Upstream Stimulatory Factors Binding to an E Box Motif in the R Region of the Bovine Leukemia Virus Long Terminal Repeat Stimulates Viral Gene Expression

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The bovine leukemia virus (BLV) promoter is located in its 5'-long terminal repeat and is composed of the U3, R, and U5 regions. BLV transcription is regulated by cis-acting elements located in the U3 region, including three 21-bp enhancers required for transactivation of the BLV promoter by the virus-encoded transactivator TaxBLV. In addition to the U3 cis-acting elements, both the R and U5 regions contain stimulatory sequences. To date, no transcription factor-binding site has been identified in the R region. Here sequence analysis of this region revealed the presence of a potential E box motif (5'-CACGTG-3'). By competition and supershift gel shift assays, we demonstrated that the basic helix-loop-helix transcription factors USF1 and USF2 specifically interacted with this R region E box motif. Mutations abolishing upstream stimulatory factor (USF) binding caused a reproducible decrease in basal or Tax-activated BLV promoter-driven gene expression in transient transfection assays of B-lymphoid cell lines. Cotransfection experiments showed that the USF1 and USF2a transactivators were able to act through the BLV R region E box. Taken together, these results physically and functionally characterize a USF-binding site in the R region of BLV. This E box motif located downstream of the transcription start site constitutes a new positive regulatory element involved in the transcriptional activity of the BLV promoter and could play an important role in virus replication.

Claire Calomme**, Thi Liên-Anh Nguyên**, Yvan de Launoit**, Véronique Kiermer**, Louis Droogmans**, Arsène Burny**, and Carine Van Lint**

From the ¤Université Libre de Bruxelles, Institut de Biologie et de Médecine Moléculaires, Service de Chimie Biologique, Laboratoire de Virologie Moléculaire, Rue des Prof Jeener et Brachet 12, 6041 Gosselies, Belgium, (CVL); Université Libre de Bruxelles, Faculté de Médecine, Laboratoire de Microbiologie, 808 Route de Lennik, 1070 Bruxelles, Belgium, (CMR); Institut Pasteur de Lille, Institut de Biologie de Lille, UMR 8526 CNRS, 1 Rue Calmette BP 447, 59021 Lille Cedex, France, and **Université Libre de Bruxelles, Laboratoire de Microbiologie, Institut de Recherche du CERIA, 1 Avenue Emile Groyon, 1070 Bruxelles, Belgium

Bovine leukemia virus (BLV)1 is a B-lymphotropic oncogenic retrovirus that infects cattle and is associated with enzootic bovine leukosis, a neoplastic proliferation of B-cells (1–6). BLV is closely related to human T-lymphotropic viruses HTLV-I and -II. The majority of BLV-infected cattle are asymptomatic carriers of the virus. Only about 30% of BLV-infected animals develop a preneoplastic condition termed persistent lymphocytosis, with 2–5% developing B-cell leukemia and/or lymphoma after a long latency period. The virus can be experimentally transmitted to sheep, in which it causes similar pathologies, providing a helpful model to understand BLV and HTLV-induced leukemogenesis. BLV infection is characterized by viral latency in the large majority of infected cells and by the absence of viremia. These features are thought to be due to the transcriptional repression of viral expression in vivo (7–9). The latency is likely to be a viral strategy to escape the host immune response and allow tumor development.

BLV transcription initiates at the unique promoter located in the 5'-long terminal repeat (5'-LTR) of the BLV genome. The 5'-LTR is composed of the U3, R, and U5 regions and transcription initiates at the U3-R junction. The U3 region contains the main sites that regulate viral transcription (Fig. 1) as follows: the promoter CAAT and TATA boxes (10, 11), a glucocorticoid-responsive element (12–15), and a large segment protected in DNase footprinting assays containing NF-xB-related sites (16, 17). Among the most important sites are three copies of an imperfectly conserved 21-bp sequence harboring in the middle a common 8-bp core sequence known as the cAMP-responsive element (CRE). At least three proteins, CRE-binding protein (CREB) and activating transcription factors-1 and -2 (ATF-1 and ATF-2), bind to these 21-bp enhancers in bovine B-lymphocytes, and the amount of generated complex correlates with the level of viral expression (18, 19). The 21-bp enhancers are also called Tax-responsive elements (TREEs) because transactivating

The abbreviations used are: BLV, bovine leukemia virus; EMSA, electrophoretic mobility shift assay; CRE, cAMP-responsive element; CREB, CRE-binding protein; ATF-1 and ATF-2, activating transcription factors-1 and -2; USF, upstream stimulatory factor; NF-xB, nuclear factor-xB; IRF, interferon regulatory factor; nt, nucleotide; LTR, long terminal repeat; HTLV, human T-lymphotropic virus; HIV-1, human immunodeficiency virus, type 1; TxAE, Tax-responsive element; HLH, helix-loop-helix; bHLH, basic HLH; ZIP, leucine zipper; PMA, phorbol myristate acetate; DAS, downstream activator sequence; PBMCs, peripheral blood mononuclear cells; EBV, Epstein-Barr virus; CaMKIV, calcium/calmodulin-dependent protein kinase IV; TK, thymidine kinase; wt, wild-type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; luc, luciferase.

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† Fellow of the FNRS-Télévie.
¶ Fellow of the Belgian “Fonds pour la Recherche dans l’Industrie et l’Agriculture (FRIA).”
†† Present address: The Gladstone Institute of Virology and Immunology, University of California, San Francisco, CA 94141.
‡‡ “Chercheurs Qualifiés” of the “Fonds National de la Recherche Scientifique” (Belgium).
¶¶ To whom correspondence should be addressed: Université Libre de Bruxelles, Institut de Biologie et de Médecine Moléculaires, Service de Chimie Biologique, Laboratoire de Virologie Moléculaire, Rue des Profs Jeener et Brachet, 12, 6041 Gosselies, Belgium. Tel.: 32-2-650-9807; Fax: 32-2-650-9800; E-mail: cvlint@dbm.ulb.ac.be.

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Regulation of the BLV Promoter through USFs

**Fig. 1. Transcription factor-binding sites in the 5'-LTR of the BLV genome.** The 5'-LTR contains the “CAAT” (nt −97/−92) and “TATA” (nt −43/−37) boxes upstream of the transcription initiation site at the U3-R junction (mRNA start site at +1 is indicated by an arrow). The TxREs are three imperfectly repeated sequences of 21 bp. These are major transcriptional enhancers, which interact with the cellular transcription factors CREB, ATF-1, and ATF-2. Transcriptional activation of the BLV LTR by the viral encoded TaxBLV transactivator requires these enhancers. Moreover, the activity of the factors CREB, ATF-1, and ATF-2 is increased by protein kinases A and CaMKIV. Each of the 21-bp enhancers contains a sequence homologous to the consensus E box-binding motif (referred to as E-box1, E-box2, and E-box3) overlapping an imperfect CRE (referred to as CRE1, CRE2, and CRE3). The U3 region also contains a glucocorticoid-responsive element (GRE) and a large segment protected in DNase footprinting assays (nt −118/−70) containing NF-xB-related sites. Outside of U3, a DAS (nt +147/−210) in the R region and an IRF-binding site (nt +246/−269) in the U5 region, interacting with IRF-1 and IRF-2, are regulatory sequences important for BLV gene expression. The E box motif identified in the present report and located in the R region is also indicated and is referred to as E-box4.

