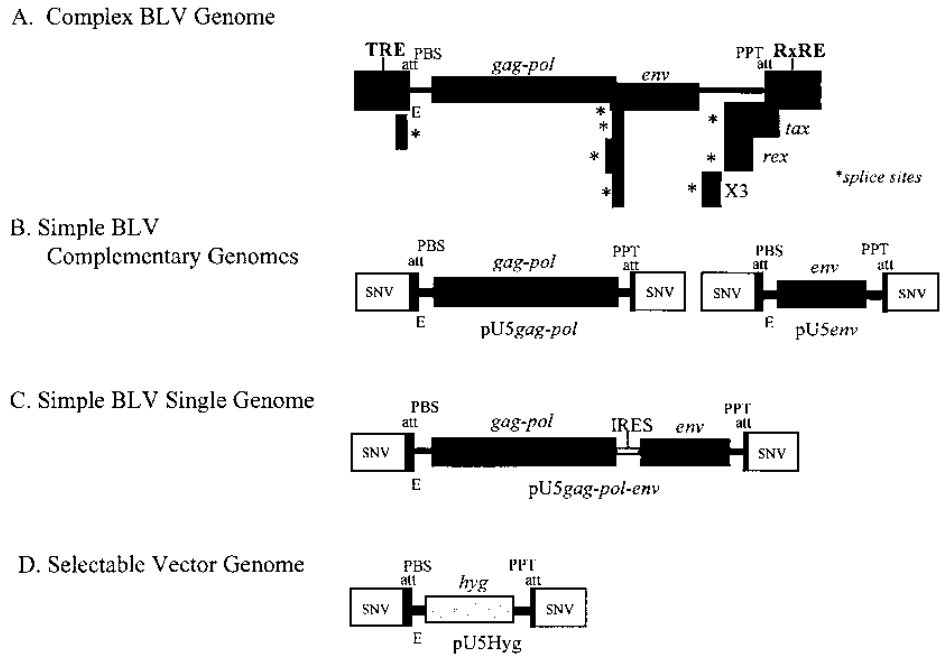

FIG. 1. Wild-type BLV, simple BLV genomes, and pHyg. (A) BLV genome. BLV sequences are shown in black. Untranslated sequences are shown with the thick horizontal black line, and genes are indicated by labeled rectangles. The locations of the Tax response element (TRE) and Rex response element (RxRE) are shown in boldface, and major BLV splice sites are denoted with asterisks. (B) Simple BLV complementary hybrid genomes, pU5*gag-pol* and pU5*env*. SNV sequences of the hybrid LTRs are shown as white, labeled rectangles, while terminal LTR sequences derived from BLV are shown as black, vertical lines and include the 5′ LTR U5 region and attR and the 3′ attL. The thick black horizontal line designates additional BLV untranslated sequences, which include the encapsidation sequence (E), PBS, and PPT. Labeled black rectangles indicate BLV genes. (C) Simple BLV single-hybrid genome, pU5*gag-pol-env* (labeled as in panel B), encoding BLV *gag*, *pol*, and *env*. The labeled, horizontal gray line denotes the poliovirus IRES fused at the *env* AUG. (D) Selectable vector genome, pHyg. The shaded box marked *hyg* indicates a hygromycin resistance gene positioned between SNV transcriptional control sequences and BLV untranslated sequences sufficient for replication, which are designated as in panel B.

control sequences and BLV *cis*-acting sequences sufficient for replication. When this vector genome is propagated in D17 cells infected with wild-type BLV and passaged on D17 target cells, the titer is 100 Hyg^r CFU/ml (Table 1).

To derive simple BLV producer cells, D17 cells were cotransfected with pU5*gag-pol-env* and pU5Hyg, and 3 days later simple BLV progeny virus was harvested and used to infect D17 target cells. Infected-cell clones were selected with hygromycin B and counted. When the Hyg vector genome was propagated with the single-genome simple BLV derivative, the titer was 30 to 40 CFU/ml, which is similar to the vector titer when propagated with wild-type BLV (Table 1). Hygromycin-resistant cell clones were screened for provirus sequences by PCR, and each clone tested was positive for the *hyg* gene and sequences between *hyg* and the PPT, which is consistent with replication of the Hyg vector (data not shown). BLV *pol* and *env* sequences were also present in 50% of these clones and indicated that they were also infected with the simple BLV derivative (data not shown). Six of the doubly infected passage 1 clones were expanded, and progeny viruses were passaged further on D17 target cells. Hyg^r passage 2 cells were observed, and the titers averaged 40 Hyg^r CFU/ml and ranged from 10 to 100 CFU/ml (Table 1). *hyg*-PPT provirus sequences were detected in all of the Hyg^r clones tested but not in uninfected D17 cells (data not shown). Further examination of infected-cell clones for simple BLV provirus sequences revealed that over 50% contained BLV *pol* and *env* sequences, while uninfected cells did not (data not shown). These results indicate that (i) the Hyg vector can be replicated in successive replication cycles, and (ii) the single-genome simple BLV derivative (pU5*gag-pol-env*) is replication competent in successive replication cycles.

