

Suboptimal Enhancer Sequences Are Required for Efficient Bovine Leukemia Virus Propagation In Vivo: Implications for Viral Latency

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Repression of viral expression is a major strategy developed by retroviruses to escape from the host immune response. The absence of viral proteins (or derived peptides) at the surface of an infected cell does not permit the establishment of an efficient immune attack. Such a strategy appears to have been adopted by animal oncoviruses such as bovine leukemia virus (BLV) and human T-cell leukemia virus (HTLV). In BLV-infected animals, only a small fraction of the infected lymphocytes (between 1 in 5,000 and 1 in 50,000) express large amounts of viral proteins; the vast majority of the proviruses are repressed at the transcriptional level. Induction of BLV transcription involves the interaction of the virus-encoded Tax protein with the CREB/ATF factors; the resulting complex is able to interact with three 21-bp Tax-responsive elements (TxRE) located in the 5' long terminal repeat (5' LTR). These TxRE contain cyclic AMP-responsive elements (CRE), but, remarkably, the "TGACGTCA" consensus is never strictly conserved in any viral strain (e.g., AGACGTCA, TGACGGCA, TGACCTCA). To assess the role of these suboptimal CREs, we introduced a perfect consensus sequence within the TxRE and showed by gel retardation assays that the binding efficiency of the CREB/ATF proteins was increased. However, *trans*-activation of a luciferase-based reporter by Tax was not affected in transient transfection assays. Still, in the absence of Tax, the basal promoter activity of the mutated LTR was increased as much as 20-fold. In contrast, mutation of other regulatory elements within the LTR (the E box, NF- κ B, and glucocorticoid- or interferon-responsive sites [GRE or IRF]) did not induce a similar alteration of the basal transcription levels. To evaluate the biological relevance of these observations made *in vitro*, the mutations were introduced into an infectious BLV molecular clone. After injection into sheep, it appeared that all the recombinants were infectious *in vivo* and did not revert into a wild-type virus. All of them, except one, propagated at wild-type levels, indicating that viral spread was not affected by the mutation. The sole exception was the CRE mutant; proviral loads were drastically reduced in sheep infected with this type of virus. We conclude that a series of sites (NF- κ B, IRF, GRE, and the E box) are not required for efficient viral spread in the sheep model, although mutation of some of these motifs might induce a minor phenotype during transient transfection assays *in vitro*. Remarkably, a provirus (pBLV- Δ 21-bp) harboring only two TxRE was infectious and propagated at wild-type levels. And, most importantly, reconstitution of a consensus CRE, within the 21-bp enhancers increases binding of CREB/ATF proteins but abrogates basal repression of LTR-directed transcription *in vitro*. Suboptimal CREs are, however, essential for efficient viral spread within infected sheep, although these sites are dispensable for infectivity. These results suggest an evolutionary selection of suboptimal CREs that repress viral expression with escape from the host immune response. These observations, which were obtained in an animal model for HTLV-1, are of interest for oncovirus-induced pathogenesis in humans.

Bovine leukemia virus (BLV) is the etiologic agent of a chronic lymphoproliferative neoplastic disease called EBL (for enzootic bovine leukosis) (9, 20). Among cattle, the majority of infected animals remain clinically asymptomatic throughout their life. Up to one-third of infected cattle will develop a persistent lymphocytosis characterized by a permanent increase in the number of peripheral blood mononuclear cells (PBMCs), and less than 5% will die from lymphomas and/or lymphosarcoma (16). Among sheep, another species that can be infected by BLV, almost all infected animals will develop tumors or leukemia within their lifetime, i.e., 1 to 5 years

(reviewed in references 39 and 43). Infection by BLV is characterized by a long latency period associated with a lack of viral expression at all stages of the disease. In fact, B lymphocytes harboring an integrated provirus do not produce *in vivo* detectable levels of viral information (either RNA or protein) (21, 22, 24). Once these cells are isolated and cultured *in vitro*, a drastic increase in viral transcription occurs, indicating that the provirus is maintained at a repressed stage *in vivo* (28). This hiding strategy, which also appears to be developed by other members of the retrovirus family such as human T-cell lymphotropic virus type 1 (HTLV-1), allows for very efficient protection against recognition by the host immune response.

BLV expression is regulated at the transcriptional level by the Tax transactivator protein encoded by the 3' end of the proviral genome (11, 40). Transcriptional activation by Tax requires an enhancer sequence located in the U3 region of the

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long terminal repeat (LTR) (12, 13). This Tax-responsive enhancer is constituted by three copies of an imperfectly conserved 21-bp sequence (also called a Tax-responsive element, or TxRE) centered at positions -148 (distal), -123 (middle), and -48 (proximal), +1 being the transcription initiation site (19, 35). These three *cis*-acting elements are essential for the responsiveness of the BLV LTR to Tax. A motif resembling the cyclic AMP-responsive element (CRE) is contained within each TxRE, but, interestingly, the "TGACGTCA" consensus is never strictly conserved. Tax does not bind directly to DNA but rather acts via cellular proteins that recognize these CRE-like motifs (1, 2, 41). These proteins, identified by UV cross-linking of lysates from *ex vivo*-isolated B lymphocytes, include three members of the activating transcription factor (ATF)/cyclic AMP response element binding (CREB) protein family: CREB, ATF-1, and ATF-2 (41). *In vitro*, Tax enhances binding of these cellular transcription factors by interacting with their bZip domains (4). In cell culture, transient transfection of expression vectors encoding these cellular proteins provokes LTR-directed gene expression in the presence of protein kinase A (PKA) or calmodulin kinase IV (CaMKIV) (1, 41). Altogether, these observations underline the importance of the TxREs and reveal a complex mode of transcriptional regulation involving protein kinases, the cellular CREB/ATF factors, and the viral Tax transactivator.

Besides the imperfect CRE consensus, each TxRE also contains an E box sequence (CANNTG) which is a potential binding site for the cellular transcription factor AP4 (36, 44) or other basic helix-loop-helix proteins such as Myc, Mad, and Max. The assumption that AP4 could be implicated in LTR activity was based on transdominant and antisense inhibition of transcription, but direct binding of this particular factor has not been reported.

In vitro (7) and *in vivo* (44) footprinting experiments revealed another important region within the LTR situated between the middle and proximal TxREs. Two major footprints delineate poorly conserved nuclear factor κ B (NF- κ B) binding sites responding to phorbol 12-myristate 13-acetate (PMA) (7). Furthermore, in transient transfection experiments, the κ B site together with a single TxRE permits strong activation of BLV transcription in the presence of p50/p65 NF- κ B proteins (6). At the 3' end of the second NF- κ B footprint, just upstream of the proximal TxRE, a glucocorticoid-responsive element (GRE) confers responsiveness to dexamethasone in the presence of the Tax transactivator (26). In the absence of Tax, mutation of the GRE significantly decreases basal LTR activity in reporter-based assays (44).

BLV transcription thus appears to be regulated by several elements (TxRE, NF- κ B, and GRE) located in the U3 region of the LTR. The R domain of the LTR also contributes to enhancement of viral expression when it is located downstream of the transcriptional start site (13). Successive deletions of these sequences identified the presence of a 64-bp enhancer element at the 3' end of R (36), but this observation was disputed in a later study (23).

Another region contributing to transcriptional activity has been described in the U5 region downstream of the CAP site (23). Progressive deletion analysis indeed revealed that a 46-bp element corresponding to the 5' half of U5 exhibited enhancer activity when inserted upstream or downstream of a heterolo-

gous promoter. Site-directed mutation of an interferon regulatory factor (IRF) binding site comprised within this region induced a twofold reduction in Tax-independent LTR basal transcription.

Functional characterization of the sites involved in LTR activity has thus led to the identification of a series of sites located mainly in U3 (TxRE, NF- κ B, and GRE) but also in R and U5. In each study reported, the experiments were based on transient transfections of reporter constructs harboring either chloramphenicol acetyltransferase or luciferase genes. In a number of cases, subfragments of the LTR were also inserted into artificial vectors containing heterologous promoters (e.g., the simian virus 40 or thymidine kinase promoter). The goal of these assays was to identify important sites within the LTR involved in transcriptional regulation, and indeed, valuable information was reported in a series of papers. The major caveat for this kind of study concerns the lack of correct nucleosomal architecture surrounding the promoter sequences, since the experiments were not performed in the context of an integrated provirus. Furthermore, LTR activity was assessed in cells exhibiting artificial or altered phenotypes, i.e., adapted to culture and/or immortalized, obtained from heterologous species (such as humans, dogs, or bats), or isolated from different tissues (such as epithelium or fibroblasts). One of the last extensive studies in this field clearly demonstrated that opposite conclusions might be drawn concerning the role of a given sequence in LTR activity depending on the cell line used (44). For example, mutation of the E box decreased or enhanced LTR-dependent transcription in human B lymphocytes (Raji) or FLK (fetal lamb kidney) cells, respectively. In other words, transient transfections into different cell lines might lead to conflicting interpretations. To circumvent these limitations, we undertook to characterize LTR promoter activity in the context of an infectious and pathogenic molecular clone of BLV. This strategy allows, data obtained from *in vitro* cell culture to be correlated with the phenotype of a recombinant provirus in an animal model.