The objective of the present study was to characterize physically and functionally the E-box4 motif located in the BLV R region. We performed competition and supershift gel shift assays with nuclear extracts prepared either from peripheral blood mononuclear cells (PBMCs) derived from a BLV-infected sheep or nuclear extracts from the human B-lymphoid cell line Raji. We demonstrated that the bHLH transcription factors USF1 and USF2 specifically interacted with the E-box4. A 2-bp mutation (central CG to TA) and another 2-bp mutation (3' TG to GA) abrogated USF binding. To assess the transcriptional regulatory function of the E-box4, we tested the effect of these 2-bp mutations by transient transfection of B-lymphoid cell lines in the context of an LTR-luciferase reporter construct in presence or absence of a TaxBLV expression vector. Both mutations caused a reproducible 25% decrease in LTR-driven basal gene expression, indicating a positive functional role of the E-box4 motif in R. Ectopically expressed USF1 and USF2a proteins had an E-box4-dependent stimulatory effect on both the homologous BLV promoter and a heterologous thymidine kinase (TK) promoter containing multiple upstream E-box4 motifs. Mutation in the E-box4 impaired the responsiveness of the BLV promoter to TaxBLV but not to other activators known to up-regulate BLV expression (overexpression of CREB2, calcium/calmodulin-dependent protein kinase IV (CaMKIV), or CREB2/CaMKIV and treatment with PMA/ionomycin). Moreover, mutation in the E-box4 in combination with a mutation in the IRF site in U5 decreased the LTR basal activity more than 2-fold. The identification of the E-box4 motif represents the first transcription factor-binding site reported in the R region of BLV.

MATERIALS AND METHODS

PBMC Isolation—The animal used in this study was a BLV-seropositive adult sheep (M298) affected with persistent lymphocytosis, presenting a persistently elevated lymphocyte count and an inverted B/T-lymphocyte ratio. This sheep was housed at the Veterinary and Agrochemical Research Center (Ucel, Belgium). Blood samples were
collected by jugular venipuncture, mixed with EDTA as an anticoagulant, and centrifuged at 1750 × g for 25 min at room temperature. The PBMCs were then isolated by Percoll gradient centrifugation (density 1.129 g/ml; Amersham Biosciences) and washed twice in phosphate-buffered saline containing 0.075% EDTA, with centrifugation steps at 1000 × g for 10 min at room temperature. At last, the cells were incubated at 4°C in 450 ml of phosphate-buffered saline (centrifugations at 200 × g for 10 min at room temperature) until the supernatant became clear.

Cell Lines and Cell Culture—The Raji and Daudi cell lines are human B-lymphoid EBV-positive cell lines derived from Burkitt’s lymphomas. The human epithelial HeLa cell line is derived from a cervical carcinoma and transfected by human papilloma virus (HPV). These lines were obtained from American Type Culture Collection (ATCC) and were maintained in RPMI 1640 medium containing 5% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 10 mM HEPES buffer, and centrifuged at 1750 × g for 5 min for three rounds of centrifugation. The last nucleotide of U3 are considered as the 5′ end (5′-GGTTTTCTGCAGCGACATTTGAAATAGATCACCGGACGAGGTTCCGACATAGGGCCG-3′). The primers PCR products were purified using the QIAQuick PCR purification kit (Qiagen) and ligated into the pCRII vector (Invitrogen). Three mutated plasmids were designated pLTR(E-box1,2-mutA)-luc, pLTR(E-box1,2-mutB)-luc, and pLTR(E-box1,2-mut)-luc, respectively, of an oligonucleotide primer oligonucleotide pairs (mutations are highlighted in bold), respectively, into the 5′ and 3′ PCR primers, and the HindIII-BamHI-restricted primer oligonucleotide corresponding to nt -118 to -83 contained an added pSel site (in bold) at the 5′ end (5′-GCCCTTCAAGGACCCCTCCACGC-3′). The 3′ primer oligonucleotide corresponding to nt +55 to +83 contained an added pSel site (in bold) at the 5′ end (5′-GGTTTTCTGCAGCGACATTTGAAATAGATCACCGGACGAGGTTCCGACATAGGGCCG-3′). The 3′ primer oligonucleotide corresponding to nt +1571 to 1598 contained an added BamHI site (in bold) at the 5′ end (5′-GCCGAGCTCT-1159ATCTAATTTTCTTATGAACAAATGAG-3′). The 3′ primer oligonucleotide corresponding to nt +1571 to 1598 contained an added BamHI site (in bold) at the 5′ end (5′-GCCGAGCTCT-1159ATCTAATTTTCTTATGAACAAATGAG-3′).

The resulting plasmid was designated pCDNA3-cMyC. The eukaryotic expression vectors pSG-TAX_BLV and pSG-CREB2 (44) were gifts from Drs. Luc Willems and Robert Kettmann. The expression plasmid pcCMV is a gift from Dr. Anthony Means (45). The USF1 and USF2 expression vectors (kindly provided by Drs. A. Kahn and M. Raymondjean) contained the human USF1 and USF2 cDNAs cloned in the pCR3 expression vector parent plasmid (Invitrogen) and were described previously (46).

Transient Transfection and Luciferase Assays—Raji cells were transfected by using the DEAE-dextran procedure as described previously (47). Briefly, cells were harvested at density of 106/ml, washed with STBS (25 mM Tris-HCl (pH 7.5), 137 mM NaCl, 5 mM KCl, 700 μM CaCl2, 500 μM MgCl2, 600 μM NaPO4), and resuspended at a concentration of 6 × 105 in 600 μl of a mixture containing 500 μg of the pGL3-BASIC-derived constructs (with or without cotransfected DNAs) and 450 μg of DEAE-dextran (Amersham Biosciences/ml in STBS. Cells were incubated for 1 h at 37°C, washed twice with STBS and once with culture medium. Cells were then lyzed and assayed for luciferase activity (Promega).

Luciferase activities derived from BLV LTRs were normalized with respect to protein concentrations using the Detergent-compatible Protein Assay (Bio-Rad).

Daudi cells were transfected by electroporation. Cells were harvested in exponential growth phase and resuspended in supplemented RPMI at a density of 5 × 104/ml in 200 μl of a mixture containing 5 × 104 per 400 μl. The 400 μl of DNA were mixed with 8 μg of pGL3-BASIC-derived constructs and 50 ng of pRL-TK (Promega) and incubated for 15 min at room temperature, transferred to electroporation vials, and electroporated at 250 V with a Bio-Rad gene pulser.