To confirm these results, infected cells were analyzed directly for viral RNA expression and protein production. Total cellular was subjected to RNA slot blot analysis with a probe complementary to BLV *pol*. As expected, BLV *pol* RNA was observed in cells infected with the simple BLV or wild-type BLV but not in mock-treated cells (Fig. 2A). Furthermore, progeny simple BLV virions (passage 3) contain encapsidated

TABLE 1. Titers of vector viruses propagated with simple BLV or BLV

Virus	Vector titer (CFU/ml)[a]	
	Passage 1	Passage 2
Hyg/simple BLV single-genome derivative		
Pool A	30 ± 9	NT[b]
Pool B	40 ± 8	NT
Clone 1		100 ± 5
Clone 2		40 ± 3
Clone 3		35 ± 15
Clone 4		30 ± 9
Clone 5		30 ± 3
Clone 6		10 ± 1
Hyg/BLV[c]	100 ± 2	NT
Mock	<MD[d]	<MD

[a] Hyg^r CFU per milliliter of cell-free medium.
[b] NT, not tested.
[c] D17 cells transfected with pHyg were infected with cell-free medium from FLK/BLV producer cells, and 3 days later progeny virus was harvested and titers were determined on D17 target cells.
[d] <MD, less than the minimum detectable.

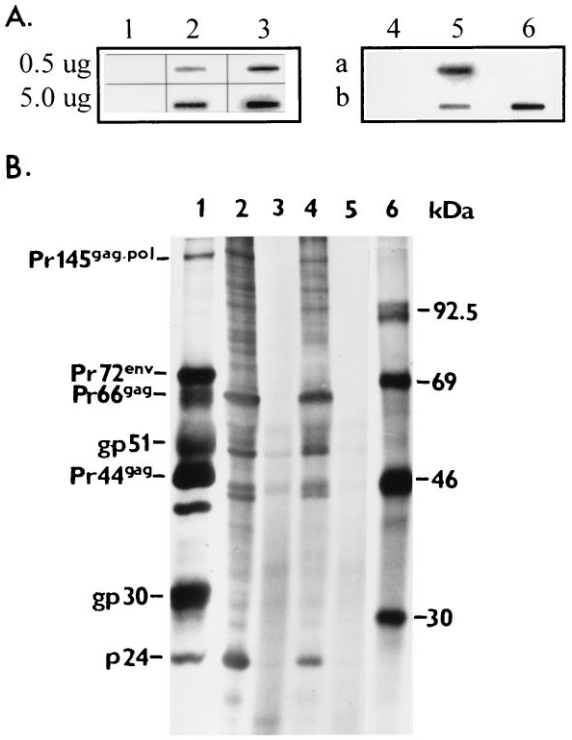

FIG. 2. (A) Analysis of viral RNA expressed in infected cells or genomic RNA from pelleted virions by RNA slot blot hybridization assay with a BLV *pol* probe and PhosphorImager analysis. Total-cell RNA was extracted from uninfected D17 cells (lane 1), pooled D17/U5*gag-pol-env* cells (passage 2) (lane 2), and FLK/BLV cells (lane 3). Virion RNA was extracted from supernatant of mock-infected D17 cells (lane 4), progeny from the D17/U5*gag-pol-env* cells (lane 5), and FLK/BLV virions (lane 6). RNA was prepared from virions harvested from 160 ml of cell-free medium (row a, lanes 4 and 5), 16 ml of medium (row b, lanes 4 and 5), 0.16 ml of medium (row b, lane 6), or a tRNA control (row a, lane 6). (B) Analysis of BLV proteins by immunoprecipitation of metabolically ^{35}S-labeled proteins in infected cells with rabbit polyclonal anti-BLV antiserum (lanes 1, 2, and 4) or preimmune serum (lanes 3 and 5). Immune complexes were recovered with protein A-Sepharose beads and analyzed by SDS–12% PAGE and autoradiography. Lane 1, FLK/BLV cells (5.4×10^7 cpm per reaction); lanes 2 and 3, D17/U5*gag-pol-env* cells (passage 2, clone 5B) (6.6×10^8 cpm per reaction); lanes 4 and 5, D17/U5*gag-pol-env* cells (passage 2, clone 31) (6.6×10^8 cpm per reaction); lane 6, molecular mass standards.

