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Attenuation of bovine leukemia virus by deletion of R3 and G4 open reading frames

(leukemogenesis/retrovirus/attenuated vaccine)

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ABSTRACT **Complex oncoviruses contain, in addition to the classical retroviral genes (*gag*, *pol*, and *env*), a region (X) located between the envelope sequences and the 3′ long terminal repeat. The X region contains two genes, *tax* and *rex*, whose protein products are involved in transcriptional and posttranscriptional regulation of viral expression. In addition to these activators, the bovine leukemia virus (BLV) and the human T-cell leukemia virus (HTLV) contain alternative open reading frames (R3 and G4 for BLV; p30, p13, and p12 for HTLV). As a virus/animal model for HTLV-induced leukemogenesis, BLV provirus can be injected intradermally into sheep, where it induced B-lymphocyte transformation. Deletion of the R3 and G4 sequences from an infectious and tumorigenic BLV provirus greatly impaired the *in vivo* propagation of the viruses as demonstrated by DNA polymerase chain reaction, RNA blots, structural-protein ELISA, and immunofluorescence analysis. Our results show that the alternative open reading frames are required for maintaining high virus loads during the course of persistent infection *in vivo*. Thus, R3 and G4 are candidates for antiviral drug development. Furthermore, viruses with a deletion in these sequences should be tested as live attenuated vaccines.**

Transactivating oncoviruses are retroviruses that induce leukemia/lymphoma after a long latency period in humans, cattle, and sheep. They share with the other retroviruses the classical genes *gag*, *pol*, and *env* required for virus particle synthesis, reverse transcription, and integration. In addition to the structural genes, the transactivating retroviruses harbor a number of regulator genes. The *tax* gene product increases the amount of viral RNA via enhancers present in the 5′ long terminal repeat (LTR) (reviewed in ref. 1). In addition to the polycistronic doubly spliced mRNA that encodes the Tax and Rex proteins, other low-abundance mRNAs transcribed from alternative open reading frames (ORFs) have been identified by cDNA cloning (reviewed in ref. 1). The role of these new proteins in the replicative cycle and in the pathogenesis of the virus remains to be identified. Because of their low level of expression, the gene products were not directly observed *in vivo*. However, experimental evidence obtained in cell culture suggests possible functions. The $p12^{I}$ protein of human T-cell leukemia virus (HTLV) cooperates with the E5 oncoprotein of bovine papillomavirus in cell transformation and binds the 16-kDa subunit of the vacuolar H^+-ATPase and the β chain of the interleukin 2 receptor (2). The role of the $p30^{II}$ and $p13^{II}$ proteins is still unknown. The R3 protein of bovine leukemia virus (BLV) could play a role in *rex*-induced transactivation[§] and G4 is a weak transactivator of LTR-directed gene expression (3). The G4 message is expressed at the beginning of the lymphoprofilerative stage of the disease, whereas the R3 mRNA is specific to the period surrounding seroconversion.

We demonstrate here that deletion of the R3 and G4 ORFs, although allowing infection of the recipient (4), leads to a drastic reduction of virus propagation.

MATERIALS AND METHODS

Plasmids and *in Vivo* Transfection of Sheep. Cloning of the proviruses pBLV344, pBLV344H, pBLVIX, pBLVX3C, pBLVDX, and pBLVRZ was previously described (4). To obtain pBLVIG4 containing a stop codon in the G4 ORF, the oligonucleotide (5′-GATCTAGGCTAGAATTCTAGCCTA-3′) was self-annealed and cloned into the *Bam*HI site [position 6997 (5)] of pBLV344. This oligonucleotide contains a translational stop codon in each of the three frames. The recombinant plasmid pBLVIG4 DNA was transfected into sheep as described (4).

The 26 sheep (see legend of Fig. 2 for numbers and infecting viruses) were maintained under controlled conditions at the National Institute for Veterinary Research (Uccle, Belgium). Total leukocyte counts were determined with a Coulter Counter, model ZN. Sera were collected and analyzed for BLV seropositivity by immunodiffusion and indirect gp51 ELISA (6, 7).