Transfected cells were plated out immediately in 4 ml of preheated medium (a 1:1 mixture of fresh culture medium and supernatant of a 24-h culture), and grown for 48 h at 37°C. All transfection mixtures contained the pRL-TK, in which a cDNA encoding Renilla luciferase is under the control of the herpes simplex virus thymidine kinase promoter region and is used as an internal control for transfection efficiency. At 48 h post-transfection, luciferase activities (firefly and Renilla) were measured in cell lysates, and firefly luciferase activities detected from the BLV promoters were normalized with respect to the Renilla luciferase activity by using the dual luciferase reporter assay system (Promega).

The pE-box1, TK-luc and pE-box4, TK-luc were generated by inserting three direct repeats in the forward orientation and seven direct repeats in the reverse orientation, respectively, of an oligonucleotide primer oligonucleotide pairs (mutations are highlighted in bold), respectively, into the 5′ and 3′ LED-PLC-gated reporter (Promega).

The pE-box4, TK-luc and pE-box4,mutB, TK-luc were generated by inserting three direct repeats in the forward orientation and seven direct repeats in the reverse orientation, respectively, of an oligonucleotide primer oligonucleotide pairs (mutations are highlighted in bold), respectively, into the 5′ and 3′ LED-PLC-gated reporter (Promega). The 5′-GGTTTTCTGCAGCGACATTTGAAATAGATCACCGGACGAGGTTCCGACATAGGGCCG-3′. The 3′ primer oligonucleotide corresponding to nt +55 to +83 contained an added pSel site (in bold) at the 5′ end (5′-GGTTTTCTGCAGCGACATTTGAAATAGATCACCGGACGAGGTTCCGACATAGGGCCG-3′). The 3′ primer oligonucleotide corresponding to nt +1571 to 1598 contained an added BamHI site (in bold) at the 5′ end (5′-GCCGAGCTCT-1159ATCTAATTTTCTTATGAACAAATGAG-3′).
addition to the DNA. Hundred microliters of this FuGENE™-0.6 serum-free medium mix was added to the DNA mixture in microcentrifuge tubes. The mixture was incubated for 15 min at room temperature and, finally, was added to each well of a 6-well plate. Transfected cells were grown in 2 ml of supplemented medium for 40–44 h. Cells were then lysed, and luciferase activities were determined by the addition of 200 μl of digestion buffer, and the single-stranded sequences were digested with RNase T1 and RNase A for 1 h at 37 °C. Following addition of 300 μl of inactivation buffer and ethanol precipitation, the protected RNA fragments were analyzed by electrophoresis through 6% urea polyacrylamide gels.

RESULTS

The BLV R Region E-box Motif Specifically Binds USF1 and USF2 in Vitro—A data base search for potential transcription factor-binding sites in the R region of the BLV LTR revealed the presence of a putative E box site (nt +173/+178 according to the sequence reported by Sagata et al. (41), 5′-CACGTTG-3′. In order to identify cellular factors that bind to this potential E box motif (designated E-box4), EMSAs were performed by using as probe a 22-bp E-box4-containing oligonucleotide corresponding to positions +165 to +186 of the 5′-LTR. This probe (referred to as E-box4-wt) was incubated with nuclear extracts from PBMCs derived from a BLV-infected sheep (BLV-infected sheep M298 presenting a persistently elevated lymphocyte count and an inverted B/T-lymphocyte ratio). A major retarded protein-DNA complex was detected with a major band indicated by an arrow and the fainter band indicated by an asterisk (Fig. 2A, lane 1). To evaluate the specificity of these interactions, unlabelled double-stranded oligonucleotides were prepared and used as competitors in the EMSAs. Binding of proteins in the major complex was shown to be sequence-specific by competition EMSAs. Indeed, the formation of this complex was competed for by an excess of the unlabeled homologous E-box4-wt oligonucleotide (Fig. 2A, lanes 2–5) but not by the same molar excess of a heterologous oligonucleotide of unrelated sequence containing the HIV-1 NF-κB sites (Fig. 2A, lanes 10–13). A well characterized E box motif from the HIV-1 promoter (51, 52) was also used as a competitor and inhibited the formation of the major complex as efficiently as the homologous oligonucleotide (Fig. 2A, lanes 6–9), demonstrating that the major complex was specific to the E box motif. In contrast, the specificity of the fainter and slower migrating band was uncertain because this band was competed by all oligonucleotides tested (the homologous and the heterologous oligonucleotides, Fig. 2A, and the two mutated E-box4 oligonucleotides, see below Fig. 3B), except by the HIV-1 E box oligonucleotide (Fig. 2A, lanes 6–9). Moreover, this fainter band was not affected by any of the E box-specific antibodies we used in supershift assays (Fig. 2B, see below) and was not further characterized. Similar results were obtained with nuclear extracts from the Raji cell line, a human B-lymphoid cell line (data not shown).

To identify directly the factors present in the specific major retarded complex, we performed supershift assays using antibodies directed against individual members of the bHLH family of transcription factors (Fig. 2B, left panel). Labeled E-box4-wt oligonucleotide probe was incubated with nuclear extracts from BLV-infected ovine PBMCs. Polyclonal antibodies directed against USF1, USF2, Max, Mad-1, Mad-2, Mad-3, Mad-4, c-Myc, and Mnt were added to the binding reaction mixture. Addition of either the anti-USF1 or the anti-USF2 antibody (Fig. 2B, left panel, lanes 2 or 3, respectively) interfered with the formation of the major complex and generated supershifted complexes of decreased mobility (Fig. 2B, left panel). Although the anti-USF1 antibody eliminated almost completely the major complex, the anti-USF2 antibody only slightly decreased the intensity of this complex. When both anti-USF1 and anti-USF2 antibodies were included in the
binding reaction, the entire complex was eliminated (Fig. 2B, left panel, lane 4). In contrast, the binding pattern was not affected by the addition of the antibodies directed against the other bHLH proteins (Fig. 2B, left panel, lanes 5–11), showing that the major retarded complex did not seem to involve these other proteins. No supershifted complex was observed with a control purified rabbit IgG (Fig. 2B, left panel, lane 1) or when the antibodies were added to the probe alone (data not shown), thus indicating the specificity of the protein-antibody interactions. Similar results were observed with Raji nuclear extracts (data not shown).

In order to establish the validity and/or the specificity of some of the antibodies other than anti-USF1/2 used in the supershift assays, control EMSAs were performed by incubating in vitro translated E box-binding proteins (Max, Mad1, and c-Myc) with a labeled CMD (specific E box) probe (50) (Fig. 2B, right panel). Addition of the anti-Mad1 or anti-c-Myc antibody interfered with formation of the Mad1-Max or c-Myc-Max complex (Fig. 2B, right panel, lanes 9–12, respectively). The anti-Max antibody supershifted the Max homodimers (Fig. 2B, right panel, lane 6) as well as the Mad1-Max (Fig. 2B, right panel, lane 8) and c-Myc-Max (Fig. 2B, right panel, lane 11) heterodimers.

Overall, our results demonstrate that both the USF1 and USF2 transcription factors interact with the E-box4 motif located in the BLV R region. Moreover, they suggest that the
predominant USF species that bind to this motif are the heterodimer USF1/USF2 and the homodimer USF1/USF1 and that a small amount of homodimer USF2/USF2 might also bind. These data are consistent with several reports (53–56) demonstrating that the major USF species present in most tissues and cell lines is the heterodimer between USF1 and USF2. USF1 homodimers are less abundant, and USF2 homodimers are usually quite scarce.