viral RNA (Fig. 2A). Similar results were observed when blots were rehybridized with a probe complementary to BLV *env* (data not shown). To look directly at the profile of BLV proteins produced in infected cells, passage 1 cells were metabolically labeled with [^{35}S]methionine-cysteine and BLV proteins were immunoprecipitated with known antibody against BLV. As expected, BLV Pr145$^{Gag\text{-}Pol}$, Pr66^{Gag}, p24^{Gag}, and gp51^{Env} proteins were observed in simple BLV- and BLV-infected cells (Fig. 2B). Although functional Env protein was produced, the amount of Pr72^{Env} and gp30^{Env} detected by Western blotting was reduced substantially in simple BLV-infected D17 cells compared to FLK/BLV cells. Env production may be less efficient in the context of the IRES as compared to the normal mode of translation from spliced RNA.

In summary, the simple BLV derivative pU5*gag-pol-env* is infectious in successive replication cycles in D17 target cells. The infected cells express viral RNA, produce BLV proteins, and release progeny that contain viral RNA. These data demonstrate that the hybrid genome pU5*gag-pol-env* produces a replication-competent simple BLV derivative in D17 cells.

Introduction of the simple BLV derivatives into rats. The rat is a tractable small animal model to test BLV immunogenicity and infectivity in vivo, although rats do not develop BLV disease (3). The simple BLV derivatives were introduced into rats by injection of D17 virus-producing cells or by injection of simple BLV DNA. The rats were monitored for seroconversion to BLV antibody production, and were screened for provirus sequences in PBMCs as an indicator of authentic virus infection and replication. Four adult Wistar rats (200 to 300 g) (designated group 1) were injected subcutaneously with heterologous D17 cells producing the complementary simple BLV at a dose of 80×10^6 live cells (8×10^9 infectious units; passage 1 simple BLV covirus set 3 in reference 5) in five sites in the back three times at 1-month intervals. Four rats were treated with uninfected D17 cells and designated group 2. In an independent part of the experiment, four additional groups of rats were inoculated subcutaneously with three 50-μg doses of DNA at 10-day intervals. These groups each received the following DNAs (Fig. 1): group 3, complementary simple BLV genomes (pU5*gag-pol* and pU5*env*); group 4, single simple BLV genome (pU5*gag-pol-env*); group 5, wild-type BLV genome (pBL913) (kindly provided by David Derse, National Cancer Institute, Frederick, Md.); and group 6, mock plasmid (pUC19).

The simple BLV derivatives induce anti-BLV antibodies. BLV typically induces an antibody response in rat against BLV structural proteins p24^{Gag} and gp51^{Env} (3, 17). Induction of antiviral antibodies in response to injection of simple BLV DNA would be consistent with virus gene expression and protein production. Sera from the treated rats were harvested over time and analyzed by Western blot analysis for the presence of

TABLE 2. Presence of antibodies against BLV p24^{Gag} and gp51^{Env} in rats inoculated with complementary virus-producing cells or various DNAs

Treatment group	Animal no.	Antibody presence[a]				
		p24^{Gag}				gp51^{Env} (6 mo)
		1 mo	2 mo	4 mo	6 mo	
1 (covirus cells[b])	342-2	3+	NT	2+	2+	2+
	326	NT	4+	3+	NT	1+
	327-1	NT	3+	2+	NT	3+
	327-3	NT	3+	NT	2+	1+
2 (uninfected cells)	344-1	−	NT	−	−	−
	344-2	−	−	−	−	−
3 (covirus DNA)	338-1	−	NT	NT	3+	2+
	338-2	3+	3+	3+	4+	1+
	338-3	3+	NT	NT	2+	2+
4 (single-genome DNA)	339-1	4+	4+	4+	−	−
	339-2	2+	NT	NT	2+	2+
	339-3	2+	NT	NT	3+	3+
	339-4	2+	NT	NT	3+	1+
5 (wild-type BLV DNA)	340-1	3+	3+	3+	3+	2+
	340-2	3+	NT	NT	3+	1+
6 (pUC19 DNA)	341-1	−	−	NT	−	−
	341-2	−	NT	−	−	−

[a] Blood was taken from the treated rats at the indicated times after inoculation, and the presence of anti-BLV antibodies against Gag and Env was tested with different serum dilutions by Western immunoblot analysis as indicated in Materials and Methods. 1+, positive reaction at a 10-fold serum dilution; 2+, positive reaction at a 50-fold serum dilution; 3+, positive reaction at a 100-fold serum dilution; 4+, positive reaction at a 500-fold serum dilution; −, negative reaction at a 10- to 50-fold serum dilution; NT, not tested.

