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In Vivo Infection of Sheep by Bovine Leukemia Virus Mutants

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Direct inoculation of a cloned bovine leukemia virus (BLV) provirus into sheep has allowed study of the viral infectivity of genetic mutants in vivo. Three BLV variants cloned from BLV-induced tumors and 12 in vitro-modified proviruses were isolated and analyzed for viral expression in cell culture. The proviruses were then inoculated into sheep in order to assess viral infectivity in vivo. Of three variants cloned from BLV-induced tumors (344, 395, and 1345), one (344) was found infectious in vivo. This particular provirus was used to engineer 12 BLV mutants. A hybrid between the 5′ region of the complete but noninfectious provirus 395 and the 3′ end of mutant 344 was infectious in vivo, suggesting that the *tax/rex* sequences were altered in virus 395. As expected, several regions of the BLV genome appeared to be essential for viral infection: the protease, *pol*, and *env* genes. Even discrete modifications in the fusion peptide located at the NH_2 end of the transmembrane gp30 glycoprotein destroyed the infectious potential. In contrast, mutations and deletions in the X3 region present between the *env* gene and the 3′ *tax/rex* region did not interfere with viral infection in vivo. This region of unknown function could thus be used to introduce foreign sequences. A BLV recombinant carrying a ribozyme directed against the *tax/rex* sequences was still infectious in vivo. Cotransfection of two noninfectious mutants carrying deletions led to infection in two of four independent injections, the infectious virus being then a recombinant between the two deletants. The experimental approach described here should help to gain insight into essential mechanisms such as in vivo viral replication, cooperation between deletants for viral infectivity, and viral superinfections. The gene products in the X3 and X4 region which are dispensable for in vivo infection could be involved in leukemogenesis, and thus proviruses deleted in these sequences could constitute the basis for a live attenuated vaccine.

Bovine leukemia virus (BLV) has provided a useful virus-animal model for the human T-cell leukemia virus (HTLV) types I and II (3). These viruses belong to the *Oncovirinae* subfamily, members of which are characterized by similar genetic organization and pathologies. They contain, in addition to the *gag*, *pol*, and *env* genes, an X region involved in the regulation of viral replication. At least two proteins are encoded by these sequences: $p18^{rex}$, involved in the post-transcriptional regulation of viral RNAs (6), and $p34^{tax}$, a transactivator of long terminal repeat (LTR)-directed gene expression that acts as an immortalizing oncogene in vitro (5, 13, 14, 19, 36, 37, 38). In addition, the intermediate sequences between the *env* gene and the *tax/rex* region contain several open reading frames of unknown functions. Transcripts corresponding to the BLV X3 reading frame were identified (1, 4). Similarly, HTLV exhibits a complex splicing pattern corresponding to the homologous intermediate region (2, 4, 18). However, no function has yet been attributed to the putative translated proteins. The genomic structure of members of the *Oncovirinae* is more complex than previously thought. In the *Lentivirinae* subfamily, multiply spliced mRNA species were also characterized (31). In addition to the standard *gag*, *pol*, and *env* genes, the lentiviruses possess a number of auxiliary genes not found in other retroviruses. These auxiliary genes include essential (*tat*, and *rev*), intermediate (*vif*), and nonessential (*nef*, *vpr*, *vpu*, and *vpx*) factors, as judged from their dispensability for replication in cell culture (8). However, the *nef* gene is essential in vivo for the maintenance of high virus loads and for the development of AIDS (15). The other nonessential genes could also be required for potential for infection and pathogenicity in vivo. When rhesus monkeys were infected with a cloned simian immunodeficiency virus provirus carrying a premature stop signal in the *nef* gene, revertants with a sense codon at this position quickly and universally came to predominate in the infected animals (15). Although deletion of the *nef* sequences had no detectable effect on virus replication in cultured cells, this modification dramatically altered the properties of the virus in infected monkeys. In addition to production of replication-competent viruses, chronic infection of a number of permanent T-cell and monocytic cell lines with different human immunodeficiency virus (HIV) isolates gave rise to cells harboring defective viral genomes (10, 11). Defective HIV-1 proviruses rapidly recombine in cell culture to produce a recombinant virus that is infectious (29). In vivo, considering the high rates of genetic variation of the HIV-1, it has become necessary to describe isolates in terms of populations of closely related genomes, referred to as quasispecies (22). High frequencies of functionally defective *tat* genes were identified. Defective mutants could act as dominant-negative repressors as observed for HIV *gag* (32) or recombine to generate infectious clones. Defective proviruses also exert a crucial role in an acute erythroleukemia induced by the Friend spleen focus-forming virus system; mutants of the membrane glycopro-

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tein (gp55) are the leukemogenic agents of Friend spleen focus-forming virus (35). Several integrated BLV proviruses harbor deletions, but the 3′ half is always present (15). A provirus deleted between the sequences coding for $p24^{gag}$ and $gp51^{env}$ was isolated from a tumor (cow 1345) (33). This virus was present at a single copy in the tumor and was unable to synthesize most of the viral proteins, including reverse transcriptase and the *tax/rex* regulatory proteins. The role of this virus in tumor induction or maintenance of the transformed state remains unclear.