MATERIALS AND METHODS

Plasmids and recombinant proviruses. To construct luciferase-based reporter plasmids (pLTR-IRF, pLTR-GRE, pLTR-NF1, pLTR-NF2, pLTR-Ebox3x, pLTR-CRE3x, pLTR-CRE148-123, pLTR-CRE148-48, pLTR-CRE123-48, pLTR-CRE148, pLTR-CRE123, and pLTR-CRE48), mutations were introduced by site-directed mutagenesis into the BLV LTR from provirus 344. These nucleotide substitutions were performed using a two-step PCR procedure essentially as described previously (42). The following sense (S) and complementary (C) oligonucleotides that contain the selected mutations were used in the PCRs: IRFS (5'-TTCTGTCTTACAGTCTGTGTCTCGCGC-3') and IRFC (5'-GCCGAGACACAGACTGTAAGACAGGAA-3'), GRES (5'-CGAAAAATCCTATCCCACAGTAGCTGACCT-3') and GREC (5'-AGGTACGACTACTGTGGATAGGATTTTCG-3'), NFIS (5'-AACCATGGGTACCTCCCCAAC TTCCC-3') and NFIC (5'-AAGGTACCGACCCAGCTGCCGTACCC-3'), NF2S (5'-AACCATGGCCGAAAAATCCACACCCCGAG-3') and NF2C (5'-AAGGTACCGAGTTGGGGAGGTACGGGGA-3'), Ebox148S (5'-ACAGACGTCAGCGACCAAGAAAGC-3') and Ebox148C (5'-GCTTTTCTGGTGCTGACGTCTCTGT-3'), and Ebox123S (5'-GGTGACGGCAGCGAGTGGCTAGAATCC-3') and Ebox123C (5'-GGATTCTAGCCACTCGTGCCGT CACC-3'), Ebox48S (5'-GCTGACCTCACCAGCTGATAAAACAA-3') and Ebox48C (5'-TTGTTTTATCAGTCGGTGAGGTACAGC-3'), CRE148S (5'-CAGACAGTGACGTACGTGCC-3') and CRE148C (5'-GGCAGCTGACGTGCTGTCTG-3'), CRE123S (5'-AAGCTGGTGACGTGACGTGGTGGCT-3') and CRE123C (5'-AGCCACCAGCTGACGTACCCAGCTT-3'), and CRE48S (5'-GAGCTGCTGACGTACCTGCTGAT-3') and CRE48C (5'-ATCAGCAGGTGACGTACGACGTCT-3').

A first round of PCRs allowed the amplification of two sequences encompassing the LTR: a 5'-end insert (using the oligonucleotide LTRSH, 5'-AAAAGC TTGTATGAAAGATCATGCGC-3', and the complementary oligonucleotide) and a 3'-end fragment (using the oligonucleotide LTRCB, 5'-TTGGATCCTT GTTGGCCGGTCTCTCTG-3', and the corresponding sense primer). The two DNA amplicons were then transferred to an agarose gel, purified using Gene Elute columns (Sigma), and amplified in a second round of PCR using the oligonucleotides LTRSH and LTRCB. The resulting DNAs, which contain the reconstituted LTR sequence, were introduced into plasmid pCR II (TA cloning kit; Invitrogen). To verify the presence of the desired substitution and the absence of *Taq* DNA polymerase errors, the mutated fragments were sequenced by the dideoxy chain termination procedure using a set of primers distributed along the LTR (T7 sequencing kit; Amersham Pharmacia Biotech). The mutated LTRs were cloned in the *KpnI-XhoI* sites of plasmid pGL3-Basic to generate pLTR-IRF, pLTR-GRE, pLTR-NF1, pLTR-NF2, pLTR-Ebox3x, pLTR-CRE3x, pLTR-CRE148-123, pLTR-CRE148-48, pLTR-CRE123-48, pLTR-CRE148, pLTR-CRE123, and pLTR-CRE48.

The pLTR-Δ21-bp mutant was obtained by digestion of the LTR with the restriction endonuclease *PvuII* and religation, resulting in a deletion of the U3 region from -148 to -124. This synthetic promoter thus contains only two TREs, the first of which is a hybrid between the distal and middle 21-bp enhancer elements.

To construct the recombinant proviruses (pBLV-IRF, pBLV-GRE, pBLV-NF1, pBLV-NF2, pBLV-Ebox3x, pBLV-CRE3x, and pBLV-Δ21-bp), reporter vectors were digested with the restriction endonucleases *EcoRI* and *BssHII* (for pLTR-IRF) or with *AatII* and *XhoI* (for pLTR-GRE, pLTR-NF1, pLTR-NF2, pLTR-Ebox3x, pLTR-CRE3x, and pLTR-Δ21-bp) and introduced into the corresponding sites of plasmid pBLV-Hind (harboring a molecular clone of wild-type BLV provirus strain 344). Restriction analysis and nucleotide sequencing were performed to verify the integrity of the resulting constructs. Plasmids were then amplified and purified by centrifugation on a cesium chloride gradient as described by Sambrook et al. (31).

Luciferase assays. D17 canine osteosarcoma cells were grown in minimal essential medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum and were transfected by the calcium phosphate coprecipitation procedure (Profection Kit; Promega). Twenty-four hours prior to transfection, the cells were cultured at a density of 3×10^5 per well (6-well dish; Nunc). Six micrograms of different pLTRLuc reporters and 10 ng of the pSGTax or pSG5 expression vector were mixed with CaCl_2 and phosphate buffer (as described by the manufacturer), incubated at room temperature for 30 min to allow DNA precipitation, and added to the cells. After incubation for 4 h in the presence of the transfection complex, the cells were washed with serum-free medium and grown at 37°C for 48 h.

Raji lymphocytes were transfected essentially as described previously (23). Briefly, 3 million cells were harvested at a density of 10^6 per ml, washed with STBS (25 mM Tris-HCl [pH 7.5], 137 mM NaCl, 5 mM KCl, 700 μM CaCl_2 , 500 μM MgCl_2 , 600 μM Na_2HPO_4), and resuspended in 300 μl of a mixture containing 250 ng of reporter plasmid and 450 μg of DEAE-dextran/ml in STBS. Cells were incubated for 1 h at 37°C, washed (twice with 1 ml of STBS and once in culture medium), and cultured for 48 h.

After transfection, the cells (D17 and Raji) were washed twice with phosphate-buffered saline (PBS), and luciferase enzyme activity was measured by using the dual-luciferase assay system (Promega) according to the manufacturer's recommendations.

Titration of the major capsid protein by ELISA. D17 cells were transfected with proviral constructs (6 μg of pBLV-IRF, pBLV-GRE, pBLV-NF1, pBLV-NF2, pBLV-Ebox3x, pBLV-CRE3x, or pBLV-Δ21-bp) by the calcium phosphate precipitation method and cultured for 48 h. The cell supernatants were recovered and analyzed for p24 protein expression using an enzyme-linked immunosorbent assay (ELISA) procedure. Briefly, 96-well microtiter plates (Maxisorb immunoplate; Nunc) were coated with monoclonal antibody 4'G9, (300 ng in PBS per well) for 4 h at room temperature. The plates were washed three times with PBS-Tween 80 (0.2%), and serial threefold dilutions of cell culture supernatants were added to the wells in the presence of bovine serum albumin (0.67%) and Tween 80 (1.33%). After overnight incubation at 4°C and three washes, the presence of the p24 antigen was revealed by using two monoclonal antibodies (2'C1 and 4'F5) conjugated with horseradish peroxidase.

Isolation of PBMCs and electrophoretic mobility shift assays (EMSA). Sheep PBMCs were isolated by Percoll gradient centrifugation as previously described (10). Briefly, venous blood was collected by jugular venipuncture and mixed with EDTA used as an anticoagulant (1 ml of 7.5% EDTA per 25 ml of blood). PBMCs were separated by Percoll density gradient centrifugation (Amersham Pharmacia Biotech) and washed (three times with PBS-0.075% EDTA and once

with PBS alone), and cell extracts were prepared as described by Sommer et al. (32). To this end, PBMCs were harvested in 300 μl of buffer (10 mM Tris-HCl [pH 7.05], 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM ZnCl_2 , 1% Triton X-100, and a cocktail of protease inhibitors [Complete; Roche Diagnostics]), incubated on ice for 10 min, mixed (vortexed) for 30 s, and centrifuged at $1,400 \times g$. The supernatants were dispensed in aliquots and stored at -80°C until use.