PCR Analysis. PCRs were performed as described (4). For the competitive PCR experiments, 2-μl aliquots of mutant proviral DNA containing lysates were mixed with 2 μl of serial (5-fold) dilutions of the wild-type (WT) DNA samples. The two DNAs to be compared were coamplified in a single reaction using two oligonucleotides: 6450 [position 6450 (5), 5′-TGGAAAGAACTAACGCTG-3′] and 8000 [position 8000 (5), 5′-CCTGCATGATCTTTCATACAAAT-3′]. The relative amounts of the DNAs were estimated after Southern blot analysis using a BLV probe (*Sac* I insert from plasmid pBLV344) as previously described (4). This probe contains the entire BLV sequence from position 143 to position 8235 (5). The size of the PCR fragments generated after pBLVDX and pBLVRZ DNA amplification allowed direct estimation of the relative amounts versus the other proviruses. Restriction digestion analysis permitted to segregate the pBLV344, pBLVIX, pBLVIG4, and pBLVX3C DNAs (as described in ref. 4).

Peripheral Blood Mononuclear Cell (PBMC) Culture, RNA Dot Blot Analysis, and p24 Titration. PBMCs were isolated by density gradient centrifugation of Ficoll/Hypaque (Histopaque; Sigma) from 200 ml of blood. Cells were cultured at

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Abbreviations: BLV, bovine leukemia virus; HTLV, human T-cell leukemia virus; LTR, long terminal repeat; ORF, open reading frame; PBMC, peripheral blood mononuclear cell; WT, wild type.

[§]Alexandersen, S., Fifth Workshop on the Pathogenesis of Animal Retrovirus, October 28–31, 1993, La Rochelle, France.

a concentration of 4×10^6 per ml of RPMI 1640 (GIBCO/BRL) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (4 mM), 10% heat-inactivated fetal bovine serum (GIBCO/BRL), and phytohemagglutinin (10 μg/ml) for 2 and 5 days.

Two days later, RNAs were extracted as described by Chomczynski and Sacchi (8). The RNAs were then denatured in 10 μl of deionized glyoxal (44%) in 0.02 M sodium phosphate (pH 6.5) for 1 hr at 50°C. After addition of 180 μl of 1.5 M NaCl/0.15 M trisodium citrate, pH 7, the mixture was spotted onto Hybond N+ membranes (Amersham). The RNAs were then fixed for 2 hr at 80°C and hybridized with an actin- or a BLV-specific probe (*Sac* I insert from plasmid pBLV344) as described (4).

Supernatant from 5-day PBMC cultures was precipitated by ammonium sulfate overnight at 4°C. The p24 major *gag*-encoded antigen was titrated from the cells or in the supernatants by ELISA (6, 7).

Immunofluorescence Assay. After culture, PBMCs were washed twice in phosphate-buffered saline (PBS), deposited on glass slides, air dried, and fixed for 5 min in cold acetone. A mixture of anti-gp51 monoclonal antibodies diluted in PBS was then incubated for 1 hr at 37°C. After washing, the slides were incubated with fluorescein-conjugated anti-mouse antibodies. At least 1000 cells were counted by immunofluorescence microscopy for determination of the percent positive cells in the preparations.

The PCR amplifications and the analyses of RNA and viral protein expression were performed 6, 12, 18, and 24 months after infection, depending on the period of inoculation.

RESULTS

***In Vivo* Infectivity of BLV Mutants in Sheep.** The sequences of the published BLV variants (5, 9) are well conserved with the exception of the 5′ end of the X3C frame located between the *env* and the *tax/rex* genes. The size of the X3C ORF indeed varies among the various isolates: viruses 344 and 395 have a termination codon at position 6768 [according to the numbering of Rice *et al.* (5)] as observed in the Belgian variant 15-2; the variant 1345 has a truncated X3C frame (ending at position 6849), and the American variant present in the FLK cell line contains the longest X3C sequence, with a translation stop codon at position 6894 (4) (Fig. 1). The provirus of pBLVX3C is isogenic to pBLV344 but contains the largest X3C frame from the FLK–BLV American variant (9). The pBLVX3C genotype is thus R3+/G4+/X3C+. Proviruses pBLVIX (R3+/G4+/X3C−) and pBLVIG4 (R3+/G4−/X3C−) contain translational stop codons inserted in the X3C (position 6614) and G4 (position 6997) frames, respectively [numbering according to Rice *et al* (5)]. Proviruses present in both pBLVDX and pBLVRZ have deletions of the R3 and G4 ORFs: their genotype is R3−/G4−/X3C−. pBLVRZ carries a ribozyme of the hammerhead type directed to *tax* and cloned into the X3C/R3/G4 frames. All of these BLV mutants derived from a single infectious and tumorigenic provirus (plasmid pBLV344) are referred to here as WT.