In this context, direct inoculation of BLV deletion mutants is a useful tool for analysis of viral replication in vivo and identification of genes involved in infection and pathogenesis. Susceptibility to BLV infection was studied in vitro. Derse and Martarano (7) constructed a recombinant BLV in which the X region was replaced by the neomycin resistance gene controlled by the simian virus 40 early promoter. This virus was able to infect cell lines of human, bovine, canine, feline, and murine origin. BLV-producing cell lines were resistant to superinfection with the recombinant. An alternative approach was used by Milan and Nicolas (23). They used recombinant proviruses in which the *gag*, *pol*, *env*, and X regions were replaced by a *lacZ* gene under the control of the BLV long terminal repeat or the simian virus 40 early promoter. The recombinants were transfected into the ovine cell line FLK-BLV, which expresses all BLV proteins from a wild-type provirus. The supernatants were used to infect cell lines of various origin (cat, sheep, rat, goat, cow, rabbit, pig, monkey, human, mouse, and chicken). Determination of the host range of the BLV vectors established that a variety of species and cell types can be infected by BLV in vitro.

In vivo infection is more restricted with respect to the target cell. The infected cell as well as the tumor clone appear to belong to the B-cell lineage. Consequently, in addition to factors involved in cell surface recognition, integration, and expression, factors involved in cell specificity play a role in vivo. The recombinant viruses carrying the neomycin resistance or *lacZ* gene are defective for replication and require transcomplementation by viral proteins. Since these viruses are suicide vectors, they allow only one round of replication and cannot be used to establish a permanent infection in vivo. In an attempt to identify genes dispensable for viral infectivity, BLV mutants were constructed and their behavior in vivo was directly analyzed in sheep.

MATERIALS AND METHODS

Plasmids. Cloning of proviruses 344, 395, and 1345 was previously described (33). Plasmid pBLV344H was obtained after excision from pBLV344 of flanking 3′ cellular sequences (1-kb *Hin*dIII-*Hin*dIII fragment). This construct is infectious in vivo (41). To obtain pBLVIPR containing a stop codon in the protease gene, the oligonucleotide 5′-CTAG GCTAGAATTCTAGCCTAGGTAC-3′ was annealed and cloned into the *Kpn*I site (position 2111 [26, 27]) of pBLV344. The provirus deleted in the *pol* gene was obtained by removal of an *Xho*I-*Xho*I (positions 3276 and 4347) fragment from pBLV344. Plasmid pBLVDENV contains an *Nco*I-*Bgl*II adaptor inserted into the corresponding sites at positions 4925 and 6101, resulting in a deletion of most of the *env* sequences. The two recombinant proviruses harboring mutated fusion peptide sequences resulted from the replacement of the internal *Nco*I-*Bgl*II (positions 4925 and 6101) fragment by the corresponding mutated fragments excised from plasmids pSC11M1 and pSC11M5, respectively (34). Plasmid pBLV344H was digested with *Xba*I, and a linker harboring a stop codon (oligonucleotide 5′-CTAGATAG GCTAGAATTCTAGCCTAT-3′) was introduced to generate pBLVIX. To construct pBLVDX, two oligonucleotides (5′-CTAGAAAGCTTG-3′ and 5′-GATCCAAGCTTT-3′) were cloned into the *Xba*I and *Bam*HI sites (positions 6614 and 6997) of plasmid pBLV344H. A cDNA corresponding to the X3 frame (39) was digested by *Xba*I and *Bam*HI, and the 383-bp fragment containing the X3 sequences was introduced between the corresponding sites of plasmid pBLV344H to generate pBLVX3C. This provirus thus contains the complete X3 frame from the FLK-BLV variant (30). Construction of the pBLVRZ vector carrying a *tax*-directed ribozyme is described elsewhere (43). The two minimal proviruses pLTRtaxLTR and pLTRrexLTR contain the *tax* and *rex* sequences isolated from the *tax/rex* cDNA and cloned between two BLV LTRs (4a). Finally, pBLV344/395 is a hybrid containing most of the pBLV395 sequences (5′ LTR *gag pol env*) and the 3′ end from plasmid pBLV344 (from the *Xba*I site at position 6614 to the *Hin*dIII site in the flanking cellular DNA).