For the EMSA, the following oligonucleotides, corresponding to a wild-type (WT) or a mutated (p, for perfect CRE consensus) CRE binding site, were used as probes: CRE148WT (5'-CAGACAGAGACGTCAGCTGCC-3'), CRE148p (5'-CAGACAGTGCAGTCAGCTGCC-3'), CRE123WT (5'-AAGCTGGTGA CGGCAGCTGGTGGCT-3'), CRE123p (5'-AAGCTGGTGCAGTCAGCTGG TGGCT-3'), CRE48WT (5'-GAGCTGCTGACCTCACCTGCTGAT-3'), and CRE48p (5'-GAGCTGCTGACCTCACCTGCTGAT-3'). (Mutated nucleotides are underlined.) These primers and the corresponding complementary oligonucleotides were end labeled with [γ - ^{32}P]ATP using T4 polynucleotide kinase (specific activity = 1×10^7 to 2×10^7 cpm/ μg). PBMC extracts (15 μg of protein in 5 μl) were incubated with 20 ng of ^{32}P -labeled oligonucleotide in 15 μl of GS buffer (20 mM HEPES [pH 7.3], 30 mM KCl, 3 mM MgCl_2 , 1 mM EDTA, 8% glycerol, 1 mM β -mercaptoethanol, 10 mM dithiothreitol) in the presence of 1 μg of sonicated salmon sperm DNA. The DNA-protein complexes were allowed to form at 25°C for 30 min and were then electrophoresed on a 5% native polyacrylamide gel in 25 mM Tris-25 mM boric acid-0.5 mM EDTA at 11 V/cm for 2 h at room temperature.

In the supershift experiments, 1 μl of the appropriate antibody was added to the DNA-protein complexes and incubated for an additional 15 min at room temperature before the reaction mixtures were loaded onto the polyacrylamide gel. G. Schutz kindly provided the polyclonal anti-CREB antiserum, whereas the monoclonal antibodies specific for ATF-1, ATF-2, CREM, USF-1, E47, Max, HEB, AP1, and AP2 were purchased from Santa Cruz Biotechnology. Quantification was performed using Image Master from Amersham Pharmacia Biotech.

Infection of sheep with recombinant proviruses. Plasmid DNA (100 μg) containing the wild-type provirus (pBLV-WT) or a mutant (pBLV-IRF, pBLV-GRE, pBLV-NF1, pBLV-NF2, pBLV-Ebox3x, pBLV-CRE3x, or pBLV-Δ21-bp) was injected into sheep as previously described (42). To this end, 100 μg of proviral DNA was mixed with 200 μl of *N*-[1-(2,3-dioleoloxyl)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Roche Diagnostics) in 1 ml of HBS (20 mM HEPES-150 mM NaCl, [pH 7.4]) and injected intradermally into the backs of BLV-free sheep. The animals were maintained under controlled conditions at the Veterinary and Agrochemical Research Centre (Machelen, Belgium). Serum samples were collected at regular time intervals and analyzed for the presence of BLVgp51-specific antibodies by the ELISA procedure (27). At 3 and 6 months postinjection, proviral loads were estimated as described previously (39). In brief, 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) and centrifuged for 20 s at $14,000 \times g$. After at least four washes in 1 ml of the same solution, the pellets were resuspended in 500 μl of PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl_2 , 50 mM KCl [pH 8.3]), incubated with 6 μl of proteinase K (5 mg/ml) for 1 h at 50°C, and boiled for 5 min to stop the proteolytic digestion. DNA in these lysates (5 μl) was amplified by PCR in the presence of 200 μM each of the four deoxynucleoside triphosphates 2 U of *Taq* DNA polymerase (Roche Diagnostics), and 100 ng (each) of primers PCRTA (5'-CTCTTCGGGATCCATTACCTGA-3') and PCRTC (5'-CCTGC ATGATCTTTCATACAAAT-3') encompassing the *tax* gene from position 6989 to 8000 according to the reported numbering (29, 30). The samples were denatured for 5 min at 95°C and then amplified by 25 cycles of PCR (30 s at 95°C, 30 s at 57°C, and 1 min at 72°C). After PCR, the amplification product was analyzed by Southern blot hybridization using a *Tax* probe (a 1-kb *EcoRI* insert from plasmid pSGTax).

For direct sequencing, LTR sequences were amplified by 36 cycles of PCR with primers LTRA (5'-TGTATGAAAGATCATGCAGGCC-3') and CHAR1 (5'-GGGTATAGGAGGGGAAT-3'). The amplicons were purified with a Sephaglass bandprep kit (Amersham Pharmacia Biotech) and sequenced by PCR with primers regularly distributed along the LTR using the double-stranded DNA cycle sequencing system (Life Technologies).

RESULTS

Increased basal transcriptional activity of an LTR harboring consensus CREs. Comparing the different BLV LTR sequences reported in the data banks as well as our unpublished results, we were intrigued by the very high conservation of the

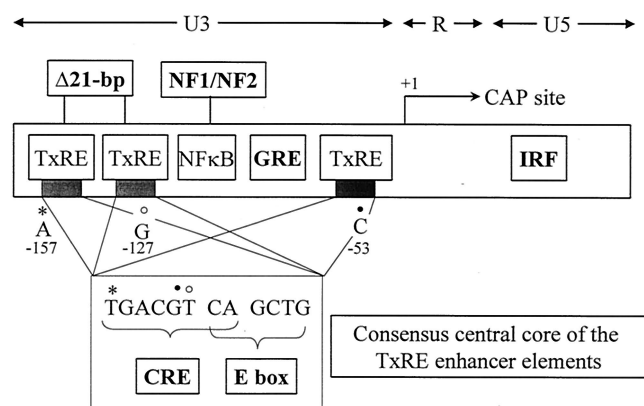


FIG. 1. Schematic representation of the mutations introduced in the LTR. LTR sequences previously shown to be involved in transcription are indicated. Three repeats of an imperfectly conserved 21-bp TxRE contain a CRE-related motif (the asterisk and solid and open circles represent variations compared to the TGACGTCA consensus; the numbers indicate the positions of nucleotide changes, with +1 being the CAP site) and an overlapping E box (CANNTG). The other sites are NF- κ B, the GRE and the IRF binding site. Mutations that were introduced within these motifs as described in Table 1 are, bold-faced (Δ 21-bp, NF1, NF2, GRE, IRF, CRE, and E box). The U3, R, and U5 regions are not drawn to scale.

transcription factor binding sites among the various isolates. Remarkably, all the strains harbor almost identical NF- κ B, E-box, GRE and IRF motifs within their LTR promoters. Such a high rate of conservation also holds true for the CRE-related sites located within the TxRE enhancers. Interestingly, none of these CRE-like motifs fit perfectly with the well-characterized consensus sequence TGACGTCA; the distal, middle and proximal TxREs were, respectively, AGACGTCA, TGACGGCA, and TGACCTCA (with differing nucleotides underlined). Therefore, we aimed to test the biological significance of these apparently minor substitutions and constructed, by site-directed mutagenesis, a recombinant LTR harboring perfect consensus CREs in all three TxREs. For comparison, we also modified other regulatory sites within the LTR either by mutation (IRF, GRE, E box) or by deletion (NF- κ B, Δ 21-bp) (schematized in Fig. 1 and summarized in Table 1). For the IRF and GRE motifs, well-defined substitutions were chosen in order to disrupt binding activity (23, 26). The LTR region

containing the NF- κ B sites was mutated by deletion of the two major protected domains as revealed by *in vitro* DNase I footprinting, generating mutants NF1 and NF2 (7). A triplicate TG $\rightarrow$ GA substitution that does not alter the overlapping CRE consensus was performed at the 3' end of the E-box motifs in each TxRE (mutant EBox3x). Finally, a large excision between residues -148 and -124 resulted in a synthetic promoter containing only two TxREs, the first of which was a hybrid between the distal and middle 21-bp enhancer elements (mutant Δ 21-bp).