Identification of Point Mutations Abolishing USF Binding to the E-box Motif in the 5′-LTR BLV R Region—To further characterize physically the E-box motif located in the R region of the BLV 5′-LTR, we studied by EMSA the effect of selected mutations on binding affinity. Two mutations were designed to abolish binding of factors to the E-box motif. The first mutation, designated E-box4-mutA, consisted of the substitution of the two central nucleotides CG with the dinucleotide TA (57). The second mutation, designated E-box4-mutB, consisted of the substitution of the 3′-TG with the dinucleotide GA (58) (Fig. 3A).

The effects of these 2-bp mutations on binding affinity were analyzed by competition EMSAs with the E-box4-wt oligonucleotide as a probe and nuclear extracts from sheep PBMCs (Fig. 3B). The E box-specific retarded complex was inhibited by competition with an excess of the homologous oligonucleotide (Fig. 3B, lanes 2–5). In contrast, the complex was not competed by the E-box4-mutA and E-box4-mutB oligonucleotides (Fig. 3B, lanes 6–9 and lanes 10–13, respectively), demonstrating that both mutations abolished USF binding to the E-box4. Moreover, we confirmed the lack of USFs binding to probes corresponding to the mutated E-box4 motifs (data not shown). Similar results were observed with Raji nuclear extracts (data not shown).

Taken together, our results identify an E box motif in the R region of the BLV 5′-LTR (nt +173 to +178). This E box motif specifically binds the bHLH proteins USF1 and USF2 in vitro. We report a couple of 2-bp mutations, referred to as E-box4-mutA and E-box4-mutB, that abrogate USF binding to this motif.

The E-box4 Motif in the R Region of the BLV 5′-LTR Is Required for Optimal Basal Gene Expression from the BLV Promoter—In order to examine the functional role of the E-box4 motif in the basal transcriptional activity of the BLV promoter, the same 2-bp mutations described above (E-box4-mutA and E-box4-mutB) were introduced by site-directed mutagenesis in the context of a pLTRBLV-luciferase reporter construct, called pLTRwt-luc and containing the firefly luciferase (luc) gene under the control of the complete 5′-LTR (nt –211 to +320) of the 344 BLV provirus. Strain 344 is an infectious and pathogenic molecular clone. The two mutated plasmids were designated pLTR(E-box4-mutA)-luc and pLTR(E-box4-mutB)-luc, respectively.

To assess the transcriptional regulatory function of the E-box4 motif, the constructs pLTRwt-luc, pLTR(E-box4-mutA)-luc, and pLTR(E-box4-mutB)-luc were transiently transfected into Raji cells. At 44 h post-transfection, cells were lysed and assayed for luciferase activity. Results presented in Fig. 4A show that both mutations, E-box4-mutA and E-box4-mutB, reproducibly reduced the basal activity of the BLV promoter by 25%. These results are consistent with the binding of a transcriptional activator, such as USF1 and/or USF2, to the E-box4 motif.

To confirm these results in another B-lymphoid cell line, the same reporter constructs (pLTRwt-luc, pLTR(E-box4-mutA)-luc, and pLTR(E-box4-mutB)-luc) were transiently transfected into the human cell line Daudi. As shown in Fig. 4B, the E-box4-mutA mutation caused a 15% decrease in basal LTR-directed gene expression. Interestingly, the E-box4-mutB mutation more strongly decreased LTR activity because it caused a 56% decrease in BLV 5′-LTR-driven basal gene expression. These functional results confirmed in another B-cell line the results observed in Raji cells. However, the E-box4-mutA and E-box4-mutB mutations decreased LTR basal activity with less strength and more strength, respectively, in Daudi cells than observed in Raji cells. These differential effects of both mutations between the two cell lines may result from differences in the expression, binding, and/or transcriptional activity of USF proteins, transcriptional cofactors, and/or other DNA-binding proteins.

Our functional results thus demonstrate a positive regulatory role of the E-box4 motif located in the R region of the BLV 5′-LTR in Tax-independent BLV promoter-driven gene expression.

Ectopic Expression of USF1 and USF2α Transcription Factors Up-regulates BLV Promoter Activity in Part through the E-box4 Motif—To examine the role of USF in regulation of the BLV promoter, we studied the effect of overexpression of USF on luciferase activity. Cotransfection of expression constructs
for USF1 and/or USF2a with the pLTRwt-luc reporter construct had no significant effect on luciferase activity in the transformed Raji cell line (data not shown). Considering that USF is abundantly expressed in most cell types, one possible explanation is that USFs would be non-limiting factors for the BLV promoter activity in Raji cells. Moreover, recent studies comparing USF function in different normal and cancerous breast cell lines have demonstrated that a partial or complete loss of USF transcriptional activity is a common event in transformed cells as compared with normal cells, even though there is no difference both in expression and DNA-binding activity of USF proteins (56, 59). These latter studies suggest a correlation between the loss of USF function and tumorigenesis. Therefore, the potential inactivation of USF in the transformed Raji cells we used is another possible explanation. Furthermore, USF was originally identified and characterized in HeLa cells (30, 60–64), and it has been demonstrated that USF proteins are transcriptionally active in this cell line (59).

Together, these observations prompted us to examine in HeLa cells whether USF1 or USF2a overexpression had an effect mediated by the E-box4 on BLV promoter activity. We performed cotransfection experiments using USF1 or USF2a expression vectors and the pLTRwt-luc reporter construct as well as the mutated derivative pLTR(E-box4-mutA)-luc. As shown in Fig. 5, USF1 and USF2a transactivated the BLV promoter in a dose-dependent manner up to 4- and 3.2-fold, respectively. Maximal activation was seen at concentrations of 2000 ng of USF1 (Fig. 5A) and 250 ng of USF2a (Fig. 5B). When the amounts of USF2a were further increased, the stimulation was reduced. When expressed at very high levels, many transcriptional activators squelched activated transcription (65). This squelching phenomenon is thought to be due to the sequestration in solution of transcriptional components, preventing their interaction at gene promoters. Moreover, cotransfection of both USF1 and USF2a together in equal amounts resulted in transactivation levels of the BLV promoter similar to those obtained with USF1 and USF2a cotransfected separately (data not shown). This observation has also been reported in numerous other studies (66–71) and is consistent with the idea that USF homodimers can function as well as heterodimers, although in purified nuclear extracts USFs were generally found as heterodimers (72).