Transfections, CAT assays, and p24 ELISA. Canine osteosarcoma cells (D17) were cultivated and transfected as previously described (40). In short, 3×10^5 cells were kept in Opti-MEM medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum. Plasmid DNA was precipitated by the calcium phosphate method and added to the cells. After 4 h, the cells were washed and cultivated for 2 days. Half of the D17 cells were then harvested, washed with phosphate-buffered saline, and lysed by three freeze-thaw cycles. After centrifugation, chloramphenicol acetyltransferase (CAT) activity was determined from the supernatants. The other half of the transfected cells were cultivated for 5 days. The p24 major *gag* antigen was titrated from the cells or in the supernatants (previously precipitated by ammonium sulfate overnight at 4°C), using an enzyme-linked immunosorbent assay (ELISA) procedure as previously described (24, 25).

In vivo transfection of sheep. The recombinant plasmid DNAs were transfected into sheep as previously described (41). In brief, sheep were injected intradermally with 100 μg of plasmid DNA mixed with 200 μg of DOTAP [*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methylsulfate; Boehringer] in 1 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (20 mM HEPES, 150 mM NaCl [pH 7.4]). The sheep were maintained under controlled conditions at the National Institute for Veterinary Research (Uccle, Belgium). Sera were collected weekly and monitored for the presence of anti-gp51 antibodies (24, 25).

PCR analysis. Blood samples (500-μl aliquots) were mixed with an equal volume of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100). After 20 s of centrifugation, the pellets were resuspended in 1 ml of lysis buffer. This step was repeated twice. The samples were then resuspended in 100 μl of polymerase chain reaction (PCR) buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 9], 0.1% Triton X-100) and incubated with 1 μl of proteinase K (5 mg/ml) for 1 h at 50°C. The digestions were stopped by boiling the sample for 5 min. Forty-microliter aliquots were amplified by PCR in the presence of 0.2 mM each deoxynucleoside triphosphate, 200 ng of primers, and 4 U of *Taq* DNA polymerase (Promega). The oligonucleotides used were A (position 4560 [26, 27]; 5′-TCCTGGCTACT AACCCCCCCGT-3′), B (position 5549; 5′-GCTAGGCC TAAGGTCAGGGCTGC-3′), C (position 4310; 5′-CCTA

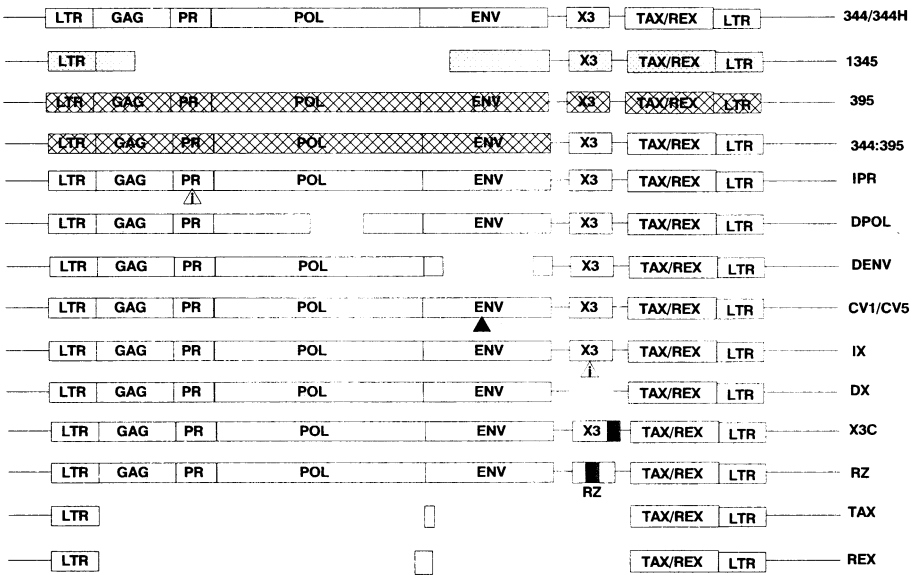


FIG. 1. Schematic representation of BLV proviruses.

GACGAACCCCACCTTCCC-3'), D (position 5614; 5'-AG GTGAGTCTCTGATGGCTAA-3'), E (position 6450; 5'-TG GAAAGAACTAACGCTG-3'), and F (position 7194; 5'-GG GTCTCGCCGGTGAGCG-3'). The reaction mixtures were overlaid with 2 drops of mineral oil, denatured for 5 min at 94°C, and amplified by 36 cycles (30 s at 94°C, 1 min at 55°C, and 2 min at 74°C). After PCR, the samples were analyzed by Southern blot hybridization using a BLV probe (*Sac*I insert from plasmid pBLV344).