To evaluate the impact of these mutations on promoter-directed transcription, the different recombinant LTRs were subcloned into plasmid pGL3-basic, a luciferase-based reporter. The resulting vectors (pLTR-CRE3x, pLTR-Ebox3x, pLTR-NF1, pLTR-NF2, pLTR-GRE, pLTR-IRF, and pLTR- Δ 21-bp) were transfected into D17 canine osteosarcoma fibroblasts. The advantage of this cell line is that it allows for the assessment of LTR activity in the absence of closely related endogenous factors specific for lymphocytes or ruminant proteins. In this cell culture system, transient transfection of the LTR reporters in the presence of Tax did not yield significant differences in promoter activity (Fig. 2A). In other words, our selected mutations did not affect Tax response, despite some minor variations without statistical significance. In the absence of Tax, however, all mutants exhibited similar levels of basal transcription, with the notable exception of pLTR-CRE3x (Fig. 2B). Besides a reduction associated with pLTR-GRE and a slight but reproducible increase induced by pLTR-Ebox3x (2-fold), the sole marked exception was the pLTR-CRE3x reporter, for which LTR-directed luciferase activity was increased as much as 10-fold. This drastic difference in basal activity became even more evident (20-fold) and highly statistically significant ($P < 0.01$) in Raji B lymphocytes, closely mimicking the BLV target cells (Fig. 2C). We conclude that, in transient transfection experiments, reconstitution of a perfect CRE motif within the TxREs provokes a strong induction of LTR activity in the absence of Tax.

To define which of the three TxREs was responsible for this phenomenon (either the distal at -148, the middle at -123, or the proximal at -48), double and single reconstitutions of the consensus CREs were designed, yielding plasmids pLTR-CRE148-123, pLTR-CRE148-48, and pLTR-CRE123-48 (harboring double mutant sites) and plasmids pLTR-CRE148,

TABLE 1. Summary of LTR mutations

Mutant name	Transcription factor binding site	Wild-type sequence ^a	Mutated sequence ^{a,b}
IRF	IRF	(+253) ACTTTCGTGTTCTC	(+253) ACAGTCTGTGTCTC
GRE	GRE	(-74) TCCACACCCCGAGCT	(-74) TCCTATCCCACAGTA
NF1	NF- κ B (first footprint)	(-116) ..CTTAGAATCCCCGT..	(-116) ..CTGCATGGGT..
NF2	NF- κ B (second footprint)	(-93) ..CTTCCCTTTTCC..	(-93) ..CTGCATGGCC..
EBox3x	21-bp enhancer (E box)	(-151) ... CAGCTG	(-151) ... CAGCGA ...
		(-126) ... CAGCTG ...	(-126) ... CAGCGA ...
		(-51) ... CACCTG ...	(-51) ... CACCGA ...
CRE3x	21-bp enhancer (CRE)	(-157) ... AGACGTCA ...	(-157) ... TGACGTCA ...
		(-132) ... TGACGGCA ...	(-132) ... TGACGTCA ...
		(-57) ... TGACCTCA ...	(-57) ... TGACGTCA ...
Δ 21-bp	Deletion of 21-bp enhancer	(-148) ..CTGCCAGAAAAGCTGGTGACGGCAG..	(-148 to -124) deletion

^a The numbers in parentheses are the nucleotide positions relative to the transcription initiation site (+1).

^b Underlining indicates mutations that were introduced by site-directed mutagenesis of the LTR.

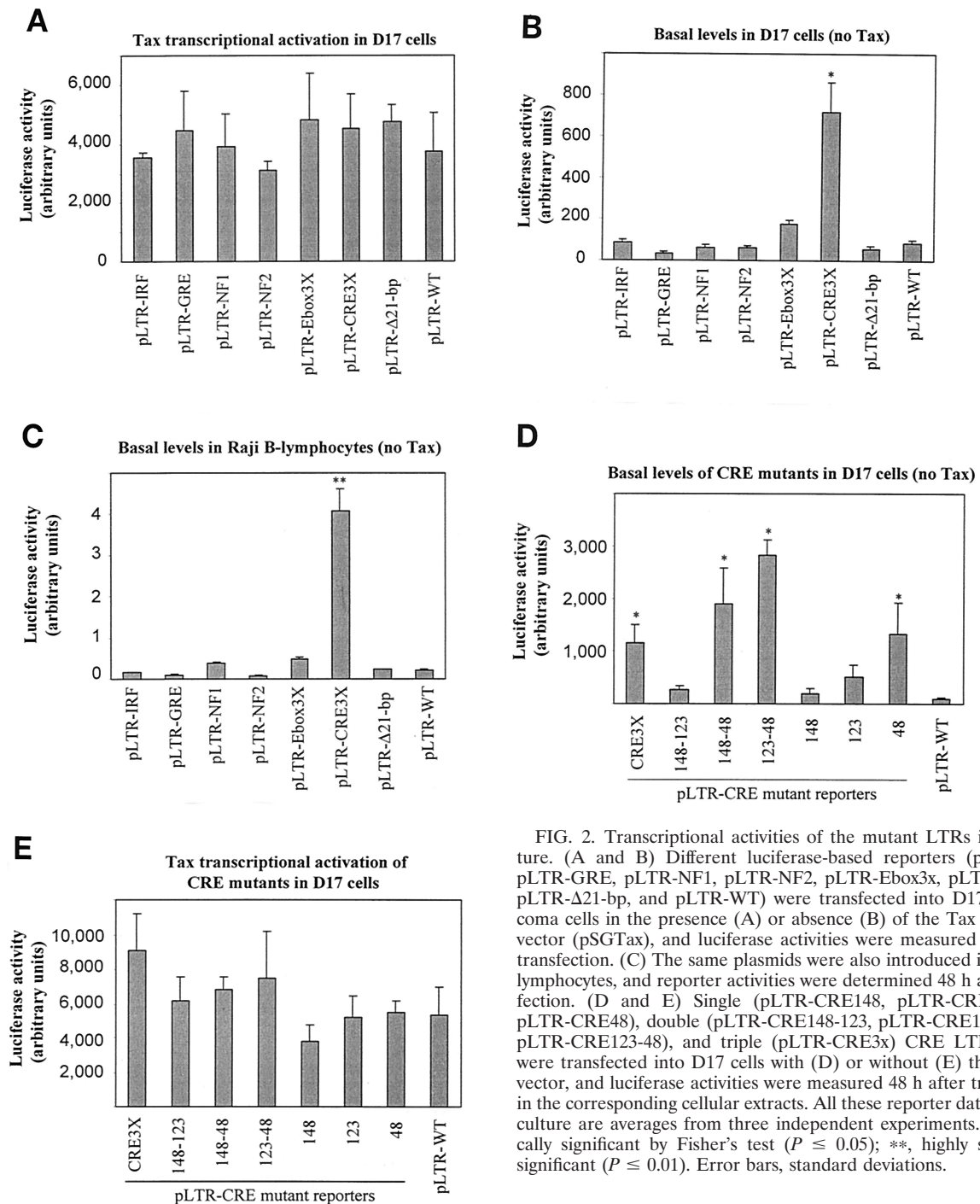


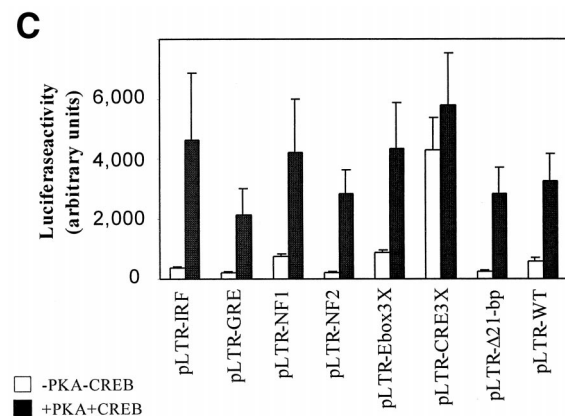
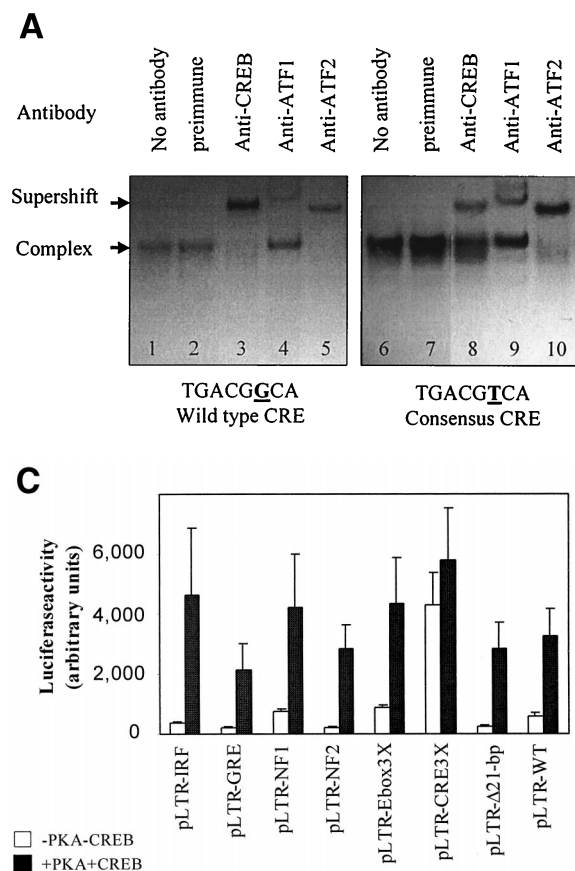
FIG. 2. Transcriptional activities of the mutant LTRs in cell culture. (A and B) Different luciferase-based reporters (pLTR-IRF, pLTR-GRE, pLTR-NF1, pLTR-NF2, pLTR-Ebox3x, pLTR-CRE3x, pLTR-Δ21-bp, and pLTR-WT) were transfected into D17 osteosarcoma cells in the presence (A) or absence (B) of the Tax expression vector (pSGTax), and luciferase activities were measured 48 h after transfection. (C) The same plasmids were also introduced into Raji B lymphocytes, and reporter activities were determined 48 h after transfection. (D and E) Single (pLTR-CRE148, pLTR-CRE123, and pLTR-CRE48), double (pLTR-CRE148-123, pLTR-CRE148-48, and pLTR-CRE123-48), and triple (pLTR-CRE3x) CRE LTR mutants were transfected into D17 cells with (D) or without (E) the pSGTax vector, and luciferase activities were measured 48 h after transfection in the corresponding cellular extracts. All these reporter data from cell culture are averages from three independent experiments. *, statistically significant by Fisher's test ($P \leq 0.05$); **, highly statistically significant ($P \leq 0.01$). Error bars, standard deviations.