We previously reported that the various proviruses mentioned above directed the synthesis of the Tax transactivator and the p24 major capsid protein in transient expression assays, these products being detected both in the cell lysates and in the supernatants (4). These data hold true for the pBLVIG4 mutant tested in various cell lines (D17, HeLa, NIH 3T3, Vero, COS, and CV-1 cells) (data not shown). In none of these cell lines was a significant difference in expression of the proviruses observed.

To test the infectivity of the mutants *in vivo*, sheep were injected intradermally with 100 μg of plasmid DNA mixed with 200 μg of cationic liposomes {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP)}. These sheep were maintained under controlled conditions at the Veterinary Institute (Uccle, Belgium). A previously described series of sheep infections (4) has been extended to obtain 26

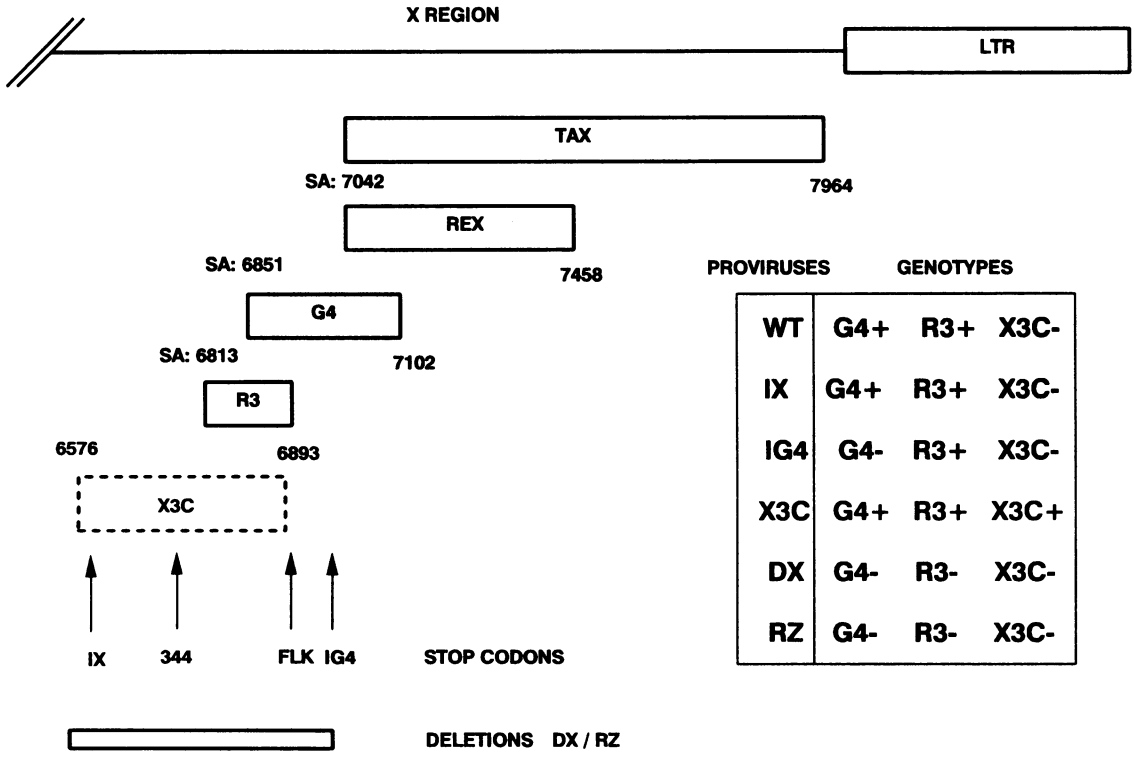

FIG. 1. Schematic representation of the 3′ end of the BLV provirus (X region and 3′ LTR). mRNAs corresponding to the Tax, Rex, G4, and R3 proteins were identified *in vivo*. The X3C frame is not conserved among the different strains: translational stop codons are indicated. The genotypes of the mutated proviruses are indicated at right. SA, splice acceptor site.