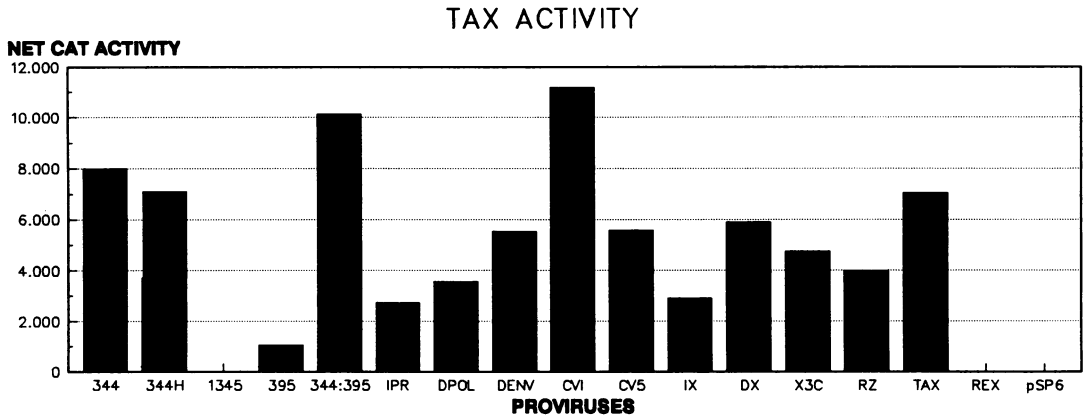
RESULTS

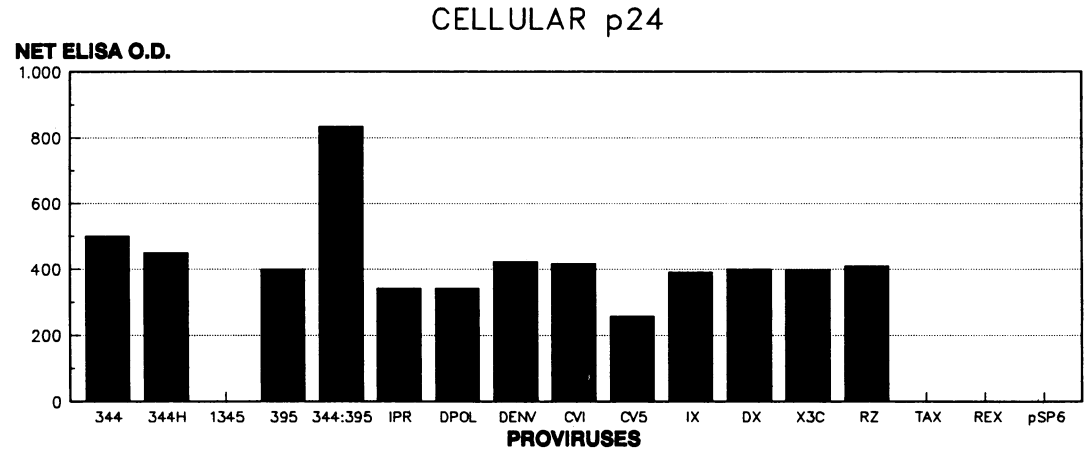
Construction of BLV mutants and viral gene expression in cell culture. Three proviruses isolated from BLV-induced tumors were previously cloned (33). Plasmid pBLV344 (previously referred as pV344) contains a complete proviral copy isolated from a sheep tumor and flanked by cellular sequences (Fig. 1). Five micrograms of this DNA was transfected in 3×10^5 D17 cells together with the pLTRCAT reporter, which contains the BLV LTR cloned upstream to the CAT gene (40). Two days posttransfection, half of the transfected cells were harvested and the CAT activity was determined from the lysates. The other half of the transfected cells were cultivated for another 3 days. The p24 major *gag* antigen was titrated from the cells or in the supernatants. The pBLV344 provirus was able to express the *tax* transactivator and the p24 *gag* protein as detected in the cells and in the supernatants (Fig. 2). Similar activities were obtained with plasmids pBLV344H (which contains the pBLV344 insert deleted in its 3' flanking cellular sequences) and pBLV395 (which contains a provirus cloned from the 395 sheep tumor). In contrast, the pBLV1345 provirus, which harbors a large deletion between the sequences coding for the $p24^{gag}$ and the $gp51^{env}$ (Fig. 1), was unable to synthesize the p24 and *tax* proteins after transfection in cell culture (Fig. 2). As controls for assay specificity, three DNAs were transfected and analyzed in parallel: pSP6 (plasmid without insert), pLTRtaxLTR, and pLTRrexLTR, the latter two of which are minimal proviruses coding for *tax* and *rex*, respectively (Fig. 1). Only pLTRtaxLTR induced detectable levels of transactivator expression after transfection into cells (Fig. 2).

BLV variants were cloned from BLV-induced tumors from sheep M344 and M395. However, these clones are closely related because they originate from the same donor cow infected with the LB285 variant (42). Their in vitro transactivating activities, however, were significantly different; the transactivation capacity of virus 344 was about 10-fold higher than that of virus 395 (Fig. 2). To investigate the role of the 3' proviral sequences, a hybrid was made between variants 344 and 395. The recombinant plasmid pBLV344:395 contains most of the provirus 395 sequences (cellular sequences and 5' LTR *gag pol env*) fused to the 3'-end sequences from provirus 344 (*tax/rex*-3' LTR and cellular sequences). In transient transfection experiments, this hybrid exhibited a transactivation potential similar to that of the parental pBLV344 (Fig. 2), indicating that the activity was restored following the replacement by the 344 3'-end region.