pLTR-CRE123, and pLTR-CRE48 (harboring single mutants) (Fig. 2D). In the absence of Tax, statistically significant increases ($P < 0.05$) were generated by pLTR-CRE148-48, pLTR-CRE123-48, and pLTR-CRE48; the common characteristic of these reporters was the presence of a perfect consensus CRE in the proximal TxRE. In the presence of Tax, these differences were abolished, indicating that all LTRs were equally responsive to the viral transactivator (Fig. 2E).

Altogether, the most straightforward conclusion that might

be drawn from these in vitro experiments is that basal transcriptional activity is drastically induced when the CRE-like motifs within the Tax-responsive enhancers are converted to a perfect consensus, with the proximal site (−48) having the largest effect.

Higher basal transcriptional activity parallels with increased binding of CREB/ATF proteins. Although the optimal consensus recognized by the CREB/ATF proteins is TGACGTCa, we have previously demonstrated that three members of this transcription factor family (CREB, ATF-1, and ATF-2) spe-



cifically interact with the imperfectly conserved CRE motif located in the TxRE enhancers (1). Conversion of these CRE-related sites into a perfect consensus might have modified the binding efficiency or the specificity of the proteins interacting with the TxREs. Therefore, we used a gel retardation approach to characterize the DNA-protein complexes generated after mutation of the wild-type CRE into a consensus motif.

To this end, a radiolabeled oligonucleotide (CRE123WT) corresponding to the middle TxRE was mixed with cellular extracts prepared with sheep PBMCs and transferred to a nondenaturing gel, generating a major complex (Fig. 3A, lane 1). This complex, which in fact comprises two closely migrating bands, was supershifted after addition of antibodies specific for CREB, ATF-1, or ATF-2 but not with addition of preimmune serum (Fig. 3A, lanes 2 to 5), confirming our previous observations (1). Under identical assay conditions, a probe corresponding to a TxRE harboring a consensus CRE (CRE123p) essentially yielded similar profiles (Fig. 3A, lanes 6 to 10), indicating that CREB, ATF-1, and ATF-2 also interact with this particular sequence. Gel shifts using oligonucleotides corresponding to the proximal or distal TxRE (probes CRE148WT, CRE48WT, CRE148p, and CRE48p) did not reveal any qualitative modification of these patterns, despite some variations in the amount of supershifted complex (data not shown). Furthermore, antibodies specific for a series of other transcription factors known to bind CRE (CREM) or E boxes (USF-1, E47, Max, HEB, AP1, and AP2) did not super-

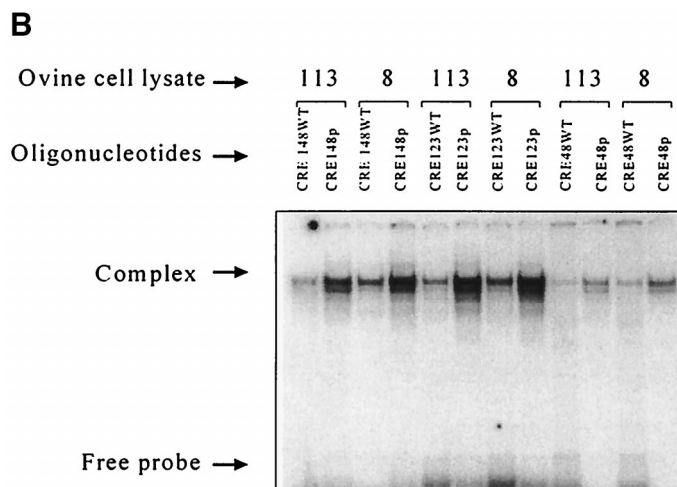


FIG. 3. In vitro interaction of the TxREs with CREB/ATF proteins and response of the mutated LTRs to the PKA pathway. (A) Interaction of CREB, ATF-1, and ATF-2 with a wild-type (containing a CRE-like sequence, TGACGGCA) and a consensus (CRE sequence TGACGTCA) middle TxRE. Gel retardation assays with lysates from infected sheep and oligonucleotide CRE123WT (lanes 1 to 5) or CRE123p (lanes 6 to 10) were performed in the absence (No antibody) or in the presence of different antibodies: a preimmune serum (lanes 2 and 7), polyclonal anti-CREB (lanes 3 and 8), and an anti-ATF-1 (lane 4 and 9) or an anti-ATF-2 (lanes 5 and 10) monoclonal antibody. (B) Binding efficiency of CREB/ATF proteins is increased when the TxRE contains a consensus CRE. ³²P-end-labeled 21-bp oligonucleotides CRE148WT, CRE123WT, and CRE48WT (wild-type TxREs) or CRE148p, CRE123p, and CRE48p (harboring a perfect CRE consensus) were incubated with cell extracts prepared from freshly isolated sheep PBMCs (uninfected sheep 113 and preleukemic sheep 8, harboring 87% of B lymphocytes) at 25°C for 30 min. The DNA-protein complexes were separated from the free probe by electrophoresis on a 5% nondenaturing polyacrylamide gel. (C) Response of the mutated LTRs to the CREB/PKA pathway. Transient transfection in D17 cells of different reporter constructs (pLTR-IRF, pLTR-GRE, pLTR-NF1, pLTR-NF2, pLTR-Ebox3X, pLTR-CRE3X, pLTR-Δ21-bp, and pLTR-WT) was carried out in the presence (solid bars) or absence (open bars) of expression vectors for CREB (pSG-CREB) and the catalytic subunit of PKA (pSG-PKA). Luciferase activities in cellular extracts were measured 48 h after transfection. Means were calculated from three independent experiments.

shift the complex under our experimental conditions (data not shown).

We conclude that conversion of the wild-type CRE into a perfect consensus does not qualitatively modify the binding of the CREB, ATF-1, and ATF-2 proteins to the TxREs. Quantitatively however, the amount of complex appeared to be increased by this substitution (Fig. 3A; compare lanes 1 to lanes 6 to 10). To confirm this observation, all probes (CRE148WT, CRE123WT, CRE48WT, CRE148p, CRE123p, and CRE48p) were tested in parallel for the ability to generate specific complexes in lysates from two sheep (seronegative sheep 113 and BLV-infected sheep 8) (Fig. 3B). Under the conditions used, the amounts of complexes induced by the wild-type probes (CRE148WT, CRE123WT, and CRE48WT) were globally similar, although the binding of the proximal TxRE (CRE48WT) was slightly reduced. Reconstitution of a consensus CRE (CRE148p, CRE123p, and CRE48p) signifi-

cantly enhanced complex formation for all three TxREs, but again, the binding was less efficient for the proximal (CRE48p) element. Proportionally, however, the relative increases in the amount of complex induced by conversion into a consensus CRE were similar for the three TxREs, as revealed by quantification of the bound probes.