However, when analyzing in the same cotransfection assays the effect of USF1 and USF2a overexpression on the mutated construct pLTR(E-box4-mutB)-luc, we observed that mutation of the E-box4 did not reduce the USF stimulatory effect. Indeed, USF1 and USF2a transactivated pLTR(E-box4-mutB)-luc as efficiently as the wild-type construct pLTRwt-luc (Fig. 5, A and B), indicating that other LTR region(s) could mediate partially or totally transactivation by USF. These other regions could mask the potential transactivation mediated by the E-box4 motif. Because three E boxes are located in the BLV LTR U3 region (E-box1, -2, and -3, Fig. 1), we evaluated the potential contribution of these motifs in the LTR response to USF1 and USF2a. Mutant reporter constructs were generated, in which the E-box1, -2, and -3 were mutated and in which all four E boxes were mutated. The plasmids were designated pLTR(E-box1,2,3-mutB)-luc and pLTR(E-box1,2,3,4-mutB)-luc, respectively, and were used in cotransfection experiments of HeLa cells with the USF1 and USF2a expression vectors. As shown in Fig. 5, A and B, mutation of the E boxes 1–3 in combination attenuated the stimulatory effect of USF1 and USF2a. There was a 28% (USF1) and 39% (USF2a) reduction of stimulation compared with the USF stimulations observed with pLTRwt-luc. Interestingly, when all four E boxes were mutated, we observed a 53% (USF1) and 57% (USF2a) reduction of stimulation, suggesting that USF proteins act in part through the E-box4 motif to stimulate BLV promoter activity. It is noteworthy that, although a mutated LTR in which all four E boxes were mutated was strongly impaired in terms of stimulation by USF, this mutated LTR was still responsive to ectopic USF.

These results thus indicate that the E-box4 plays a role in the transactivation of the BLV promoter by USF but that the
three other E boxes located in U3 are also responsible for part of the USF response. Moreover, besides these four E boxes, other cis-elements that are directly or indirectly affected by USF seem to contribute to the residual USF induction observed with the total mutant construct pLTR(E-box1,2,3,4-mutB)-luc.

**FIG. 5.** Response of the BLV promoter to ectopically expressed USF transcription factors. HeLa cells were transiently cotransfected with 500 ng of an LTR luciferase reporter construct wild type or mutated in the different E boxes (as indicated) and increasing amounts (0, 100, 250, 500, 1000, and 2000 ng) of either the USF1 or the USF2a expression vector, pCR3-USF1 or pCR3-USF2a (A or B, respectively).

**FIG. 6.** Ability of multimerized BLV E-box4 motifs to confer USF stimulation to a TK minimal promoter. HeLa cells were transiently cotransfected with 500 ng of a TK luciferase reporter construct devoid or not of upstream wild-type or mutated E-box4 motifs (as indicated) and increasing amounts (0, 100, 250, 500, 1000, and 2000 ng) of either the USF1 or the USF2a expression vector, pCR3-USF1 or pCR3-USF2a (A or B, respectively). To examine the USF inducibility of these reporter constructs, HeLa cells were transiently cotransfected with 500 ng of a TK luciferase reporter construct containing wild-type or mutated E-box4 motifs (as indicated) and increasing amounts (0, 100, 250, 500, 1000, and 2000 ng) of either the USF1 or the USF2a expression vector, pCR3-USF1 or pCR3-USF2a (A or B, respectively). The control pTK-luc construct was moderately transactivated by USF1 (up to 3.5-fold) and by USF2a (up to 7.7-fold). Cotransfection of USF1 or USF2a expression vectors with the reporter constructs containing the wild-type E-box4 motifs resulted in a stimulation of luciferase activity that was dependent on the number of E boxes [p(E-box4)TK-luc versus p(E-box4)TK-luc]. Addition of three copies of the E-box4 motif upstream of the TK promoter resulted in a dose-dependent increase in luciferase activity by ectopically expressed USF1 (up to 8.2-fold) and
USF2a (up to 21.1-fold), thus representing a 2.3- and 2.7-fold up-regulation when compared with the USF response of the control pTK-luc devoid of upstream E-box4 motifs. Seven copies of the E-box4 further increased the USF stimulation: 4.5-fold (USF1) and 4-fold (USF2a) up-regulation compared with the pTK-luc. This effect required intact E-box4 motifs, because mutations in these motifs [p(E-box4-mutB)3TK-luc (Fig. 6, A and B) and p(E-box4-mutA)3TK-luc (data not shown)] resulted in levels of USF-mediated transactivation similar to those obtained with the control pTK-luc. Moreover, using each TK reporter construct, the combination of USF1 and USF2a transfected together in equal amounts had no more effect than either factor transfected alone (data not shown).

We conclude from these experiments that ectopic USF1 and USF2a proteins have an E-box4-dependent stimulatory effect on the heterologous TK promoter containing multiple upstream E-box4 motifs. These results thus establish the functional significance of USF through the BLV E-box4 motif.

The E-box4 Motif in the R Region of the BLV 5’-LTR Regulates Tax-dependent BLV Promoter-driven Gene Expression—Efficient transcription and replication of the BLV genome require both the viral LTR and the virus-encoded transcriptional activator TaxBLV, which functions through the TxREs in U3. In the next experiments, we studied the effect of the E-box4-mutA and E-box4-mutB mutations on the responsiveness of the BLV promoter to TaxBLV. Toward this end, cotransfections of Raji cells were performed using either pLTRwt-luc, pLTR(E-box4-mutA)-luc, or pLTR(E-box4-mutB)-luc and increasing amounts of both pSG-CREB2 (5, 15, 30, 60, and 120 ng) and pCaMKIV (0, 50, 150, 300, and 600 ng). To maintain the same amount of transfected DNA and to avoid squelching artifacts, the different amounts of pSG-CREB2-pCaMKIV cotransfected were complemented to 1200 ng of DNA by using the empty plasmids pSG5 and a pCMV-based vector, respectively. Luciferase activity was measured in cell lysates 44 h after transfection. The results are presented as histograms indicating luciferase activities (arbitrary units) normalized to protein concentrations (top panel) or indicating the induction by TaxBLV (in fold) with respect to the activity of the same reporter construct in the absence of TaxBLV, which was assigned a value of 1 (bottom panel). Means and standard errors of the means from four independent transfections performed with at least two different DNA preparations are shown.

**Fig. 7.** Effects of the E-box4 mutations on Tax-dependent BLV promoter-driven gene expression. A, Raji cells were transiently cotransfected with 500 ng of either pLTRwt-luc, pLTR(E-box4-mutA)-luc, or pLTR(E-box4-mutB)-luc and increasing amounts of the TAXBLV expression vector pSG-TAXBLV (0, 1, 2, 4, and 8 ng of plasmid DNA). To maintain the same amount of transfected DNA and to avoid squelching artifacts, the different amounts of pSG-TAXBLV cotransfected were complemented to 8 ng of DNA by using the pSG5 empty plasmid. Luciferase activity was measured in cell lysates 44 h after transfection. The results are presented as histograms indicating luciferase activities (arbitrary units) normalized to protein concentrations (top panel) or indicating the induction by TaxBLV (in fold) with respect to the activity of the same reporter construct in the absence of TaxBLV, which was assigned a value of 1 (bottom panel). Means and standard errors of the means from six independent transfections performed with at least two different DNA preparations are shown.
by 21–26 and by 29–44%, respectively. These results demonstrate a positive regulatory role of the E-box4 motif in Tax-dependent BLV promoter-driven gene expression.