In an attempt to screen for regions in the BLV provirus dispensable for viral infectivity, a series of nine mutants was constructed as described in Materials and Methods and schematically represented in Fig. 1. Linkers containing translation stop codons were inserted in the protease (pBLVIPR) and in the X3 frame present between the *env* gene and the *tax/rex* region (pBLVIX). Large regions were deleted in the *pol*, *env*, and X3 frames (constructs pBLVD POL, pBLVDENV, and pBLVDX, respectively). The pBLVRZ vector carries a ribozyme of the hammerhead type directed to *tax* and cloned into the X3 frame (43).

Two mutations were performed in the sequences corresponding to the fusion peptide located at the NH_2 part of the transmembrane glycoprotein gp30. This wild-type peptide adopts an oblique configuration responsible for lipid bilayer destabilization leading to cell fusion (34). These sequences were modified to obtain a parallel orientation (pBLVCV1) of the BLV fusion peptide or an oblique insertion with a fusion peptide from simian immunodeficiency virus (pBLVCV5). It has been shown that the CV1 peptide does not allow efficient





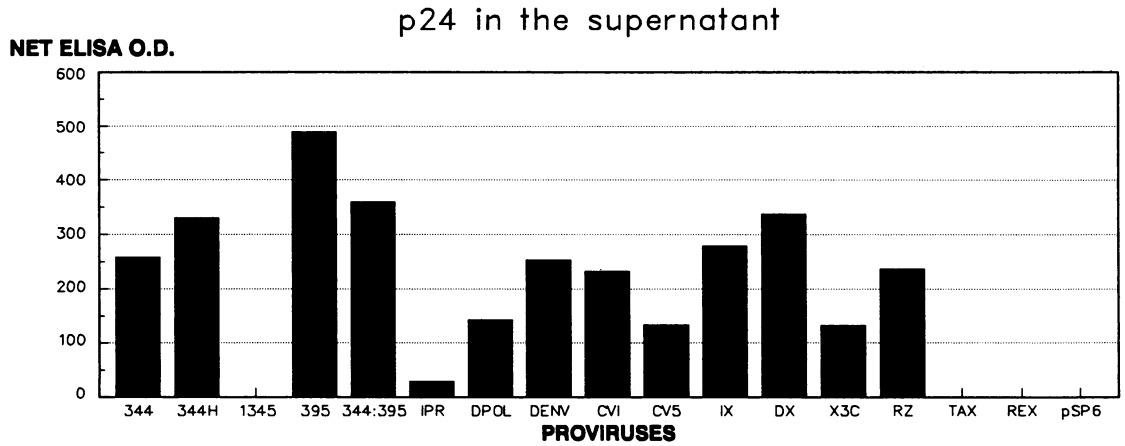


FIG. 2. Functional analysis of BLV proviruses in cell culture. Canine osteosarcoma cells (D17) were transfected with the different proviruses by the calcium phosphate method. Two days posttransfection, half of the cells were harvested and CAT activity was determined. The other half of the transfected cells were cultivated for another 3 days. The major $p24^{gag}$ antigen was titrated from the cells or in the supernatants (previously precipitated by ammonium sulfate overnight at 4°C) by an ELISA procedure (25). The data are from three independent experiments (with a maximum of 10% variation between experiments).

TABLE 1. In vivo transfection of modified proviruses

pBLV provirus	No. of seroconversions (anti-gp51)/no. of transfections
344/344H	6/6
1345	0/2
395	0/3
344:395	2/2
IPR	0/2
DPOL	0/2
DENV	0/2
CV1	0/3
CV5	0/3
IX	3/5
DX	3/5
X3C	5/5
RZ	2/2
CV1 + IPR	0/1
CV5 + IPR	0/1
DENV + DPOL	2/2

fusion (15% of wild-type levels), in contrast to CV5, which behaves normally in the in vitro syncytium assay (34).

The open reading frames of the published BLV variants (26, 27, 30) are well conserved with the exception of the X3 region intermediate between the *env* and the *tax/rex* genes. The sizes of the X3 open reading frames vary among the various isolates; viruses 344 and 395 have a termination codon at position 6768 (according to the numbering of Rice et al. [26, 27]) as observed in the Belgian variant, variant 1345 has a truncated X3 frame (ending at position 6849), and the American variant contains the longest X3 sequence, with a translation stop codon at position 6894 (our unpublished data). The provirus of pBLVX3C is isogenic to pBLV344 (LB285 variant closely related to the Belgian virus [42]) but contains the largest X3 frame from the FLK-BLV American variant (30).