It thus appears that TxREs harboring either consensus or wild-type CREs interact with the same CREB/ATF-1/ATF-2 transcription factors but that the efficiency of their binding differs quantitatively between the two motifs. The CREB/ATF transcription factors are essential mediators of BLV expression and act as final intermediates in pathways that also include protein kinases such as PKA or CaMKIV (1, 41). Increased affinity of CREB/ATF-1/ATF-2 for the perfect consensus CRE site could thus, in principle, correlate with a better response to the activation induced by these protein kinases. To test this hypothesis, we transfected our luciferase-based reporters (pLTR-IRF, pLTR-GRE, pLTR-NF1, pLTR-NF2, pLTR-Ebox3x, pLTR-CRE3x, pLTR- Δ 21-bp, and pLTR-WT) in the presence of expression vectors for CREB and PKA (pSG-CREB and pSG-PKA) into D17 cells. Luciferase activities generated by the various reporters did not differ significantly in response to CREB and PKA, although some variations were indeed observed (Fig. 3C). In other words, the absolute levels of CRE3x-induced transcription in response to CREB and PKA are similar to the activities associated with the other reporters, as observed after triggering with the Tax transactivator (Fig. 2A and E). More importantly, the relative increase in activity in the presence, of stimulation with CREB plus PKA to that in its absence was abrogated in the case of the perfect consensus CRE reporter (plasmid pLTR-CRE3x [Fig. 3C]). This reporter vector was apparently already fully activated, and additional triggering of the PKA pathway did not increase LTR-dependent transcription.

Collectively, our results show that correcting the mismatches of the wild-type CRE into a perfect consensus does not modify the specificity of the proteins interacting with the TxRE, but drastically increases the amount of the complexes formed, as revealed by gel shift assays. Enhancement of CREB/ATF interaction parallels a higher basal promoter activity in heterologous D17 cells, but the relative response to the PKA pathway is abrogated, since the consensus CRE3x promoter is already fully activated even in the absence of exogenous CREB and PKA.

Role of the LTR mutations in vivo. As stated in the introduction, the main goal of this report is to clarify the roles of different LTR motifs that have been suggested by cell culture experiments to be important in transcriptional regulation. In the first part of this report, we have characterized in vitro a series of novel mutants (pLTR-NF1, pLTR-NF2, pLTR-Ebox3x, pLTR-CRE3x, and pLTR- Δ 21-bp) and others already described in the literature (pLTR-IRF and pLTR-GRE), based on an LTR isolated from the pathogenic provirus 344 (23, 26). Since opposite conclusions might be drawn concerning the role of a given sequence in LTR activity depending on the cell line used (44), we decided to evaluate the impact of these mutations in the context of an infectious BLV molecular clone in the sheep model. To this end, a series of recombinant proviruses harboring the LTR mutations (pBLV-IRF, pBLV-GRE, pBLV-NF1, pBLV-NF2, pBLV-Ebox3x, pBLV-CRE3x,

pBLV- Δ 21-bp, and pBLV-WT) were constructed based on the backbone of a pathogenic BLV strain (clone 344). To assess the integrity of the mutated proviruses, we first performed two preliminary experiments designed to evaluate their capacity to express viral proteins in cell culture. Plasmid DNAs containing the different proviruses were cotransfected into D17 cells with the pLTR-WT reporter (containing the wild-type LTR inserted upstream of the luciferase gene). Forty-eight hours posttransfection, luciferase activity was measured in order to indirectly determine Tax expression and the amount of p24 major capsid antigen was titrated by ELISA. It appears that the eight proviruses expressed similar levels of Tax activity (Fig. 4A) and p24 (Fig. 4B), indicating that the LTR mutations introduced in the 3' LTR did not, as expected, impede viral protein synthesis in cell culture. More importantly, these assays, in addition to preliminary restriction analysis and sequencing (see Materials and Methods for construction details), support the ability of the proviruses to be correctly transcribed in vitro.

Each recombinant provirus was then injected intradermally into two seronegative sheep, and as a control, two animals were also infected in parallel with the wild-type strain 344. Surprisingly, all the sheep seroconverted a few weeks postinfection, indicating that none of the mutations or deletions interfered with viral infectious potential. It thus appears that even large deletions in the NF- κ B sites (mutants NF1 and NF2) or between the distal and middle TxREs (Δ 21-bp) did not abolish infectivity in vivo. To evaluate the capacities of the mutants to persist and propagate within the host, proviral loads were estimated by semiquantitative PCR 3 and 6 months after seroconversion. Cell lysates were prepared from blood isolated by jugular venipuncture of all infected sheep, and the *tax* sequences were amplified by PCR. As a control for quantification, serial dilutions (10- and 100-fold) of DNA isolated from an infected sheep (sheep 2664) were amplified in parallel. After PCR, the amplicons were hybridized by Southern blotting using a *tax* probe (Fig. 5). No *tax* sequences were amplified from DNA isolated from an uninfected sheep (sheep 115) and used as a negative control. Amplification of viral sequences from the infected sheep confirmed that all the recombinant viruses were indeed infectious in vivo. At 3 months, mutants IRF (sheep 2670 and 2671), GRE (sheep 2660 and 2661), NF1 (sheep 2666 and 2667), NF2 (sheep 2658 and 2659), Ebox (sheep 2668 and 2669), and Δ 21-bp (sheep 2664 and 2665) propagated at levels similar, to those of the wild-type provirus (sheep 2672 and 2673) or even slightly faster (Fig. 5). In contrast, proviral loads in sheep, 2662 and 2663, infected with the consensus CRE3x mutant, were drastically reduced at 3 months and almost disappeared at later times (6 months).

Together, these data demonstrate that all BLV recombinants were infectious in the animal model and, most interestingly, that the consensus CRE3x mutant was drastically impaired in viral propagation. In other words, our results indicate that the presence of perfect CRE sites in the LTR correlates with an attenuation phenotype during infection of sheep.

Before a final conclusion can be drawn, our experiments require an essential control demonstrating the lack of reversion of the mutants into a wild-type sequence. To answer this question, LTR fragments were amplified by PCR and the resulting amplicons were directly sequenced. As partly shown in

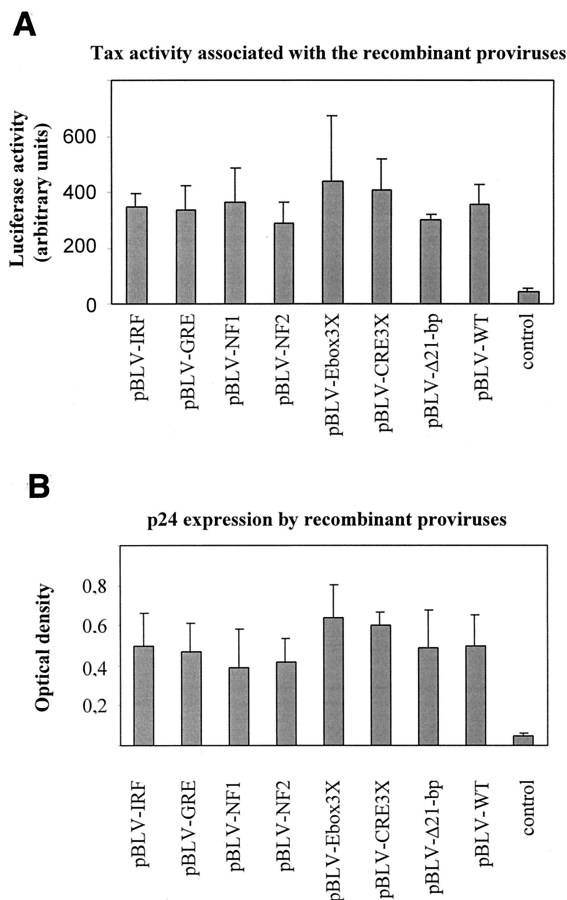


FIG. 4. Abilities of recombinant proviruses to express Tax-associated activity and p24 proteins in cell culture. Mutant proviruses (pBLV-IRF, pBLV-GRE, pBLV-NF1, pBLV-NF2, pBLV-Ebox3x, pBLV-CRE3x, pBLV-Δ21-bp, and pBLV-WT) and a control plasmid (pSG5) were cotransfected with pLTRWT reporter (in which the 5' LTR sequence of the wild-type 344 provirus is inserted upstream of the luciferase gene). Forty-eight hours posttransfection, luciferase activities in the cell extracts were determined (A) and the major capsid p24 antigen was titrated in the supernatants using an ELISA procedure (B). Data derive from three independent experiments.

Fig. 6 (data not shown for animals 2659, 2661, 2662, 2665, 2667, 2669, 2671, 2672, and 2673), amplification and sequencing of the LTRs clearly demonstrated that the mutations were perfectly preserved in the corresponding infected sheep.

We conclude that the phenotypes associated with the recombinant viruses (infectivity and propagation) do not result from a reversion of the expected mutation.

DISCUSSION

The aim of this report was to evaluate the role of specific transcription factor binding sites in LTR promoter function as well as to establish a correlation between these *in vitro* activities and viral replication *in vivo*. A series of interesting data were obtained from transient transfection experiments in cell culture, and several unexpected observations were derived from the sheep model (summarized in Table 2).