[b] D17/co-virus set 3 (5), 8×10^9 infectious units.

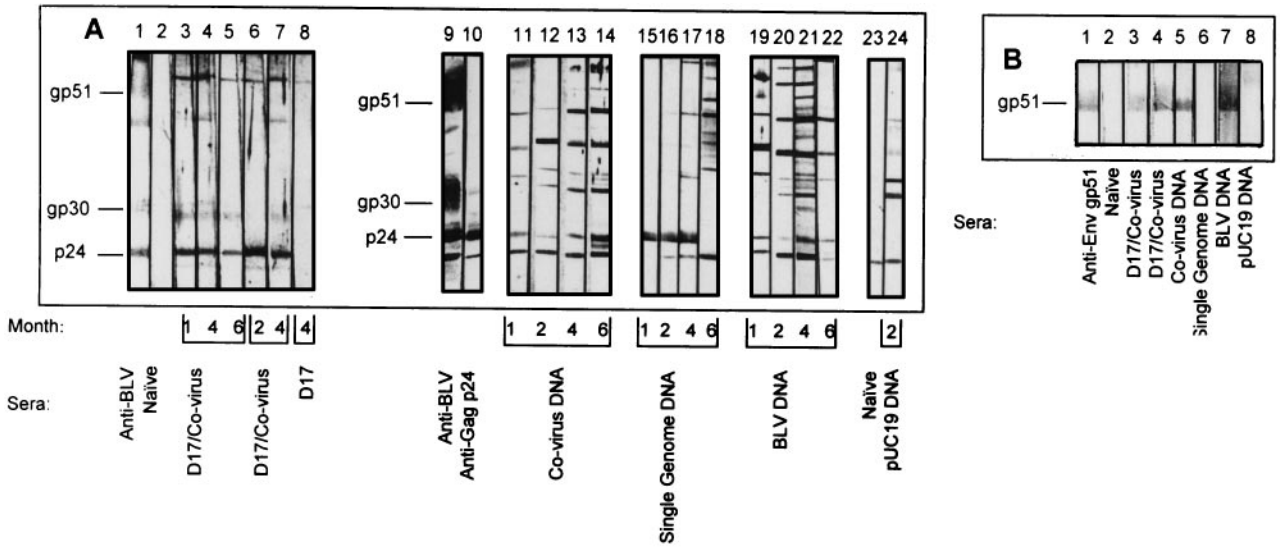

FIG. 3. Detection of antiviral antibodies in simple BLV-treated rats by Western blot analysis. Whole BLV proteins or gp51^{Env} was analyzed by electrophoresis, transferred to a membrane, incubated with sera from the experimental rats or with control serum, and reacted with corresponding alkaline phosphatase-conjugated antibodies and Western blue stabilizer. Positions of BLV-specific proteins are indicated on the left. (A) Representative data showing the time course appearance of p24^{Gag} antibodies in experimental rats. All sera were diluted 50-fold. Lane 1, polyclonal, polyspecific anti-BLV rabbit serum; lane 2, naive rabbit serum; lane 3, group 1 rat 342-2 1 month after inoculation; lane 4, rat 342-2 at 4 months; lane 5, rat 342-2 at 6 months; lane 6, group 1 rat 327-1 at 2 months; lane 7, rat 327-1 at 4 months; lane 8, group 2 rat 344-1 at 4 months; lane 9, polyclonal, polyspecific anti-BLV rabbit serum; lane 10, polyclonal, monospecific anti-p24^{Gag} serum; lane 11, group 3 rat 338-2 at 1 month; lane 12, rat 338-2 at 2 months; lane 13, rat 338-2 at 4 months; lane 14, rat 338-2 at 6 months; lane 15, group 4 rat 339-1 at 1 month; lane 16, rat 339-1 at 2 months; lane 17, rat 339-1 at 4 months; lane 18, rat 339-1 at 6 months; lane 19, group 5 rat 340-1 at 1 month; lane 20, rat 340-1 at 2 months; lane 21, rat 340-1 at 4 months; lane 22, rat 340-1 at 6 months; lane 23, preimmune serum; lane 24, group 6 rat at 2 months. (B) Representative data showing antibodies against gp51^{Env} 6 months after inoculation. Lane 1, monospecific anti-gp51^{Env} rabbit serum; lane 2, naive rabbit serum; lane 3, group 1 rat 342-2; lane 4, group 1 rat 327-1; lane 5, group 3 rat 338-1; lane 6, group 4 rat 339-1; lane 7, group 5 rat 340-1; lane 8, group 6 rat 341-1.