All of these mutant proviruses were analyzed for viral gene expression in cell culture (Fig. 2). It appeared that in transient expression assays, all of them were able to synthesize the *tax* transactivator and the p24 major capsid protein, these products being detected in the cells lysates and in the supernatants.

In vivo transfection of the mutants in sheep. To analyze the infectivity of the different mutants in vivo, sheep were injected intradermally with 100 μg of plasmid DNA mixed with 200 μg of cationic liposome (DOTAP). The sheep were maintained under controlled conditions at the Veterinary Institute (Uccle, Belgium). Sera were collected weekly and monitored for the presence of anti-gp51 antibodies (24, 25). Table 1 shows the number of injected sheep versus the number of animals that had seroconverted. It appeared that of the three viruses (344, 395, and 1345) cloned ex vivo, only variant 344 is infectious. Since variant 1345 is deleted in most of the sequences required for structural gene expression, it was expected that this virus would not be able to replicate in sheep. Virus 395 appeared wild type according to the data obtained from transient cell culture assays except for a lower transactivating potential (Fig. 2). The lack of infectious potential of this provirus could therefore result from an altered *tax* gene. When hybrid pBLV344:395 was transfected, infection occurred, indicating that the 3′ end of provirus 344 can correct the defect of variant 395 for infectivity. The 3′ ends of proviruses 344 and 395 (from the *Xba*I site at position 6614 [26, 27] to the 3′ LTR) were entirely sequenced and compared. Five point mutations were present: two in the X3 frame (positions 6755 and 6798), two in the *tax* gene (positions 7719 and 7944), and one in the LTR (position 8313 [26, 27]). The nucleotide sequence modifications in *tax* corresponded to an amino acid change of a glutamine to a lysine in virus 395.

In vivo injection of mutants pBLVIPR, pBLVDPOL, and pBLVDENV demonstrated that the protease, *pol*, and *env* genes are absolutely required for viral infection (Table 1). Discrete modifications in the fusion peptide sequences (pBLVCV1 and pBLVCV5) eliminated the virus infectivity potential. This finding indicates that syncytium formation is a crucial step in vivo. Since it has been reported that the CV1 peptide is not able to induce syncytia in vitro, it is therefore not surprising that the virus is also inactive in vivo. However, the CV5 peptide with an oblique insertion in the lipid bilayer induced cell fusion in vitro (34). Surprisingly, provirus pBLVCV5 was unable to infect sheep. This result highlights the importance of the amino acid composition of the fusion peptide in addition to its general structural conformation.

In contrast, the introduction of a translational stop codon in the X3 region (provirus pBLVIX) did not prevent the virus from infecting sheep in vivo (Table 1). Even a deletion of 383 bp removing most of the X3 frame did not destroy infectivity (plasmid pBLVDX; rate of infection, 3/5; Table 1). This is in sharp contrast with the low variation tolerance in the *env* fusion peptide sequences. Swapping of the X3 frame of variant 344 with the complete corresponding region of the American variant (pBLVX3C) also generated an in vivo infectious virus (rate of infection, 5/5).

It should be mentioned that the transfection efficiency rate (number of seropositive animals/number of transfections) is below 1 for proviruses pBLVIX and pBLVDX. The modification of the intermediate X3 sequences could thus interfere with the infectious potential. However, since most of the sheep transfected with the proviruses mutated in the X3 region became infected, we can conclude that these sequences are dispensable for virus infectivity in vivo. This region can thus be used as a cloning site for exogenous sequences such as ribozymes (pBLVRZ). Despite efficient cleavage in vitro (43), this recombinant provirus remained infectious in sheep (Table 1).

In vivo, many BLV proviruses harbor deletions, but the 3′ end is always present (16, 17). The role of these defective proviruses is unknown. The transfection methodology allows the study of a possible in vivo cooperation process. Could two deletants unable to grow separately in vivo cooperate for the establishment of an infection? Four sheep were coinjected with different combinations of defective mutant proviruses: pBLVCV1 and pBLVIPR (in sheep M237); pBLVCV5 and pBLVIPR (in sheep M240); and pBLVDENV and pBLVDPOL (in sheep M238 and M241). Infection succeeded in two sheep (M238 and M241) in which anti-gp51 antibodies were detected (Table 1).

PCR amplifications of viral sequences. BLV is able to infect cattle and sheep, in which it induces a lymphoproliferative disease, and rabbits, in which it causes immunodeficiency (3, 20, 44). Natural transmission between animals is observed only in cattle, although the main infections are due to iatrogenic manipulations. The absence of natural transmission between sheep is a great advantage in our study. To ensure that the infecting virus corresponds to the transfected mutant, PCR amplifications were performed.