Dispensability of the NF-κB sites *in vivo*. DNase footprint experiments have identified two major protected areas located between the middle and proximal TxREs (7). Although poorly conserved κB binding sites comprised within this region confer responsiveness to p50/p65 NF-κB proteins (6), site-directed mutations had opposite effects *in vitro* depending on the cell line used (44). Our results show that deletion of the sequences corresponding to the first (mutant NF1) or the second (mutant NF2) footprint had no effect, either on LTR basal transcription *in vitro* or on viral spread *in vivo*. It thus appears that quite large modifications might be introduced within this region without affecting viral replication in sheep. Furthermore, two of the animals infected with mutants NF1 (sheep 2667) and NF2 (sheep 2658) did develop leukemia 15 and 19 months postseroconversion, respectively, providing further support for the dispensability of these sequences during pathogenesis. However, we cannot exclude the possibility that the footprints exert a mutual compensatory effect and that simultaneous deletion of both regions would yield a tangible phenotype. In any case, our results demonstrate that strict conservation of these NF-κB binding sites is not essential either for LTR activity *in vitro* or for viral propagation *in vivo*.

No effect of the IRF motif in the context of an infectious molecular clone. The U5 region of the LTR contains an IRF consensus sequence that interacts, as shown by gel shift assays, with the IRF-1 and -2 proteins (23). Site-directed mutation of this motif disrupting the interactions induced a slight (twofold) reduction in basal transcription of an LTR isolated from the T15 BLV strain (23). We did not confirm this observation using an LTR derived from another molecular clone (provirus 344); the variations were not statistically significant (Fig. 2B and C). At present, we do not understand the reason for this discrepancy, but our experiments underline the importance of the viral strain in revealing the role of the IRF motif. Of note, the T15 clone, which was isolated from a tumor in cattle, has not been shown to be infectious and could constitute a dead-end provirus, as is frequently observed at late stages of pathogenesis. In any case, the IRF site appears to be dispensable both *in vitro* and *in vivo* in the context of an infectious and pathogenic molecular clone (strain 344).

The GRE: reduction in basal transcription *in vitro*. Just upstream of the proximal TxRE, a GRE confers responsiveness to dexamethasone in the presence of the Tax transactivator (26), and mutation of this GRE site significantly decreases basal LTR activity in reporter-based assays (44). We confirmed this observation using the 344 backbone despite a variation in the GRE sequences, (a C-to-T transition at position -65). *In vivo*, mutation of the GRE abrogating the dexamethasone response did not modify viral infectivity or propagation in sheep. However, it should be mentioned that our experimental infection protocol in sheep is based on direct intradermal injection of cloned proviral DNA, and we cannot exclude the possibility that specific steps occurring during natural infection require the integrity of the GRE motif. In particular, the GRE could be necessary for transmission via mammary epithelial cells (as suggested by Buehring et al. [8]). Our protocol based on proviral DNA injection, however, is probably a more relevant mimic of other infection routes such as skin contact, biting insects, or blood transfer via contaminated needles.

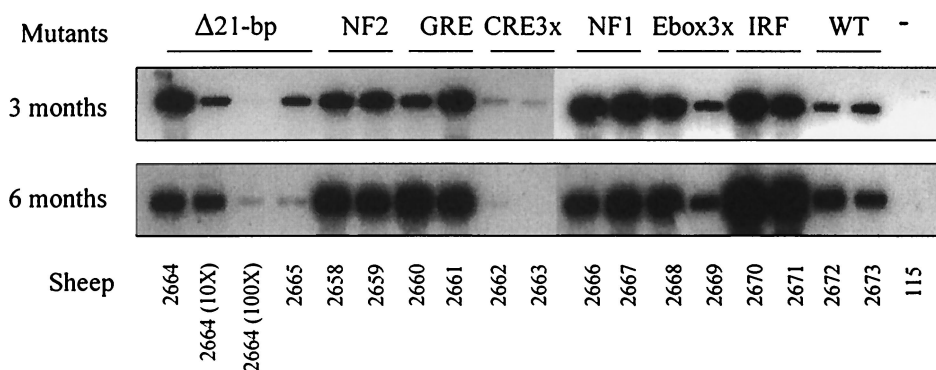


FIG. 5. Proviral loads in sheep infected with BLV mutant proviruses. The wild-type (pBLV-WT) provirus and each recombinant (pBLV- Δ 21-bp, pBLV-NF2, pBLV-GRE, pBLV-CRE3x, pBLV-Ebox, and pBLV-IRF) provirus was injected into two different sheep as indicated. Three and six months after seroconversion, blood was isolated by jugular venipuncture and the corresponding lysates were amplified by 25 cycles of PCR using two primers flanking the *tax* gene. Under these conditions, PCR amplifications yielded semiquantitative estimation of the proviral loads as shown by 10-fold dilutions (10 $\times$ and 100 $\times$) of a lysate from an infected sheep (sheep 2664). As a negative control, a lysate from an uninfected sheep was tested in parallel (sheep 115). After PCR, the resulting amplicons were analyzed by Southern blotting using a *tax* probe.

The E-box motif: increase in basal transcription. The E box is a dual-function element binding numerous proteins, such as AP4 or the Myc/Max/Mad complex, acting either as transcriptional activators or as repressors (17, 18). Interestingly, this type of motif overlaps the CREs in each of the three TxREs that constitute the enhancer of the BLV LTR. A subtle mutation (CANNTG $\rightarrow$ CANNGA), which does not affect the overlapping CRE, provokes a slight, but significant (by the Student *t* test) and reproducible, increase in LTR basal promoter activity (Fig. 2B and C). This enhancement of transcription indicates that the E-box element acts as a repressor, perhaps directly regulating the overlapping CRE. Our conclusion contrasts with the interpretation provided in the report of Unk and colleagues (36) but not with their data, since the E-box mutation designed by these authors also disrupted the CRE motif. Of note, concerning the proteins interacting with the E-box motif of the TxRE, we were unable to identify AP4 using an antiserum cross-reacting with the ovine homologue (kindly provided by R. Gaynor) (data not shown). In contrast, antibodies against USF-1 and -2 specifically supershifted the TxRE complex using a modified version of the gel retardation assay (E. Adam, unpublished data). Further experiments will be required to characterize the different factors involved in the interaction with the E box.

Concerning the role of the E box in the context of a molecular clone, mutation of this motif within all three TxREs of the U3 region did not lead to any alteration of viral spread in sheep. Perhaps a disruption of an additional E-box motif situated in the R region of the LTR will be required to induce a phenotype *in vivo*. Currently ongoing experiments indeed show that mutation of this fourth E-box site decreases LTR activity during transient transfection experiments, indicating that this motif acts as an activator sequence (C. Calomme *et al.*, unpublished data). Our results here demonstrate that the three other E boxes located in the TxREs rather mediate a repressor effect *in vitro*. In sheep, however, simultaneous mutation of the three E boxes of U3 does not alter viral spread and pathogenesis. One of the animals infected with provirus pBLV-Ebox3x (sheep 2668) indeed developed leukemia 19 months postinfection, whereas sheep 2669 died from unrelated causes.

The excision of a TxRE still permits infection *in vivo*. The TxREs are major regulators of LTR function; a deletion of the internal CRE leads to a decrease in transcriptional activity (44). In fact, only two of these TxRE enhancers are required to confer a Tax response on heterologous promoters (13), and it is thus not surprising that our Δ 21-bp mutant exhibits a wild-type phenotype in transient reporter assays. The Δ 21-bp promoter indeed contains two complete copies of TxREs, the first of which is a hybrid between the distal and middle 21-bp enhancer elements. Our data thus indicate that, in the absence of a mutated version of a TxRE (generated, for example, by a deletion of the CRE, leaving the flanking E box intact), the LTR (44) harboring two complete TxREs is fully active. What was far less expected is that a provirus carrying this kind of truncated promoter is infectious in sheep. We conclude that a drastic deletion (between residues -148 and -124) within this crucial region of the LTR does not interfere with infectivity. Furthermore, viral propagation also appears to be unaffected in one out of two sheep (in sheep 2664 but not in sheep 2665). This intermediate phenotype does not permit us to draw a final conclusion concerning the dispensability of one of the three TxREs in BLV-associated pathogenesis. However, the wild-type behavior of the Δ 21-bp mutant in sheep 2664 demonstrates that two full copies of TxREs are sufficient to maintain efficient viral spread, perhaps depending on the host genetic background. Most importantly, primate T-lymphotropic proviruses harboring only two TxRE copies have been isolated (37, 38). Since enhancer duplications of LTR sequences have been implicated in pathogenesis induced by murine retroviruses (14,

