Journal of Virology, Mar. 1996, p. 1990–1999 Vol. 70, No. 3
0022-538X/96/$04.00+0
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The CREB, ATF-1, and ATF-2 Transcription Factors from Bovine Leukemia Virus-Infected B Lymphocytes Activate Viral Expression

EMMANUELLE ADAM,[1,2] PIERRE KERKHOFS,[3] MARC MAMMERICKX,[3] ARSÈNE BURNY,[1,2] RICHARD KETTMANN,[2] AND LUCAS WILLEMS[2]*

Department of Molecular Biology, University of Brussels, B1640 Rhode-St-Genèse,[1] National Institute for Veterinary Research, B1180 Brussels,[3] and Faculty of Agronomy, B5030 Gembloux,[2] Belgium

Received 14 September 1995/Accepted 28 November 1995

Efficient transcription and replication of the bovine leukemia virus (BLV) genome require both the viral long terminal repeat (LTR) and the virus-coded transcriptional activator Tax, which functions through a 21-bp sequence (Tax-responsive element [TxRE]) which is repeated three times within the LTR. Since Tax does not bind directly to DNA, host cell transcription factors play a central role in BLV expression. Electrophoretic mobility shift assays with nuclear extracts prepared with infected bovine B lymphocytes revealed five TxRE-specific complexes (C1, C2, C3, C4, and C5). Here, by using a UV-induced indirect labeling technique (UV cross-linking) in conjunction with mobility shift assays, eight major polypeptides of 31, 33, 42, 46, 51, 57, 87, and 119 kDa were identified within these five complexes. Immunoprecipitation experiments identified the 57- and 119-kDa proteins as cyclic AMP response element-binding (CREB) proteins, the 46- and 51-kDa proteins as activating transcription factor-1 (ATF-1), and the 87-kDa as protein ATF-2. All of these proteins (except the ATF-1 protein of 51 kDa) belong to the complex C1, which is the major complex identified in freshly isolated BLV-infected lymphocytes from cattle with persistent lymphocytosis. In transient-cotransfection experiments, these three transcription factors were able to activate LTR-directed gene expression in the presence of protein kinase A or Ca^{2+}/calmodulin-dependent protein kinase IV. CREB protein, ATF-1, and ATF-2 thus appear to be the major transcription factors involved in the early stages of viral expression.

Bovine leukemia virus (BLV) is a type C lymphotropic retrovirus which is the etiological agent of enzootic bovine leukosis. Enzootic bovine leukosis is a chronic lymphoproliferative neoplasic disease of cattle and is often associated with persistent lymphocytosis (PL) and/or the development of B-cell lymphomas after irregular, long incubation periods (13, 24, 42, 59). PL is characterized by an increase in circulating B-lymphocyte counts (53) and appears in 30 to 70% of BLV-infected cattle. Lymphoid tumors are found in 2 to 5% of BLV-infected cattle that may have PL, whereas other infected cattle remain asymptomatic or aleukemic (13, 45, 59, 69). Infected B lymphocytes circulating in the blood of cattle with PL contain multiple copies of proviruses and represent a subset of the polyclonally expanded B-cell population, whereas cells from individual tumors are clonal with one to three copies of the provirus per cell (43, 46, 60). Usually, infected lymphocytes or tumor cells rarely express BLV antigens in vivo. However, these cells express BLV information after cultivation in vitro. Although BLV expression in vivo is thought to be blocked at the transcriptional level (4, 29, 44, 72), little is known about specific cellular events responsible for this viral latency and the derepression after in vitro culture. A plasma-blocking factor present in some virus-infected animals could be implicated in the inhibition of viral synthesis in vivo (29, 80). However, the nature of this factor and its mechanism of action have not yet been established.