BLV-transfected animals. Sera were collected weekly and monitored for the presence of anti-gp51 antibodies. The delays for seroconversion varied between 18 and 210 days (data not shown). No significant difference between these delays could be correlated with the type of provirus. However, the frequency of a successful infection varied among the mutants. Indeed, both the WT provirus (pBLV344) and pBLVX3C were able to induce infection in five out of five transfections (100% efficiency each). In contrast, the insertion or deletion mutants displayed lower infectious potential: pBLVIX, three out of six (50% efficiency); pBLVIG4, two out of three (67%), pBLVDX, three out of six (50%); pBLVRZ, two out of three (66%). Two animals resisted a second DNA injection: sheep 247 (injected with provirus pBLVIX) and 245 (injected with provirus pBLVDX). However, all the uninfected sheep could be inoculated by using BLV-containing blood (data not shown).

These results suggest that the proviruses harboring mutations in the R3/G4 ORFs are less infectious after direct inoculation of proviral DNA.

Natural transmission among different animals is observed only in cattle, the main sources of infection being, however, the iatrogenic manipulations. Natural transmission between sheep does not seem to occur and was never observed in our experimental herd (4). PCR amplifications using leukocyte DNAs demonstrated that the infecting virus corresponded to the transfected mutants (data not shown).

The relative amounts of the mutant proviruses were evaluated by competitive PCR analysis (as described in *Materials and Methods*). When serial dilutions of the WT provirus DNA were mixed with the pBLVDX, pBLVIG4, or pBLVRZ samples, the PCRs allowed us to evaluate the relative amounts of the proviruses in the blood samples. It appeared that the proviruses deleted in the R3 and G4 frames (pBLVDX, pBLVIG4, or pBLVRZ) were 20- to 1000-fold less abundant than the WT virus in the infected sheep (data not shown).

***Ex Vivo* Viral RNA Expression.** All experimentally infected sheep were kept over a 2-year period. The infection persisted as verified by serological tests and PCR amplifications. To compare the *in vivo* behavior of the various mutants, peripheral blood leukocytes were isolated by density gradient centrifugation on Ficoll/Hypaque and the cells were cultured in the presence of phytohemagglutinin, an activator of BLV expression. After extraction, total RNAs were denatured in glyoxal, spotted onto nylon membranes, and hybridized with a BLV-specific probe (Fig. 2). A specific hybridization signal was obtained with RNAs from PBMCs of WT-infected sheep (animals 234, 235, 236, 244, and 252). These results demonstrated that, as expected, the provirus was transcribed after short-term culture. PBMCs from pBLVX3C- and pBLVIX-infected sheep (nos. 253, 254, 265, 266, 267, 248, 260, and 261) also expressed viral information. In contrast, only very weak signals (if any) were obtained for the other BLV mutants: pBLVDX (sheep 246, 263, and 264), pBLVIG4 (sheep 240 and 249), and pBLVRZ (sheep 255, 256, and 257). As negative controls, no hybridization was observed with RNAs isolated from uninfected sheep (nos. 247, 259, 245, 262, and 237). For quantitation, the hybridization spots were counted by liquid scintillation. Counts per minute (cpm) ranged from 1000 to 10,000 for pBLV344 (WT), pBLVX3C, and pBLVIX, depending on the individual animals and the period after infection, the highest values being obtained with the WT virus. In contrast, the pBLVDX, pBLVIG4, and pBLVRZ RNAs yielded cpm values close to background levels (100–250 cpm). As a control for individual variations in the sample preparations, the RNAs were hybridized with an actin probe (Fig. 2 *Bottom*).

This particular experiment was repeated during the course of infection (data not shown). All the data were consistent with a strongly reduced viral transcription in the sheep infected with proviruses mutated in R3 and G4. These results thus contrast with data from transient *in vitro* transfection, in which no significant difference in expression was measured among the proviruses tested (4).