DNAs extracted from blood samples (500-μl aliquots) were amplified by PCR and analyzed by Southern blot

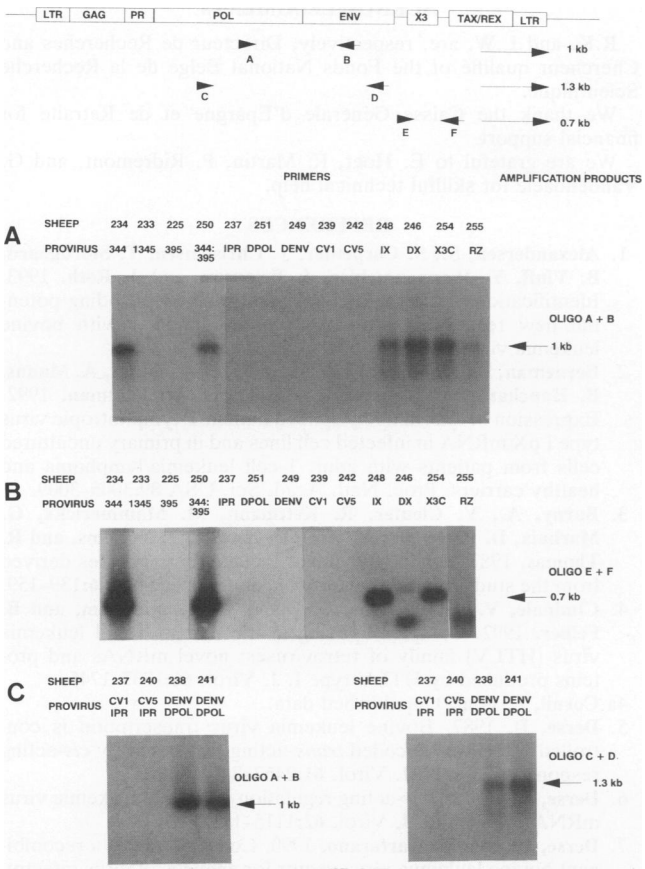


FIG. 3. PCR amplifications of BLV sequences in blood samples from infected sheep. DNA was extracted from 500-μl aliquots of blood samples and amplified by PCR using oligonucleotides A to F (for sequences, see Materials and Methods). After PCR, the samples were analyzed by Southern blot hybridization using a BLV probe.

hybridization using a BLV-specific probe. Amplification of the *pol/env* sequences was performed with primers A and B (Fig. 3A). Amplification products were identified in sheep transfected with provirus 344, hybrid 344:395, and X3 mutants pBLVIX, pBLVDX, pBLVX3C, and pBLVRZ. These data confirm the positive serological analysis. No fragments were amplified when no anti-gp51 antibodies were detected (proviruses 1345, 395, pBLVIPR, pBLVDPOL, pBLVDENV, pBLVCV1, and pBLVCV5) (Fig. 3A). Oligonucleotides E and F surround the X3 sequences (Fig. 3). When these primers were used for PCR, specific fragments (0.7 kb) were identified in sheep transfected with provirus 344, hybrid 344:395, and X3 mutants pBLVIX and pBLVX3C (Fig. 3B). However, a smaller fragment of 0.4 kb was amplified in samples corresponding to proviruses pBLVDX and pBLVRZ (Fig. 3B). This difference of 0.3 kb corresponds to the deletion of the X3 sequences. Nucleotide sequence analysis confirmed that these fragments indeed contained the mutated X3 genes (data not shown).

In the cooperation experiments with two deletion mutants, the *pol/env* sequences could be amplified with primers A and B from leukocyte DNA of sheep 241 (Fig. 3C). Using primers located in the deleted fragments (C in the *pol* sequences excised from provirus pBLVDPOL and D in the *env* gene absent in provirus pBLVDENV), a fragment of 1.3 kb was amplified, demonstrating that the two deleted proviruses recombined in sheep 241. The pBLVDPOL and pBLVDENV parental proviruses could not be amplified in this sheep (by using primers A and F, for example) (data not shown). Similar data were obtained for sheep 238. It thus appeared that the infecting virus in sheep 241 results from a recombination event between the parental viruses which, separately, were unable to infect the animal.