Expression of BLV is regulated at the transcriptional level by its own gene product, Tax. The Tax protein activates in *trans* the transcription of the BLV genome through an enhancer sequence located in the U3 region of the viral long terminal repeat (LTR) (17, 75). The Tax-responsive enhancer contains three copies of an imperfectly conserved 21-bp sequence (also called Tax-responsive element [TxRE]). These repeats are centered at positions −148, −123, and −48 with respect to the RNA cap site and share the cyclic AMP (cAMP)-responsive element (CRE) core sequence (TGACGTCA). The CRE-like motifs found in each of the BLV 21-bp repeats play an essential role in Tax-mediated transactivation. Indeed, mutation of the CRE motif abolishes Tax responsiveness (17, 41). There is no evidence for direct binding of the Tax protein to the 21-bp enhancer DNA. Therefore, cellular proteins should mediate the Tax-induced transactivation. In cell culture, the Tax effect was shown to be mediated, at least in part, by the bovine CREB2 protein (77). This factor has been cloned from a BLV-induced tumor and identified by its ability to bind to the TxRE element (77). In addition to the TxRE elements, an NF-κB binding site has been identified between nucleotides −118 and −70 upstream of the RNA start site (12). NF-κB may also be a critical nuclear protein that regulates viral expression.

Ex vivo, BLV expression can be up-regulated by several lymphocyte activators, including lipopolysaccharides (46), anti-immunoglobulin M antibodies (46), phytohemagglutinin (PHA) (4, 66), pokeweed mitogen (46), and concanavalin A (46, 74). This suggests that immune activation of a latently infected cell could initiate viral expression in vivo (46, 66). Therefore, regulatory factors that normally control the expression of certain cellular genes involved in B-cell activation and growth could also direct BLV transcription. Recently, we analyzed the proteins binding the 21-bp enhancer by electrophoretic mobility shift assays (EMSA). Using nuclear extracts from ex vivo-isolated bovine B lymphocytes, we have identified five TxRE-

* Corresponding author. Mailing address: Molecular Biology and Animal Physiology Unit, Faculty of Agronomy, 13, Avenue Maréchal Juin, B5030 Gembloux, Belgium. Phone: 32-81-61.21.57. Fax: 32-81-61.38.88.

specific complexes (designated C1 to C5) (1). One of these complexes (C1) is the major complex identified in freshly isolated BLV-infected lymphocytes from cattle with PL. The CREB protein is one of the components of this C1 complex (1). This extends our previous observation showing that the bovine CREB2 protein in the presence of the cAMP-dependent protein kinase A (PKA) is able to induce BLV LTR-directed expression in the absence of the Tax protein (77). However, activation of the PKA pathway by using either forskolin or dibutyryl-cAMP (db-cAMP) did not enhance viral synthesis in B lymphocytes (1). In contrast, the phorbol esters (e.g., phorbol 12-myristate 13-acetate [PMA]) are able to upregulate viral protein synthesis (1, 37). Since PMA is known to activate protein kinase C (PKC), it is likely that the increase in BLV expression induced by PMA is mediated by this kinase. However, nothing is known about the precise role of PKC in the signaling pathway leading to the regulation of BLV expression. In vitro, PKC has been shown to phosphorylate and stimulate the dimer formation of CREB protein (79). In contrast, this kinase has not yet been implicated in the phosphorylation and transcriptional activation of CREB in vivo. The signaling pathways that mediate phosphorylation and activation of CREB are beginning to be defined. CREB was originally characterized as a transcription factor mediating responses to cAMP via phosphorylation by PKA at a single phosphoacceptor site, Ser-133 (28, 33, 54, 79). However, several recent studies have provided evidence that CREB can also mediate transcriptional responses to changes in intracellular Ca^{2+} concentrations. The effects of Ca^{2+} are mediated by the activation of members of the calmodulin-dependent kinase family (15, 62, 63). These kinases have been implicated in the phosphorylation of CREB Ser-133 in vivo by agents that increase the Ca^{2+} concentration (15, 63). Since the PKA pathway does not seem to be involved in the induction of BLV expression, the calmodulin-dependent kinases could play a role in the regulation of BLV expression in vivo by activating a cellular transcription factor specific for TxRE, i.e., the CREB protein. The Ca^{2+}-dependent signaling pathway could thus propagate a BLV LTR activation signal from the cytoplasm to the nucleus in a response of the infected cell to extracellular stimuli.