BLV-specific antibodies. A whole BLV preparation enriched in p24Gag or a gp51^{Env} preparation was fixed on membranes that were used for rat antibody detection. To confirm the specificity of viral bands detected, control membranes were incubated with rabbit polyclonal and polyspecific anti-BLV serum, polyclonal and monospecific anti-p24Gag serum, polyclonal and monospecific anti-gp51Env serum, or corresponding preimmune serum.

In each of the four group 1 rats (D17/covirus), antibodies against p24^{Gag} were detected 1, 2, 3, and 6 months postinoculation (Table 2; representative data are shown in Fig. 3A). Antibodies against gp51^{Env} were also detected when assayed at 6 months postinoculation (Table 2; representative data are shown in Fig. 3B). Antiviral antibodies were not detected in group 2 rats, consistent with a lack of BLV proteins in mock-treated controls. These results indicate that inoculation with D17 cells that produce simple BLV coviruses induces production of antibodies against BLV Gag and Env. Seroconversion is sustained for at least 6 months (the duration of the study), which is consistent with the sustained presence of antigen.

Similar antibody responses were observed for the group 3 rats injected with the complementary simple BLV genomes (Table 2; representative data are shown in Fig. 3). Each group 3 rat seroconverted, and p24^{Gag} and gp51^{Env} antibodies were sustained at 6 months postinoculation. These data indicate that introduction of simple BLV coviruses by virus-producing cells or by direct injection of proviral DNA results in antibody responses that are similar in magnitude.

Group 4 rats injected with the single hybrid genome displayed similar patterns of seroconversion to BLV Gag and Env. However, one of the rats (rat 339-1) failed to produce antibodies against Gag and Env at 6 months postinjection. Interestingly, this rat initially had the highest anti-Gag antibody titer in the group. The lack of sustained antibody production is consistent with elimination of the producer cell population or with the possibility that the immunological response resulted from transient expression from the injected DNA.

All rats inoculated with the heterologous simple BLV producer cells or proviral DNA, except rat 339-1, exhibited antibody titers similar to those of the group 5 rats inoculated with wild-type BLV DNA. As expected, sera from mock-treated rats (group 6) were consistently negative for anti-BLV antibody. In summary, these analyses indicate that (i) introduction of simple BLV producer cells or proviral DNA induces production of anti-BLV antibodies; (ii) injection of simple BLV proviral DNA leads to transcription and translation, and the proteins are immunogenic; (iii) production of antiviral antibody is sustained for 6 months (the duration of the study) in most of the virus-treated rats, consistent with sustained presence of antigen (antibody was no longer detected in one group 4 rat [339-1] at the 6-month time point); and (iv) anti-BLV antibody titers were similar among the virus-treated rats.

The simple BLV derivatives infect PBMCs. Induction of anti-BLV antibodies in response to the simple BLV genomes is consistent with virus gene expression and protein production. The virus proteins may assemble into virions and be released from the cell as infectious virus. The natural target for BLV is PBMCs (8, 13, 15, 18), and one indicator that infectious simple BLV is produced would be the presence of provirus sequences in PBMCs. The input D17 virus-producing cells are destroyed within weeks after injection because they are immunologically foreign to the host (3). We performed PCR on PBMC DNA prepared from the rats 6 months postinoculation to screen for provirus sequences. BLV *pol* sequences were detected in PBMCs from group 1 rats (inoculated with D17 covirus-producing cells) but not in those from group 2 rats (mock-treated con-

TABLE 3. Presence of BLV *pol* sequences in PBMCs of rats 6 months after inoculation with complementary virus-producing cells or various DNAs

Treatment group	Animal no.	Presence of BLV *pol*[a]
1 (covirus cells)	342-2	+
	326	NS[b]
	327-1	+
	327-3	+
2 (uninfected cells)	344-1	−
	344-2	NS
3 (covirus DNA)	338-1	NS
	338-2	+
	338-3	+
4 (single-genome DNA)	339-1	−
	339-2	+
	339-3	+
	339-4	+
5 (wild-type BLV DNA)	340-1	+
	340-2	+
6 (pUC19 DNA)	341-1	−
	341-2	−

[a] PBMC DNA was isolated and screened for BLV *pol* sequences by PCR and analyzed by Southern blotting as described in Materials and Methods.
[b] NS, sample not tested because harvest of PBMCs was not sufficient for PCR.

trols) (Table 3). These results indicate that the simple BLV covirus infected PBMCs and formed a provirus.