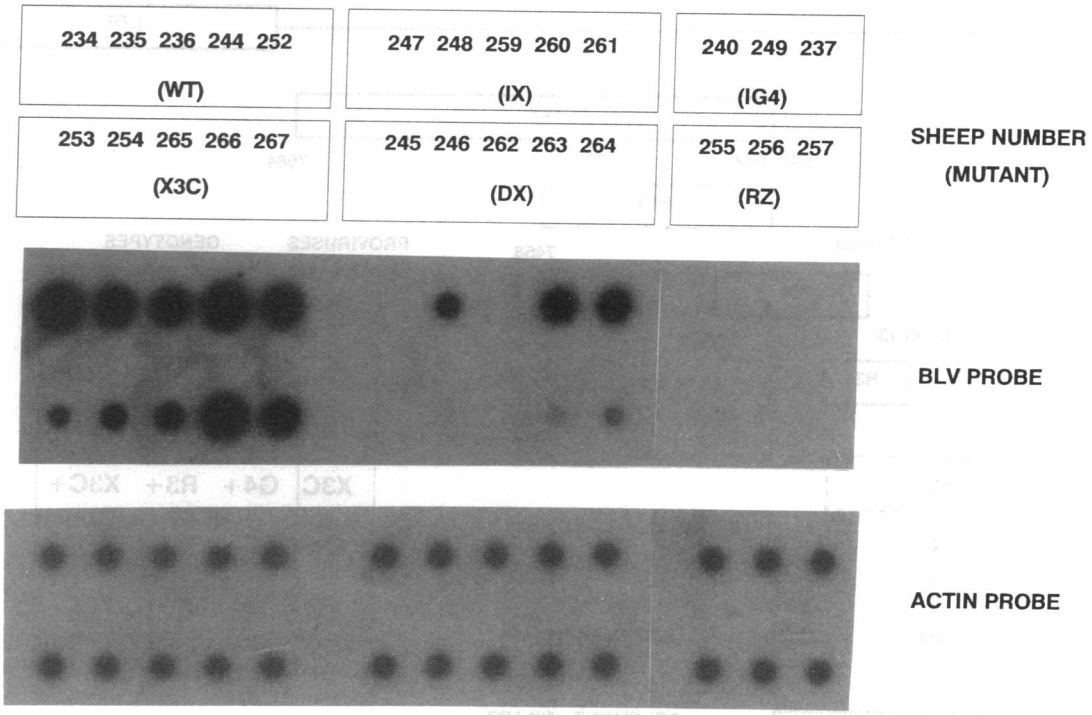

FIG. 2. Viral RNA expression. Peripheral blood leukocytes were cultured for 2 days. RNAs were extracted, denatured in glyoxal, spotted onto nylon membranes, and hybridized with an actin-specific (*Bottom*) or a BLV-specific (*Middle*) probe [positions 143 to 8235 (5)]. Positions of samples are indicated by animal number (with transfected provirus in parentheses) (*Top*). Sheep 247, 259, 245, 262, and 237 did not seroconvert after injection of the corresponding proviruses.

***Ex Vivo* Viral Protein Expression.** Expression of the various proviruses was measured by viral protein titration after *ex vivo* culture. Peripheral blood leukocytes were isolated by density gradient centrifugation on Ficoll/Hypaque and were cultured in the presence of phytohemagglutinin for 5 days. The virus particles in the supernatants were precipitated by ammonium sulfate and recovered by centrifugation. The cells were collected and washed by PBS. The p24 major *gag* antigen was titrated in the cells (Fig. 3) or in the supernatants (data not shown) by ELISA. The p24 protein was detected in PBMC cultures infected with the WT provirus (sheep 234, 235, 236, 244, and 252), the pBLVIX (sheep 248, 260, and 261) and pBLVX3C (sheep 253, 254, 265, 266, and 267). Expression was observed in the cells as well as in the supernatants. A quantitative difference was measured between WT and pBLVIX which contains a translational stop codon at the 5′ end of the X3C frame. However, a similar decrease in expression efficacy was also obtained with pBLVX3C, which contains the complete X3C ORF. As a negative control, no p24 antigen was detected in PBMC cultures from uninfected sheep (nos. 247, 259, 237, 245, and 262). Background levels were measured in PBMCs infected with the other BLV mutants: pBLVDX (sheep 246, 263, and 264), pBLVIG4 (sheep 240 and 249), and pBLVRZ (sheep 255, 256, and 257) (Fig. 3).