We next wanted to test the effect of the E-box4-mutA and E-box4-mutB mutations on the activation of the BLV LTR by other stimuli. It has been reported previously (19, 44) by transient transfections of D17 osteosarcoma cells that the bovine CREB2 protein is able to transactivate the BLV LTR in the absence of the TaxBLV protein and that the cAMP-dependent protein kinase A or the calcium/calmodulin-dependent protein kinase IV (CaMKIV) substantially increases the ability of CREB2 to stimulate gene expression. Moreover, ex vivo, BLV expression can be up-regulated by several lymphocyte activators, including phorbol ester PMA (73) and calcium ionophore ionomycin (74).

We performed transient cotransfections of Raji cells with either pLTRwt-luc, pLTR(E-box4-mutA)-luc, or pLTR(E-box4-mutB)-luc and increasing amounts of both the CREB2 expression vector, pSG-CREB2, and the CaMKIV expression vector pCaMKIV. At 44 h post-transfection, luciferase activity was measured in cell lysates. As shown in Fig. 7B, CREB2/CaMKIV activation of the wild-type LTR ranged from 61.7- to 2201-fold. In comparison, CREB2/CaMKIV activation of the pLTR(E-box4-mutA)-luc and of the pLTR(E-box4-mutB)-luc ranged from 76.4- to 2656-fold, and from 95.1- to 2836-fold, respectively. Thus, in contrast to what we observed with TaxBLV, the E-box4-mutA and E-box4-mutB mutations did not reduce CREB2/CaMKIV responsiveness of the BLV LTR compared with the wild-type construct pLTRwt-luc. Similar results were observed in cotransfection experiments using either pSG-CREB2 alone, pCaMKIV alone, or after treatment with PMA/ionomycin (data not shown).

Thus, our results demonstrate that mutations of the E-box4 motif located in the BLV R region impaired the responsiveness of the viral promoter to the viral protein TaxBLV, but not to other activators known to up-regulate BLV expression.

The Effects of the Mutations in the E-box4 Motif Occur at the Transcriptional Level—In order to demonstrate that the amount of transcription (i.e. RNA levels) has been affected by the point mutations in the E-box4 motif, transcript levels in transiently transfected Raji cells were measured by RNase protection assays using probes proximal and distal to the BLV promoter (Fig. 8). The proximal probe, which overlaps the start of transcription in the BLV reporter plasmids, stretches from nt. –118 to +83 and therefore hybridizes to all transcripts that initiate at the BLV LTR to produce a protected species of 83 nt. The distal probe, producing a 225-nt protected luciferase product, can only detect transcripts that have extended into the luciferase gene and therefore provides a measure of elongation. We failed to observe any reporter transcripts in the absence of the transactivator TaxBLV with both the BLV promoter-specific probe and the luciferase gene-specific probe (data not shown), probably as a consequence of the weak BLV promoter activity in absence of TaxBLV and of the weak transcription efficiency of the DEAE-dextran procedure. Because TaxBLV is known to increase considerably the BLV LTR transcriptional activity and because the E-box4-mutA and E-box4-mutB mutations reduced both the basal and TaxBLV-activated BLV-driven gene expression (see top of Figs. 4 and 7A, respectively), we decided to analyze the RNA synthesis in the presence of TaxBLV to facilitate the evaluation of the luciferase gene expression directed by the wild-type and mutated LTRs. To this end, we performed RNase protection assays using RNAs extracted from Raji cells transiently cotransfected with either pLTRwt-luc, pLTR(E-box4-mutA)-luc, or pLTR(E-box4-mutB)-luc and 25 ng of the TaxBLV expression vector, pSG-TAXBLV. As expected, the E-box4-mutA and E-box4-mutB mutations reduced the luciferase activity by approximately 2-fold (Fig. 8A). In the same experiment, analysis of the steady-state mRNA level showed that these mutations also reduced transcript production, as detected with the proximal BLV promoter-specific probe or with the distal luciferase gene-specific probe (Fig. 8B, lanes 2 and 3). As an internal control, RNase protection analysis of the same RNA samples using an antisense riboprobe corresponding to the GAPDH gene showed no change in the level of mRNA. We thus demonstrate that the mutations A and B introduced in the E-box4 motif decrease the amount of transcription directed by the BLV promoter. These results are consistent with those of the LTR-luciferase assays and show that the effects of the E-box4 mutations occur at the level of transcription.

Mutations of Both the R Region E-box4 Site and the U5 Region IRF Site Decrease Basal Gene Expression from the BLV Promoter to a Greater Extent Than the Individual Mutations—Previous studies (22) from our laboratory have identified a binding site for the interferon regulatory factors IRF-1 and
Raji cells were transiently transfected with 500 ng of either pLTRwt-luc, the single mutants pLTR(E-box4-mutA)-luc, pLTR(E-box4-mutB)-luc, and pLTR(IRFmut)-luc, or the double mutants pLTR(E-box4-mutA/IRFmut)-luc and pLTR(E-box4-mutB/IRFmut)-luc. Luciferase activity was measured in cell lysates 44 h after transfection. The results are presented as histograms indicating luciferase activities (arbitrary units) normalized to protein concentrations. Means and standard errors of the means from four independent transfections performed with at least two different DNA preparations are shown.

IRF-2 in the first half of the BLV U5 region and have shown that mutation in this site causes a decrease in Tax-independent BLV LTR gene expression. Together with the E-box4 site described in this report, the IRF site in U5 constitutes the only two characterized transcription factor-binding sites located downstream of the BLV transcription start site. Therefore, double mutant constructs were generated in which both the E-box4 and the IRF site were mutated. The constructs pLTRwt-luc, pLTR(E-box4-mutA)-luc, and pLTR(E-box4-mutB)-luc were used as substrates to introduce a 3-bp mutation in the IRF motif. The three mutated plasmids were designated pLTR(IRFmut)-luc, pLTR(E-box4-mutA/IRFmut)-luc, and pLTR(E-box4-mutB/IRFmut)-luc, respectively. We tested the functional effects of the E-box4-mutA and E-box4-mutB mutations in combination with the mutation of the IRF-binding site by transient transfection of these plasmids into Raji cells. As shown in Fig. 9, mutation of the E-box4 motif resulted in a 22% reduction and a 35% reduction (depending on the mutation) of LTR-directed luciferase expression. Mutation of the IRF motif by itself decreases basal BLV promoter activity to a greater degree than the individual mutations.

In this report, we clearly demonstrated a significant decrease in BLV promoter activity accompanied by the R region E-box4 motif and indicate that transcription factor-binding sites at the R/U5 junction are critical for optimal basal BLV promoter activity.

**DISCUSSION**

In this report, we have physically and functionally characterized an E box motif (referred to as E-box4) located in the R region of the BLV 5′-LTR. We have demonstrated by competition and supershift EMSAs that the bHLH transcription factors USF1 and USF2 bound in a sequence-specific manner to the E-box4-containing sequence. Mutations abolishing USF binding caused a reproducible decrease in BLV promoter-driven gene expression in transient transfection assays of B-lymphoid cell lines. Cotransfection experiments showed that the USF1 and USF2a transactivators were able to act through the BLV R region E-box4. Moreover, combined mutation of both this E-box4 motif and the IRF site in U5 decreased the LTR basal activity to a greater degree than the individual mutations. This E box motif represents the first transcription factor-binding site reported in the BLV R region.