The present study was undertaken to identify the nuclear factors binding to the BLV 21-bp enhancer repeat and to characterize their effect on the regulation of BLV expression in cell culture.

MATERIALS AND METHODS

Cells isolation and culture conditions. The animals used in this study included one BLV-seronegative cow (B78) and one BLV-seropositive adult cow (B163) affected with PL and presenting a persistently elevated lymphocyte count (17.3×10^3 lymphocytes/mm^3) and an inverted B/T lymphocyte ratio (65% B cells and 12% T cells). These cattle were kept at the National Institute for Veterinary Research (Uccle, Belgium). By using EDTA as an anticoagulant, venous blood was collected by jugular venipuncture. Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation over Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.). The mononuclear cell layers were harvested, washed three times with phosphate-buffered saline (PBS), and suspended at a concentration of 2×10^6 cells per ml in culture medium (RPMI 1640 with 10% heat-inactivated horse serum [Gibco BRL], 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml). The cells were then cultured for 24 or 48 h in the presence of PMA (Sigma Chemical Co.) at a final concentration of 0.1 μM and PHA (BACTO-PHA-P) (Difco Laboratories) at 1 μg/ml. All cultures were incubated at 37°C in a 5% CO_2 air atmosphere.

Preparation of nuclear extracts. Nuclear extracts were prepared from bovine PBMCs or from B-cell-enriched populations prepared by negative selection as described previously (1). Briefly, cells were washed in PBS and resuspended in buffer A (10 mM Tris-HCl [pH 7.9], 1.5 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 0.5 M sucrose, 10 mM Na_2MoO_4, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were pelleted and resuspended in 4 volumes of buffer A. Nonidet P-40 was added at a concentration of 0.1%, and the cell suspension was incubated for 10 min at 4°C. The cells were washed once in the same buffer. Nuclear proteins were extracted in a high-salt buffer (20 mM Tris-HCl [pH 7.9], 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.6 M KCl, 10 mM Na_2MoO_4, 0.5 mM dithiothreitol [DTT], 0.5 mM PMSF) overnight at 4°C with gentle shaking. The nuclear extracts were then centrifuged at $126{,}300 \times g$ for 30 min, and the supernatants were dialyzed for 5 h against a low-salt buffer (50 mM Tris-HCl [pH 7.9], 0.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.1 M KCl, 10 mM Na_2MoO_4, 1 mM DTT, 0.1 mM PMSF). Precipitates were removed by centrifugation at $126{,}300 \times g$ for 30 min. The supernatants were dispensed in aliquots and stored at −80°C until used.

Probes for gel retardation assays (EMSA). The TxRE oligonucleotide (5′AAGCTGGTGACGTCAGCTGGT3′) was end labeled with polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and then purified by Sephadex G-25 chromatography (Eurogentec). For the UV cross-linking experiments, the probe was obtained by hybridization of two DNAs: a chemically synthesized oligonucleotide harboring 5-bromodeoxyuridine (5-BrdU) (Pharmacia) (5′AAGC-BrdU-GG-BrdU-GACG-BrdU-CAGC-BrdU-GG-BrdU-3′) and the oligonucleotide TxRE(Δ6) (5′ACCAGCTGACGTCAC3′). This probe was then ^{32}P labeled by using the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP (800 Ci/mmol; Amersham).

Gel retardation assays. The assays were carried out as previously described (64) with a modified running buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA [pH 8.3]). The nuclear extract (1.2 μg of total protein) was preincubated with 0.5 μg of poly(dI-dC) in 20 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) at room temperature for 30 min before addition of the labeled probe. The reaction mixture with the probe was further incubated for 30 min at room temperature and electrophoresed on a 6% nondenaturing polyacrylamide gel in 25 mM Tris (pH 8.3)–190 mM glycine–1 mM EDTA (pH 8.3) at 132 V (11 V/cm) for 2 h at room temperature. All gels were dried and exposed to an autoradiography film.