When addressing whether proviruses were formed in PBMCs from rats injected with the hybrid genomes, we considered the fate of injected DNA, which has been examined in mice (11). In mice, plasmid DNA (50 μg complexed with liposomes) in peripheral blood was rapidly degraded, with a half-life of less than 1 h. Quantification of plasmid levels by PCR in various tissues showed that by 6 months, the plasmid is not detectable in peripheral blood or in tissues, except for muscle (at a level of less than 0.012 copies/genome). Thus, although the DNA may be retained at low levels in muscle for up to 6 months, injected BLV DNA that reaches peripheral blood is degraded. Replicated BLV DNA integrated into PBMC DNA would be protected from degradation. We isolated PBMC DNA at 6 months postinoculation and screened for the unlikely presence of residual plasmid DNA in blood by PCR with primers complementary to pUC19 (the plasmid backbone). All samples were negative for pUC19 sequences (data not shown). In contrast, when we screened for BLV *pol* sequences, BLV provirus sequences were observed in the BLV control rats (group 5) but not in mock-treated controls (group 6) (Table 3). Furthermore, BLV sequences were detected in each group 3 rat (covirus DNA) and in three of four group 4 rats (single-genome DNA) (Table 3), consistent with virus infection and provirus formation. Provirus sequences were not detected in the group 4 rat that was negative in the Western blot assay at 6 months (rat 339-1). These results demonstrate that residual plasmid DNA is not detectable in PBMCs and that BLV provirus is not detectable in rat 339-1.

To obtain further evidence of authentic virus infection and replication, we performed RT-PCR to detect viral RNA in the PBMCs. PBMC RNAs were prepared from blood harvested by cardiac venipuncture of rats in group 4 (single-genome simple BLV DNA) and group 6 (pUC19 DNA) at 14 months after the last DNA inoculation. The RNAs were reverse transcribed

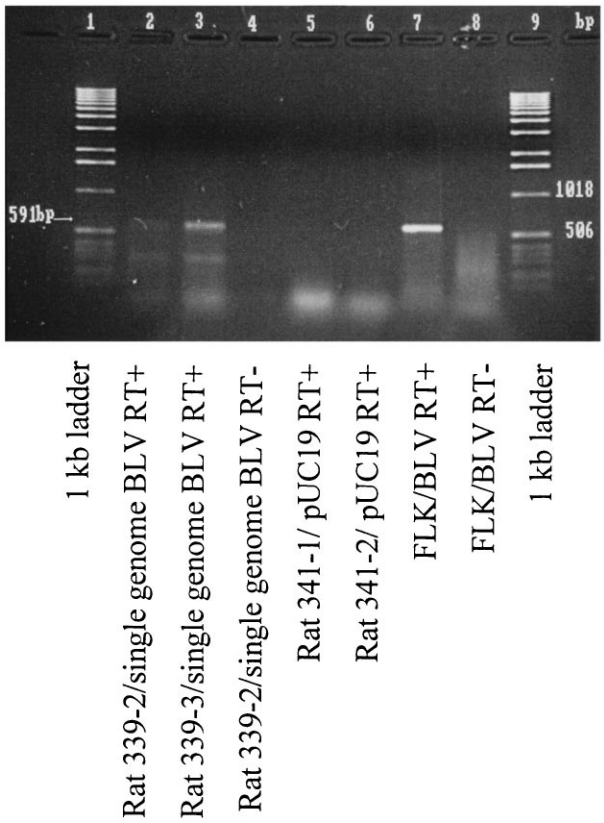

FIG. 4. RT-PCR analysis of viral RNA in PBMCs. PBMC RNA was prepared from blood samples harvested by cardiac venipuncture 14 months after the final inoculation and was treated with avian myeloblastosis virus RT. BLV-specific cDNAs were PCR amplified by thermostable Tf1 DNA polymerase with BLV *pol* primers (Access RT-PCR System; Promega). The size of the expected DNA product (591 bp) is indicated. Lanes 1 and 9, molecular size standards; lane 2, RNA of group 4 rat 339-2 (single-genome simple BLV); lane 3, RNA of group 4 rat 339-3 (single-genome simple BLV); lane 4, RNA of rat 339-2 without RT; lane 5, RNA of group 6 rat 341-1 (mock); lane 6, RNA of group 6 rat 341-2 (mock); lane 7, RNA of FLK/BLV cells; lane 8, RNA of FLK/BLV cells without RT.

with avian myeloblastosis virus RT, and BLV-specific cDNAs were amplified by PCR with thermostable Tf1 DNA polymerase and BLV *pol* primers (Access RT-PCR System; Promega). As shown in Fig. 4, viral RNA is detected in PBMCs of simple BLV-treated rats (lanes 2 and 3) but not in those of mock-treated rats (lanes 5 and 6). As expected, the length of the PCR product is equal to that of the BLV control RNA (Fig. 4, lane 7) (591 bp). A signal was not detected in the absence of RT (Fig. 4, lanes 4 and 8), which demonstrates that the positive RT-PCR signal was in response to template RNA, not DNA. These results indicate that simple BLV RNA is expressed 14 months after DNA inoculation. These results are consistent with authentic simple BLV infection of PBMCs and at least one cycle of virus replication.