DISCUSSION

Because of the lack of a propagation method for BLV in normal B-lymphocyte cultures (absence of selection procedure and/or bovine B-cell specific growth factors), it has been difficult to analyze the mechanisms involved in regulation of infectivity and replication in vitro. Expression of in vivo-transfected BLV sequences provided a powerful tool to determine sequences required for infection. The X3 frame present between the *env* gene and the *tax/rex* region appeared to be dispensable for in vivo infection. If the delay for seroconversion is not significantly different among the mutants tested (between 3 to 10 weeks), their transmission efficiency appeared to be affected in our transfection protocol. The X3 mutants pBLVIX and pBLVDX remained in the host for at least 10 months. The anti-gp51 antibody titers rose gradually, and the virus was expressed in these animals (as demonstrated by reverse transcription-PCR; data not shown). The infection thus appeared to be permanent and indistinguishable from a wild-type infection. Being dispensable for infection, the X3 sequences can thus be deleted and replaced by exogenous genes such as ribozymes (28). This region could also be used to insert marker genes like the *lacZ* or β-glucuronidase gene to allow direct analysis of the target cell in vivo. Alternatively, a recombinant provirus carrying a neomycin resistance gene should allow, after ex vivo biopsies, to select for infected cells in vitro. An other possibility could be the introduction of a thymidine kinase gene into a replication-competent BLV mutant to provide an evaluation method for a curative protocol. Treatment of a sheep transfected with this recombinant with ganciclovir should kill the infected cells. Alternatively, the diphtheria toxin gene could provide a tool for constructing a suicide provirus able to infect the animal and induce an immune response before killing the target cell. The experiments described here thus open new routes for the study of the BLV target cell in vivo and the principle of a curative protocol in our experimental model system.

In light of the experiments performed with hybrid pBLV344:395, it appeared that the transactivation potential could be crucial in the establishment of the infection. Our assays show that the lack of infection of variant pBLV395 can be complemented by 3′ sequences of an infectious provirus (pBLV344). Among the five differences observed between the two viruses in the 3′-end fragments, the two mutations in the X3 region do not appear to be essential since this region can be deleted without affecting in vivo infection. In contrast, the two amino acid changes (*tax* mutations from glutamine to lysine) appear crucial to the transactivation capacity and the in vivo infectious potential. We cannot, however, exclude the possibility that the 3′-end cellular flanking sequences (which are different in plasmids pBLV344 and pBLV395) or the mutation in the 3′ LTR of the provirus (position 8313 [26, 27]) plays a role in the infectious potential observed in vivo. The defect of provirus 395 could be due to mutations that occurred during the ex vivo cloning procedure. Alternatively, the provirus was defective in vivo like variant 1345. Since these proviruses were present in

single copy in the tumors, it seems reasonable to hypothesize that they were associated with the induction of leukemogenesis. After pushing the target cell to a crucial step leading to irreversible transformation, viral replication is no longer required and the virus can become defective. Since the animal has a strong immune response directed toward the virus, this nonexpression is favorable to the growth of transformed cells. The inhibition of expression could occur by deletion (provirus 1345), by decrease of the transactivation potential (provirus 395), or by repression mechanisms in the case of replication-competent proviruses viruses (provirus 344).

Many tumors harbor proviruses with deletions in vivo (17). Are such proviruses still functional? In light of our experiments, it appears that two proviruses with deletions are unable to cooperate to establish infection unless they recombine to generate a replication-competent virus. Hu and Temin (12) have recently shown that as many as 30 to 40% of dimeric viral genomes recombine during a single cycle of infection. However, the copy number of BLV proviruses in infected peripheral blood mononuclear cells being low, functional complementation of defective genomes would not be a frequent event. Therefore, complementation is unlikely to be important in BLV pathogenesis. However, it remains possible that some deleted proviruses can express specific tumor functions if they are complemented in *trans*, for instance by extracellular *tax*. In the HIV and HTLV systems, soluble transactivator proteins are able to stimulate specific target cells when provided extracellularly (9, 21). It is possible that a similar mechanism exists in the BLV system. Alternatively, the deleted viruses possess the transforming potential but require in vivo complementation by a replication-competent virus, as observed for the acute erythroleukemia induced by the Friend spleen focus-forming virus complex (35). We performed cotransfection trials using the defective 1345 and wild-type 344 proviruses (data not shown). Three months posttransfection, we were unable to amplify the 1345 sequences, indicating that the defective virus did not remain in the host. A transcomplementation in the BLV system seems thus unlikely.

When a sheep becomes infected by BLV, it develops a strong immune response directed toward the viral structural proteins (24, 25). No case of BLV infection without serologic reaction has been reported so far. The presence of high titers of neutralizing antibodies together with the cellular immunity should prevent superinfection by another BLV virus. Vaccination trials using inactivated virus or recombinant vaccinia viruses expressing BLV structural proteins showed that infection can be prevented (24). Furthermore, passive immunization by colostrum is able to protect calves from infection. The use of identifiable mutants allows evaluation of the superinfection of a BLV-infected sheep by another mutant. Our preliminary results suggest that the superinfection of an inoculated sheep by another variant is not possible. These data support a vaccination strategy based on the use of proviruses deleted in genes essential for tumorigenesis but not for replication. The X3 and X4 frames being dispensable for in vivo infection, it remains to be established whether this region is involved in leukemogenesis. Our experimental injection protocol not only allows infection but can also be useful to study cellular transformation. At present, two sheep transfected with provirus 344 have died from leukemia. The sheep inoculated with the X3 and X4 mutants will be analyzed during tumorigenesis if it occurs. If these sequences are essential, our mutants could constitute the basis for a live attenuated vaccine.

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