USF proteins belong to the bHLH-ZIP family of transcription factors, which includes c-Myc, Max (75), Mad (76), Mxi1 (77), AP4 (78), TFE2 (29), TFE3 (27), MiTF (79, 80), and ADD1 (81). USF was initially identified from HeLa cell nuclei and was shown to be necessary to stimulate transcription from the adenovirus major late promoter through the core sequence CACGTG (30, 60–62). USF factors are encoded by two distinct genes (the USF1 and USF2 genes) (53, 54, 63, 64, 82). The USF1 gene encodes a single 43-kDa protein. The USF2 gene, due to alternative splicing, encodes two proteins, USF2a and USF2b of 44 and 38 kDa, respectively. The three USF isoforms differ in their N-terminal moieties, whereas they have highly conserved bHLH-ZIP domains (53, 54). USF proteins form homo- and heterodimers both in vitro and in vivo (53, 82). Dimerization with other bHLH proteins has not been observed (27, 33, 75, 76, 83). Although expression of the USF1 and USF2 species is ubiquitous, different ratios of USF homo- and heterodimers are found in different cell types (53). The major USF species present in most tissues and cell lines is the USF1-USF2 heterodimer. USF1 homodimers are less abundant, and USF2 homodimers are usually quite scarce (53, 54). USF proteins are ubiquitous transcription factors that were initially considered to play a role in housekeeping functions (61). Furthermore, these USF proteins have also been recognized as important players in the transcriptional regulation of tissue-specific genes (26, 84–88) and in the specific response of genes to external modulators (89), as glucose (46, 90, 91). The mechanisms by which USF proteins contribute to these specific functions are yet unclear and may overlay several phenomena. USF-binding sites have been found, and the involvement of USF in transcriptional regulation has been studied in a number of cellular and viral genes (46, 90, 92–95).

In this report, we clearly demonstrated a significant decrease in BLV promoter activity accompanying mutation in the E-box4, indicating a positive functional role of this motif. Moreover, cotransfection experiments using USF1 and USF2a expression vectors demonstrated the ability of a DNA fragment corresponding to multiple E-box4 copies to confer USF inducibility to a minimal TK promoter in an E-box4-dependent manner. We also showed that ectopic USF1 and USF2a transcription factors up-regulated BLV promoter activity. This USF responsiveness of the BLV promoter was mediated in part through the E-box4 motif. However, the three other E boxes located in U3 were also responsible for part of the USF response. We are currently investigating the identity of the transcription factors binding to these three E boxes. Interestingly, we observed that mutation of all four E boxes did not com-
pletely attenuate the stimulatory effect of USF1 and USF2a in cotransfection experiments. Rather, there was a reduction of stimulation to $\sim 50\%$ that observed with the wild-type BLV promoter reporter construct. The presence of other yet unidentified E boxes in the BLV LTR could be a first explanation. However, computer analysis searches of the complete BLV LTR did not reveal any other CANNTG consensus sequence (data not shown), suggesting that non-E box cis-elements within the BLV promoter are directly or indirectly responsive to USF. A report by Desbarats et al. (96) documented a similar situation with the rat prothymosin-$\alpha$ intron enhancer that contains a critical E box; ectopic expression of USF was shown to equally transactivate reporter plasmids carrying this sequence with the E box either intact or mutated, indicating that activation did not occur entirely through the E box element. Similarly, activation by USF has been reported for a number of other promoters either lacking an E box or containing a mutated E box (93, 97, 98). Our results are consistent with these previous studies and suggest that USF may interact directly and indirectly with non-E box cis-elements in the BLV promoter region. Alternatively, overexpression of USF may be modulating the level or activity of other proteins or basal transcription factors that mediate the activity of the BLV promoter.

Studies by other investigators (60) have reported that USF is able to bind sequences other than CACGTG such as the initiator element in the adenovirus major late promoter. Moreover, some studies have shown that USF factors can stimulate transcription by direct interaction with members of the basal initiation complex as follows: the basal factor TFIID (60, 63), the initiator-binding protein TFIIF-I (99, 100), and TBP-associated factor TAFII55 (101). USF proteins have also been shown to interact with other transcription factors, such as Fra1 (102), c-Maf (103), Ets1 (104), and Sp1/Sp3 (105). Thus, substantial evidence indicates that USF is involved in regulating gene expression by direct binding to E boxes and/or initiator elements and by functionally interacting with basal and/or specific transcription factors other than USF. The present study showed that USF regulated the expression of BLV in part through four E boxes (the E-box-1–3 in U3 and the E-box4 in R). The residual USF induction observed with the LTR construct mutated in all four E boxes could result from USF action directly or indirectly through other cis-regulatory elements. Additional experiments will be necessary to test this hypothesis.

Our in vitro binding studies demonstrated that both USF1 and USF2a specifically interacted with the E-box4 motif. Our ex vivo functional studies showed that ectopic USF1 and USF2a proteins activated transcription of a reporter gene driven by either the homologous BLV promoter or a minimal heterologous promoter containing multiple upstream E-box4 motifs. In these latter studies, both USF1 and USF2a exerted their stimulatory effect with similar efficiency. Although from different genes, USF1 and USF2 display a high degree of homology in their C-terminal DNA binding domain and USF-specific region that is thought to play a role in transcriptional activation of promoters (53, 106). Moreover, studies with USF null mice (55) and specific genes such as L-type pyruvate kinase (54), liver fatty-acid synthase (66), and human dipeptidyl peptidase IV (107) have shown that USF1 and USF2 have overlapping, redundant activities and can transactivate some promoters with similar efficiency. Therefore, our results are consistent with these previous observations, because they demonstrate that the BLV LTR is another promoter activated equally well by USF1 or USF2a, suggesting that both USF genes are involved in BLV transcriptional activation.

The members of the bHLH transcription factor family have been divided into four classes (A–D) depending on the sequence of the canonical bHLH-binding site CANNTG (25). Class B proteins, which include c-Myc, Max, MyoD, myogenin, and USF, bind to CACGTG or CATGTC. The E-box4 motif identified here in the BLV R region is an E box class B consensus motif and accordingly was found to bind the class B proteins USF1 and USF2. We could not observe the binding of c-Myc to this motif by supershift experiments, even though Myc/Max heterodimers share the same binding site requirements as USF dimers. Interestingly, in vivo, the myc oncogene is overexpressed in B-lymphocytes from tumors (108) and PBMCs isolated from BLV-infected animals with persistent lymphocytosis (109). Moreover, it has been reported that levels of Myc/Max proteins in nuclear extracts are very difficult to detect, whereas USF is the major binding activity detected by in vitro assays with crude nuclear extracts from several cell types and species (98, 110–114), even from cells that are transformed by c-Myc (112). For all these reasons, although we did not observe Myc binding under our in vitro conditions, we considered the possibility that, under physiological conditions, the E-box4 motif would be nevertheless permissive for Myc interactions. Toward this end, we decided to analyze the consequences of c-Myc overexpression on the activity of the BLV promoter. We cloned the ovin c-Myc cDNA (43) in the eukaryotic expression vector pCDNA3, and we performed transient cotransfection experiments into both B-cell lines (Raji and Daudi) and non-B-cell lines (HeLa) of this Myc expression construct with the LTRBLV-luciferase vector in the absence or presence of a Max expression vector. Our results did not reveal any stimulatory effect of Myc/Max on the BLV promoter. Therefore, our data strongly suggest that USF proteins but not Myc-Max complexes are the functional transcription factors that can activate the E-box4 in the R region of BLV. However, it should be stressed that we did not investigate the factors that bind to the E-box4 motif in vivo, and therefore the binding of factors different from both USF and Myc cannot be excluded at this time.