The quantitative transcriptional difference between the R3 and G4 deletion mutants and the WT provirus is thus extended at the protein level. The intracellular amounts of p24 correlated with the levels measured in the supernatants, indicating that protein export and viral particle formation were not affected in the mutant proviruses pBLVIX and pBLVX3C (data not shown).

Mutations in the R3 and G4 ORFs do not interfere with viral expression in transient *in vitro* assays (4) but almost completely abrogate viral protein synthesis after *ex vivo* culture of peripheral blood leukocytes. This lack of expression could be due to a general lower amount of viral protein per cell or to a decrease in the number of infected cells (or to a combination of both). Therefore, the number of infected PBMCs isolated from the sheep was determined by immunofluorescence.

After culture, PBMCs were fixed on glass slides and incubated with a mixture of anti-gp51 monoclonal antibodies (gp51 is the external envelope glycoprotein). BLV-expressing cells were then revealed with an anti-mouse fluorescein conjugate. At least 1000 cells were counted by immunofluorescence microscopy for determination of the percent positive cells in the preparations. Ten to 27 immunofluorescence-positive cells could be detected in pBLV344 (WT)-, pBLVX3C-, and pBLVIX-infected PBMCs (mean values depending on the individual animals and the period after infection), the highest values being obtained with the WT virus. In contrast, immunofluorescence values were close to the background levels for the pBLVDX, pBLVIG4, and pBLVRZ mutants (≤1 virus-expressing cell out of 1000 PBMCs).

It thus appears that the deletion of the R3 and G4 ORFs decreased the viral loads in the infected sheep as shown by reduction in the number of virus-expressing cells.

DISCUSSION

Direct injection of proviral DNA into sheep has allowed the study of the biological properties of BLV mutants *in vivo*. 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 is 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 and propagation. The different frames (X3C, R3, and G4) present between the *env* gene and the *tax/rex* region appeared to be dispensable for *in vivo* infection. However, virus transmission appeared less efficient when modifications were introduced in the X3C, R3, and G4 ORFs. More convincingly, virus loads were drastically decreased, indicating that viral propagation was impaired. The level of this blockage is unknown. At this stage, it is not possible to analyze the relative efficiency