DISCUSSION

The aims of this study were to construct a second-generation simple BLV genome that is replication competent, to test the immunogenicity of the simple BLV derivatives in a BLV animal model, and to assess the ability of these viruses to infect

the natural BLV target cell population, PBMCs. We accomplished the first aim by combining parts of previously described Tax- and Rex-independent complementary hybrid genomes to express BLV *gag-pol* and *env* from a single hybrid genome (Fig. 1). A poliovirus IRES was inserted adjacent to the *env* ATG to facilitate cap-independent translation of BLV *env*. IRESs have been used previously to construct selectable bicistronic retrovirus vectors in which selection for a downstream drug resistance marker guarantees expression of the upstream gene(s) (1, 5, 10, 14). In this study, the purpose of the IRES in pU5*gag-pol-env* was to drive translation of BLV *env* from a polycistronic genomic RNA. We found that pU5*gag-pol-env* produces virions that are replication competent upon two passages in D17 cells (Table 1). RNA and protein analyses of the infected cells confirmed directly that proviral RNA is expressed, the expected viral BLV proteins are produced, and progeny virions contain viral RNA (Fig. 2).

The second aim of this work was to determine if viral proteins produced from the simple BLV genomes induce antibodies against the major BLV antigenic determinants, Gag and Env. We compared two approaches for introduction of the virus into rats: injection of heterologous virus-producing cells and direct injection of proviral DNA. Previous reports have demonstrated BLV seroconversion in rats or sheep in response to introduction of BLV-producing cells and seroconversion in sheep upon direct injection with BLV proviral DNA (3, 17, 21). Our experiments confirm seroconversion in response to virus-producing cells and demonstrate seroconversion in rats injected with viral DNA (Table 2; Fig. 3). The titers of anti-BLV antibodies were similar in simple BLV- or BLV-treated rats (Table 2). Furthermore, these results indicate that BLV proteins are produced in vivo from simple BLV derivatives that lack Tax, Rex, RIII, and GIV open reading frames.

The third aim of this study was to assess the ability of the simple BLV derivatives to infect the natural BLV target cells, PBMCs. Retrovirus infection and replication result in the permanent integration of provirus DNA into the chromosome of the infected cell. We screened PBMC DNA for provirus sequences at 6 months posttreatment, since by 6 months, injected D17 virus-producing cells and injected DNA are eliminated from the circulation (3, 11). PBMCs from each rat treated with heterologous virus-producing cells and from several of the rats treated with viral DNA contained replicated BLV *pol* sequences, whereas those from mock-treated rats did not. The lack of BLV *pol* sequences in rat 339-1 (group 4 [single-genome DNA]) demonstrates that residual input DNA was absent (Table 3). Furthermore, viral RNA was expressed in PBMCs of simple BLV-treated, but not mock-treated, rats. These results are consistent with simple BLV infection of PBMCs and at least one cycle of replication.

This study used small groups of rats to establish the immunogenicity and infectivity of simple BLV derivatives that lack the regulatory genes, *tax* and *rex*, and the RIII and GIV open reading frames. These data validate simple BLV derivatives as useful reagents to study the roles of Tax, Rex, RIII, and GIV in BLV persistence and pathogenesis. The small test groups did not reveal differences in response among the simple BLV derivatives and BLV. Future studies with larger test groups will compare virus load and persistence for simple BLV derivatives and wild-type BLV. Evaluation of the simple BLV derivatives in BLV models with a disease endpoint (4, 6, 21, 22) will also contribute toward the evaluation of simple retroviruses as preventative vaccines.

ACKNOWLEDGMENTS

We thank Jeff Jones and Jiayou Zhang for comments on the manuscript, and we are grateful to D. Portetelle for rabbit polyclonal anti-BLV antiserum and to H. Chorvatova for help with animals.

This work was supported in part by a grant from the Slovak Academy of Sciences and by NIH grant RO3-TW00065-03.