Our data showed that the E-box4 positively regulated BLV LTR-directed gene expression both in the absence and presence of TaxBLV ex vivo and could therefore be involved in the early and late stages of viral infection, respectively. USF proteins, in conjunction with CREB, ATF-1, ATF-2 (18, 19), IRF-1, and IRF2 (22), could thus be important transcription factors involved in the initiation of BLV transcription. Indeed, the E-box4 motif may with others initiate a low level of transcription from the BLV LTR promoter and lead to the synthesis of small amounts of the TaxBLV transactivator, which could then amplify transcription of the viral genome. Although a 64-bp DAS at the 3′ end of the R region (+147 to +211) was reported previously (23), no transcription factor-binding site had been identified in or close to the DAS. The E-box4 motif described in this report is located right in the center of DAS and could therefore be, at least in part, responsible for the positive regulatory activity of DAS. Moreover, we have demonstrated that mutation of both the R region E-box4 site and the U5 region IRF site decreased TaxBLV-independent LTR-driven gene expression to a greater degree than mutation of either site separately. These results indicate that the two transcription factor-binding sites identified to date downstream of the transcription start site play a critical role in the basal transcriptional regulation of the BLV promoter. Downstream regulatory sequences have also been identified in the HTLV-I LTR. Indeed, a 45-bp element, which is located at the boundary of R-U5 and binds the YB-1 transcription factor, is required for TaxHTLV-LTR-indepen-
the Sp1 and Sp3 transcription factors to the HTLV-1 U5 region has been associated with transcriptional repression of the LTR (117, 118). Furthermore, it has been suggested that the interaction of CREB and ATF-2 with the R region of the HTLV-1 LTR is associated with viral latency (119, 120).

We have shown that the E-box4 motif also positively regulates BLV LTR-directed gene expression in the presence of TaxBLV, and could therefore be involved in the late stage of viral replication. How USF affects the responsiveness of the BLV LTR to TaxBLV, and whether this effect is mediated by direct or indirect interactions of USF with TaxBLV, remains to be established. Interestingly, the cellular transcription factor USF has been reported to cooperate with other viral transactivators, such as the E2 proteins of human papillomavirus type 16 and bovine papillomavirus type 1 (121), and the immediate-early protein 62 of varicella-zoster virus (93). USF has been demonstrated to interact with the adenovirus E1A protein, which stimulates transcription of adenovirus genes as well as a wide variety of other viral and cellular genes (122). Moreover, a few studies show that the Tax protein of HTLV-1 represses the expression of cellular genes through the bHLH transcription factors. First, Uittenbogaard et al. (123, 124) have demonstrated that members of the bHLH protein family mediate repression by the HTLV-1 Tax protein, including the transcriptional repression of the p53 gene. They show that this repression of p53 by TaxHTLV1 is dependent upon the p53 promoter E box element and is mediated by the class B bHLH proteins c-Myc and USF (124). Second, TaxHTLV1 has been shown to mask c-Myc function through a c-AMP-dependent pathway (125). The region in c-Myc perturbed by TaxHTLV1 is contained within a highly conserved transcription/activation domain. Their data provide evidence that the N-terminal portion of c-Myc is conformationally altered by TaxHTLV1, without additional effects on overall protein stability. A conformational disruption of this protein could account for an abrogation of the transactivation and transformation properties of Myc (125).

Third, the biological behavior of p56^ck is modulated by the expression of the viral regulatory tax gene in HTLV-1-infected T-cells through a mechanism of repression that involves the E box DNA recognition sequence encountered in the lck gene distal promoter (126). Fourth, a molecular mechanism for TaxHTLV1-mediated repression of the transcriptional activity of the bHLH myogenic MyoD protein has been proposed recently (127). In this study, the authors show that TaxHTLV1 binding to the KIX domain of the cellular coactivator p300 prevents MyoD from contacting this N-terminal domain of p300, thus resulting in repression of MyoD-dependent transcription (127). Regarding the results reported in the present study, it would be interesting to study the possibility that TaxHTLV1 may inhibit the transcriptional activation function of bHLH proteins, thereby interfering with cellular gene expression and/or BLV expression.

We have characterized a novel E box motif in the R region of the BLV LTR. Functionally important E box motifs have been identified in the regulatory regions of other viruses. Latency-associated promoter 1 (LAP1) of herpes simplex virus type 1 is required to generate a series of latency-associated transcripts in sensory neurons of latently infected animals, and a USF element and a CRE site contribute to LAP1 function during latency (128). The transcriptionally regulatory regions of the lymphomagenic Akv and SL3–3 murine leukemia retroviruses contain E box motifs that are important determinants for murine leukemia retrovirus transcriptional activity in lymphocytic cells (129). The murine sarcoma virus enhancer has six E box targets for MyoD family proteins (130). Activation of the Epstein-Barr virus (EBV) DNA polymerase promoter by the BRLF1 immediate-early viral transactivator is mediated through USF and E2F (131). The latent membrane protein 1 (LMP1) gene promoter in the EBV genome contains a silencing activity overlapping with a transcriptional enhancer in a region that contains an E box motif (71). The latter study shows that bHLH transcription factors Max, Mad1, USF, E12, and E47 and the corepressor mSin3A bind in vitro to this E box (71). The silencing and enhancer activities correlate with the binding of Max-Mad1 and of USF, respectively (71). Finally, four E boxes are present in the promoter region of human immunodeficiency virus type 1 (HIV-1) (51, 52, 132–135), and cooperative interaction of USF1 with Ets-1 is required for full transcriptional activity of the HIV-1 LTR in T-cells (104).

In summary, we provided evidence that the BLV R region E-box4 is required, and USFs are components for BLV promoter regulation by correlating functional assays and USF binding activities to the E-box4. Our findings contribute toward a clearer understanding of the transcriptional regulation of BLV. Further studies will focus on characterizing the physiological role of the R region E-box4 site in the context of the complete infectious BLV provirus. A BLV provirus mutated in this site will be constructed. This mutated provirus will be injected into sheep in order to test for infectivity and ability to induce pathogenesis. These in vivo experiments should provide new insight into the molecular mechanisms of BLV transcriptional activation.

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