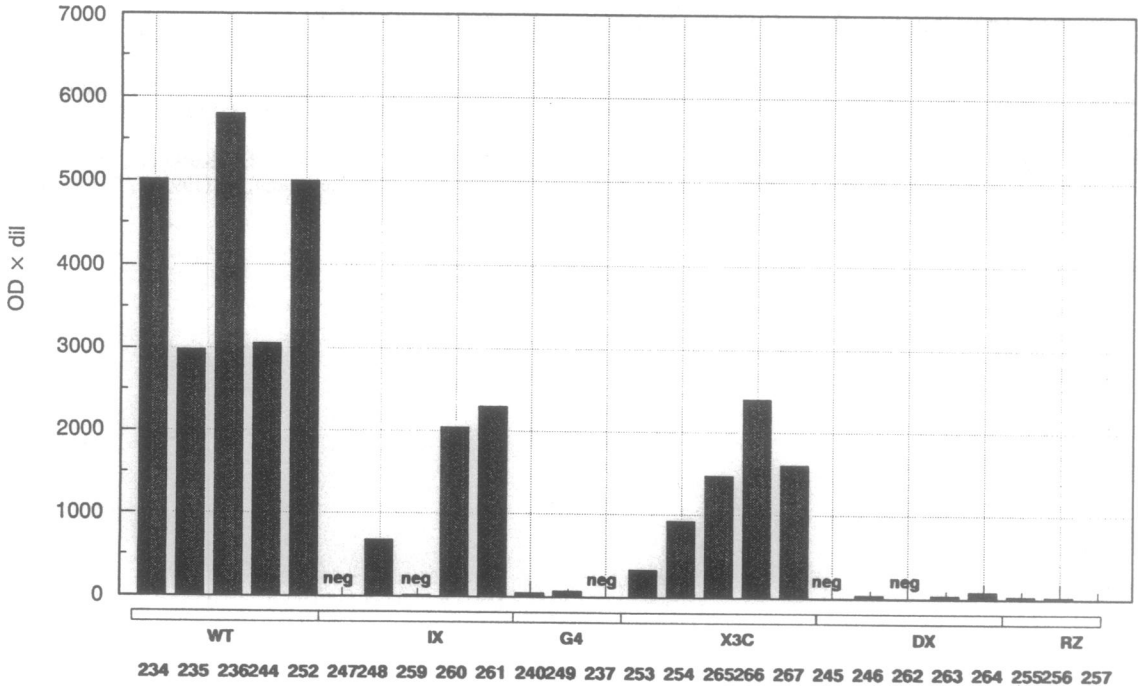

FIG. 3. Viral protein expression. The p24 major *gag* antigen was titrated from the cells by ELISA. OD × dil values are equivalent ELISA optical densities according to the dilutions. neg, uninfected sheep.

between the WT and mutated viruses, because very few cells are infected *in vivo* when the X3C, R3, and G4 ORFs are deleted. Should the mutated proviruses be able to propagate, it would then be possible to isolate enough infected PBMCs and analyze the infectious potential *in vitro*. As such, equivalent amounts of leukocytes could be cocultivated with CC81 indicator cells. The number and the size of the induced synctia should allow quantitation of the infectious viral particles. The X3C, R3, and G4 ORFs could possibly play a role in the infectivity of the virus. Alternatively, the deletions could interfere with viral protein maturation and export. The levels of intra- and extracellular p24 for the pBLVDX-, pBLVIG4-, and pBLVRZ-infected PBMCs should therefore be compared when sufficient numbers of infected cells become available. However, in transient-transfection experiments using several cell lines, we did not observe a difference in protein export. Since R3 shares sequences with *rex* and inhibits *rex* function *in vitro*,[§] deletion of the R3 frame could induce an overproduction of structural proteins and a continuous expression of the provirus. This should be deleterious to the infected cell, since the animal develops a strong immune response directed to the viral proteins. The virus mutated in R3 should not be hiding in host cells and could not escape immunological attack. This should interfere with its propagation potential inside the sheep. The G4 protein acts as a weak activator of LTR-driven transcription (3). The availability of PBMCs infected with the pBLVIG4 mutant should help to unravel the biological relevance of this *in vitro* transactivation. Our data show that the G4 ORF is important *in vivo* although its product is dispensable to establish an infection. Since G4 mRNAs are detected preferentially at the beginning of lymphocytosis (3), the behavior of pBLVIG4 will be particularly interesting to analyze for pathogenesis.

In contrast to cattle, all BLV-infected sheep die as a consequence of leukemia. The virus, although initially mandatory, appears dispensable later on, since many tumors contain only proviruses harboring deletions (reviewed in ref. 1). However, when one considers the number of infected cells, the onset of transformation appears to be a very rare event. A significant decrease in the number of infected cells should reduce the risk of developing leukemia. Since the virus loads of the pBLVDX and pBLVRZ mutants are greatly diminished, one may speculate that the risk of developing pathogenesis would be decreased to a level insufficient to allow the onset of leukemia during a normal sheep lifespan. Over a 2-year experimental period, we did not observe revertants of the mutated proviruses. This corroborates our previous studies on the genetic variability during the asymptomatic phase of the disease (10). This genetic stability, together with the biological properties of the X3C/R3/G4 deletion mutants, opens the route for making live attenuated strains of BLV for experimental vaccine testing.

BLV shares many properties of genomic structure, viral replication, and pathology with HTLV. The R3 and G4 proteins share some homologies with regulator proteins of HTLV ($p12^{I}$, $p13^{II}$, and $p30^{II}$ or Rof and Tof) and human immunodeficiency virus (Vpr) (3). This suggests that all these proteins belong to a common family of complex retrovirus regulatory proteins. To our knowledge, there is to date no infectious HTLV clone able to induce a leukemia in an animal system. BLV appears to be a good animal model for the study of the related HTLVs. In this context, direct inoculation of BLV deletion mutants is a useful tool to analyze viral replication *in vivo* and to identify genes involved in infection and pathogenesis.

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