REFERENCES

1. **Adams, M. A., N. Ramesh, A. D. Miller, and W. R. A. Osbourne.** 1991. Internal initiation of translation in retroviral vectors carrying picornavirus 5′ untranslated regions. J. Virol. **65:**4895–4990.
2. **Altaner, C., M. Merza, V. Altanerova, and B. Morein.** 1993. Envelope glycoprotein gp51 of bovine leukemia virus is differently glycosylated in cells of various species and organ origin. Vet. Immunol. Immunopathol. **36:**163–177.
3. **Altanerova, V., D. Portetelle, R. Kettmann, and C. Altaner.** 1989. Infection of rats with bovine leukaemia virus: establishment of a virus-producing rat cell line. J. Gen. Virol. **70:**929–932.
4. **Altanerova, V., J. Ban, and C. Altaner.** 1989. Induction of immune deficiency syndrome in rabbits by bovine leukemia virus. AIDS **3:**755–758.
5. **Boris-Lawrie, K., and H. M. Temin.** 1995. Genetically simpler bovine leukemia virus derivatives can replicate independently of *tax* and *rex*. J. Virol. **69:**1920–1924.

5a. **Boris-Lawrie, K., and H. M. Temin.** Unpublished data.

6. **Djilali, S., and A. L. Parodi.** 1989. The BLV-induced leukemia-lymphosarcoma complex in sheep. Vet. Immunol. Immunopathol. **22:**233–244.
7. **Hanafusa, H., T. Hanafusa, and H. Rubin.** 1963. The defectiveness of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **49:**572–580.
8. **Jensen, W. A., J. Rovnak, and G. L. Cockerell.** 1991. In vivo transcription of the bovine leukemia virus *tax/rex* region in normal and neoplastic lymphocytes of cattle and sheep. J. Virol. **65:**2484–2490.
9. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4:**1172–1174.
10. **Koo, H.-M., A. M. C. Brown, Y. Ron, and J. P. Dougherty.** 1991. Spleen necrosis virus, an avian retrovirus, can infect primate cells. J. Virol. **65:**4769–4776.
11. **Lew, D., S. E. Parker, T. Latimer, A. M. Abal, A. Kuwahara-Rundell, S. G. Doh, Z. Yang, D. Laface, S. H. Gromkowski, G. J. Nabel, M. Manthorpe, and J. Norman.** 1995. Cancer gene therapy using plasmid DNA: pharmacokinetic study of DNA following injection in mice. Hum. Gene Ther. **6:**553–564.
12. **Mansky, L. M., A. E. Krueger, and H. M. Temin.** 1995 The bovine leukemia virus encapsidation signal is discontinuous and extends in the 5′ end of the *gag* gene. J. Virol. **69:**3282–3289.
13. **Mirsky, M. L., C. A. Olmstead, Y. Da, and H. A. Lewin.** 1996. The prevelance of proviral bovine leukemia virus in peripheral blood mononuclear cells at two subclinical stages of infection. J. Virol. **70:**2178–2183.
14. **Morgan, R. A., L. Couture, O. Elroy-Stein, J. Ragheb, B. Moss, and W. F. Anderson.** 1992. Retroviral vectors containing putative internal ribosome entry sites: development of a polycistronic gene transfer system and applications to gene therapy. Nucleic Acids Res. **20:**1293–1299.
15. **Paul, P. S., K. A. Pomeroy, D. W. Johnson, C. C. Muscoplat, B. S. Handwerger, F. F. Soper, and D. K. Sorenson.** 1977. Evidence for the replication of bovine leukemia virus in the B lymphocytes. Am. J. Vet. Res. **38:**873–876.
16. **Pelletier, J., G. Kaplan, V. R. Racaniello, and N. Sonenberg.** 1988. Cap-independent translation of poliovirus mRNA is conferred by sequence elements within the 5′ noncoding region. Mol. Cell. Biol. **8:**1103–1112.
17. **Radke, K., D. Grossman, and L. C. Kidd.** 1990. Humoral immune response of experimentally infected sheep defines two early periods of bovine leukemia virus replication. Microb. Pathog. **9:**159–171.
18. **Radke, K., T. J. Sigala, and D. Grossman.** 1992. Transcription of bovine leukemia virus in peripheral blood cells obtained during early infection *in vivo*. Microb. Pathog. **12:**319–331.
19. **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:**677–681.
20. **Temin, H. M.** 1993. A proposal for a new approach to a preventive vaccine against human immunodeficiency virus type I. Proc. Natl. Acad. Sci. USA **9:**4419–4420.
21. **Willems, L., D. Portetelle, P. Kerkhofs, G. Chen, A. Burny, M. Mammerickx, and R. Kettmann.** 1992. In vivo transfection of bovine leukemia provirus into sheep. Virology **189:**775–777.
22. **Wyatt, C., D. Wingett, J. White, C. Buck, D. Knowles, R. Reeves, and R. Magnuson.** 1989. Persistent infection of rabbits with bovine leukemia virus associated with development of immune dysfunction. J. Virol. **63:**4498–4506.