TABLE 2. Conclusions drawn from *in vitro* and *in vivo* experiments

Mutant	Basal activity <i>in vitro</i>	Viral spread <i>in vivo</i>
NF1	Wild type	Wild type
NF2	Wild type	Wild type
IRF	Wild type	Wild type
GRE	Slight decrease	Wild type
EBox3x	Slight increase	Wild type
Δ 21-bp	Wild type	Intermediate
CRE3x	Drastic increase	Attenuated

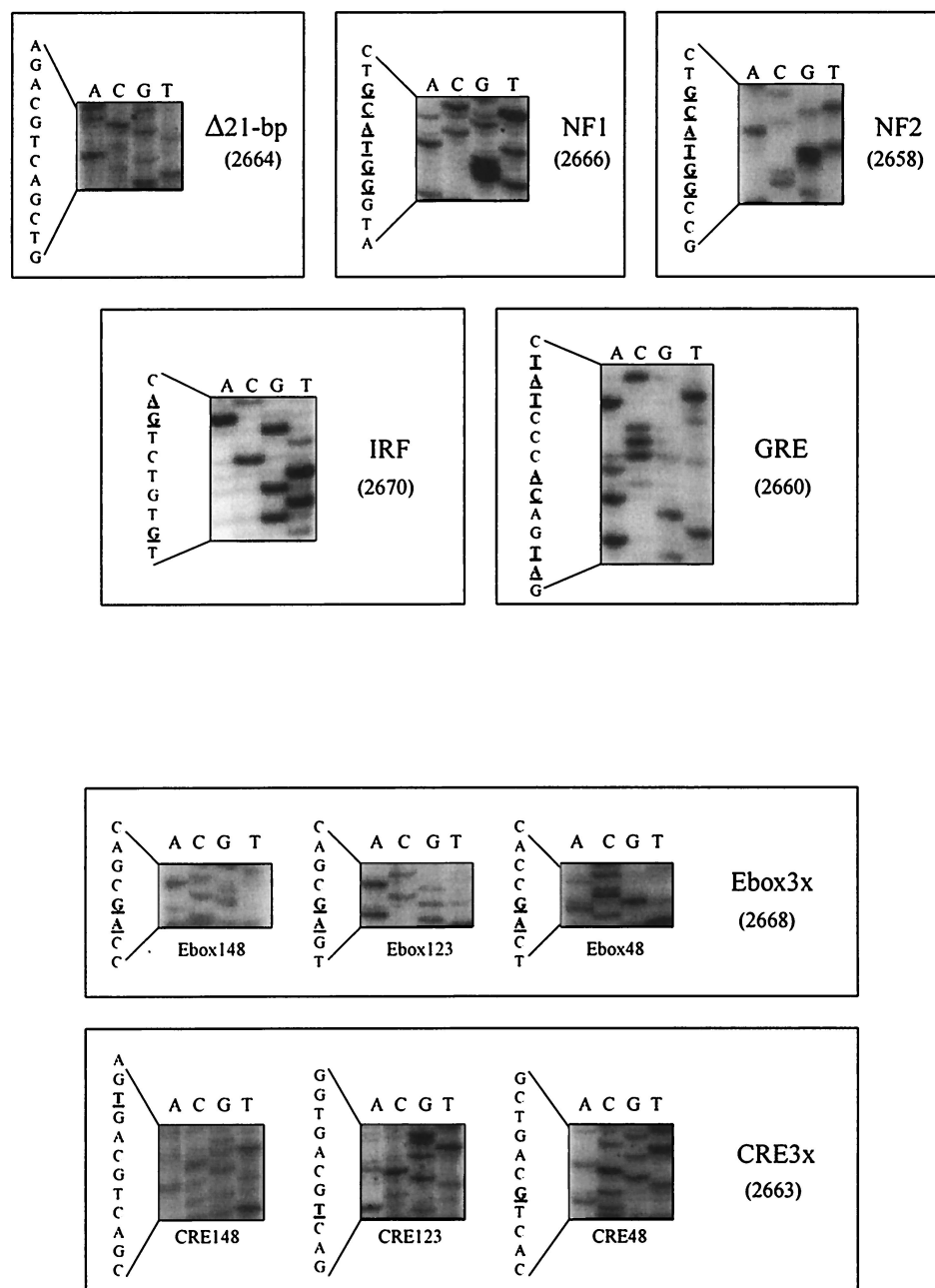


FIG. 6. Direct sequencing of 5' LTRs in sheep infected with BLV recombinants. At 6 months postseroconversion, cell lysates were prepared from the blood of sheep (sheep 2664, 2666, 2658, 2670, 2660, 2668, and 2663) infected with mutant proviruses (pBLV- Δ 21-bp, pBLV-NF1, pBLV-NF2, pBLV-IRF, pBLV-GRE, pBLV-Ebox3x, and pBLV-CRE3x, respectively) and the 5' LTRs were amplified by PCR. The amplicons were then directly sequenced by PCR and transferred to a denaturing polyacrylamide gel. The sequences surrounding the mutations introduced into the LTR are displayed.

25, 33, 34), it would be interesting to compare the Δ 21-bp mutant with the wild-type provirus 344 during the process of leukemogenesis, for instance, at the level of cell target specificity or the length of the latency period preceding tumor induction.

Mutant CRE3x: drastic increase in basal transcription parallels with viral attenuation. Perhaps the most striking result of our study is provided by the CRE3x mutant, which contains a triple substitution of the imperfectly conserved CRE. Recon-

stitution of a consensus CRE has a drastic effect in transient transfection experiments, leading to a 20-fold induction in basal transcriptional activity in lymphocytes. This enhancement in LTR promoter function correlates with an increase in complex formation using lysates prepared from freshly isolated sheep PBMCs. It makes sense that an increase in CREB/ATF binding correlates with a reconstitution of a perfect CRE; similar observations have been made in the HTLV-1 system (5, 45). These results support a model in which transcriptional

silencing of the LTR results from the presence of nonconserved CRE motifs associated with a reduction in the formation of complexes between the TxREs and the CREB/ATF factors. Single CRE mutations, as well as their combinations, indicate that the proximal TxRE (−48) exerts a major effect in the induction of basal transcription (Fig. 2D). Since deletion of the CRE of this particular element also has a more drastic effect than deletion of the distal or middle TxRE in terms of Tax responsiveness (44), it is possible that the close proximity of the TATA box and the CAP transcriptional initiation site might augment the relative importance of the proximal 21-bp enhancer. Interestingly, the −48 TxREs (both wild type and consensus) display lower complex formation efficiency than the −123 and −148 enhancers (Fig. 3B). The mechanisms involved in LTR functioning appear to be somewhat different in the HTLV system, in which a central role has been assigned to the middle CRE (3). It should be mentioned here that a major difference between HTLV and BLV TxREs concerns the sequences flanking the CREs: an E box in the case of BLV and GC-rich residues for HTLV. For both viruses, however, the TxREs always harbor imperfectly conserved CREs, further supporting the biological relevance of this modification. In one of the recently sequenced BLV clones, the distal TxRE even contains a dual mutation (underlined) in the CRE: AAACG TCA (15). Together these observations suggest that a selection pressure occurred during evolution to maintain this type of modification.

Using our experimental animal model, we were able to test the importance of these CRE alterations *in vivo*, based on only three nucleotide changes introduced into the LTR of an infectious and pathogenic BLV molecular clone. Interestingly, reconstitution of a perfect CRE consensus within the LTR does not abrogate infection of sheep but induces a drastic reduction in the efficiency of viral propagation (Fig. 5). Direct sequencing of the 5′ LTRs amplified from the expanding viruses demonstrates that no reversion of the initial mutation occurred, even in a subpopulation of the clones (Fig. 6). More importantly, since the mutations were introduced in the 3′ LTRs (see Materials and Methods for details of the constructions), the identification of the perfect CREs in the 5′ LTR demonstrates that the recombinant virus indeed replicated *in vivo*. Although the first round of replication of the transfected provirus is under the control of a wild-type promoter, subsequent cycles generated an infectious virus harboring the mutations in both LTRs. Furthermore, the continuous presence of significant levels of anti-BLV antibodies within animals 2662 and 2663 supports the idea of a permanent infection by the CRE3x mutant. Altogether, it appears that the CRE3x mutant is infectious but that its ability to propagate inside the sheep host is drastically impaired.

In conclusion, our data establish a link between an increase in basal transcriptional activity and a concomitant reduction in viral spread *in vivo*. Our results suggest that suboptimal CRE sequences were selected during evolution in order to avoid transcriptional activation by cellular CREB/ATF factors. We propose that these alterations would have allowed a better silencing of viral transcription in the presence of various stimuli activating the B lymphocyte. This strategy would thus also permit hiding from recognition by the host immune response.

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