In the supershift experiments, the appropriate antibody (2 μl per sample) was added to the reaction mixtures 30 min after addition of the probe and incubated for an additional 15 min. The monoclonal anti-CREB-1 antibody (24H4B) (raised against the recombinant human CREB-1 protein), the antipeptide CREB-2 antibody (C-20) (raised against a synthetic peptide corresponding to amino acid residues 330 to 350 mapping to the carboxy terminus of human CREB-2), the monoclonal anti-ATF-1 antibody (C41-5.1) (raised against the recombinant human ATF-1 protein), the antipeptide ATF-2 antibody (C-19) (raised against a synthetic peptide corresponding to amino acid residues 487 to 505 mapping to the carboxy terminus of human ATF-2), the monoclonal anti-ATF-2 antibody (F2BR-1) (raised against the recombinant human ATF-2 protein), and the antipeptide ATF-3 antibody (C-19) (raised against a synthetic peptide corresponding to amino acid residues 194 to 212 mapping to the carboxy terminus of human ATF-3) were purchased from Santa Cruz Biotechnology, Inc. The polyclonal anti-CREB antibody was kindly provided by G. Schutz and W. Schmid.

UV cross-linking. In the UV cross-linking reactions, 10 to 20 μg of nuclear extract prepared from bovine PBMCs (or B-cell-enriched populations) was first incubated with 1 μg of poly(dI-dC) in 50 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) at room temperature for 30 min. The BrdU-substituted, ^{32}P-labeled TxRE probe was added, and the incubation was continued for an additional 30 min. The reaction mixture was then irradiated for 1 h in a 1.5-ml Eppendorf tube placed at 2 cm from the surface of a hand-held UV lamp (302 transilluminator [Lab-Center]). After irradiation, 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 mM Tris-HCl [pH 7.0], 2% SDS, 1.5 M β-mercaptoethanol, 5% glycerol, 0.01% bromophenol blue) was added. The mixture was boiled for 5 min and then electrophoresed at 45 V overnight on an SDS-polyacrylamide gel with 10 or 15% polyacrylamide. This gel was then dried and exposed to an autoradiography film.

To identify the proteins in the specific complexes, the protein-DNA mixture was electrophoresed after irradiation on a 6% nondenaturing polyacrylamide gel in 25 mM Tris (pH 8.3)–190 mM glycine–1 mM EDTA (pH 8.3) at 132 V (11 V/cm) for 2 h at room temperature (gel retardation assay). Specific complexes were visualized by autoradiography of the wet gel and excised with a razor blade. After incubation at room temperature for 1 h in SDS-PAGE loading buffer, the gel slices were "stuffed" into the stacking wells of an SDS-polyacrylamide gel with 10 or 15% polyacrylamide and electrophoresed at 45 V overnight. The gel was then dried and exposed to an autoradiography film. This second gel electrophoretically resolved the covalently linked protein-DNA complexes, as determined from the migration properties of the protein components.

Immunoprecipitation analysis. Nuclear proteins were labeled by using the UV-induced indirect labeling technique described above. After irradiation, the appropriate antibody (2 μl per sample) was incubated for 30 min at 37°C with the reaction mixture. After a 10-fold dilution with radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% Triton X-100, 1‰ sodium deoxycholate, 0.1% SDS, 40 mM PMSF, and 1% NaN_3), the reaction mixtures were incubated overnight at 4°C. After addition of 100 μl of slurry protein A-Sepharose CL4B (Pharmacia) and incubation for 2 h at room temperature, the Sepharose beads were washed four times with radioimmunoprecipitation assay buffer and twice with Last buffer (50 mM Tris-HCl [pH 7.2], 50 mM NaCl). The

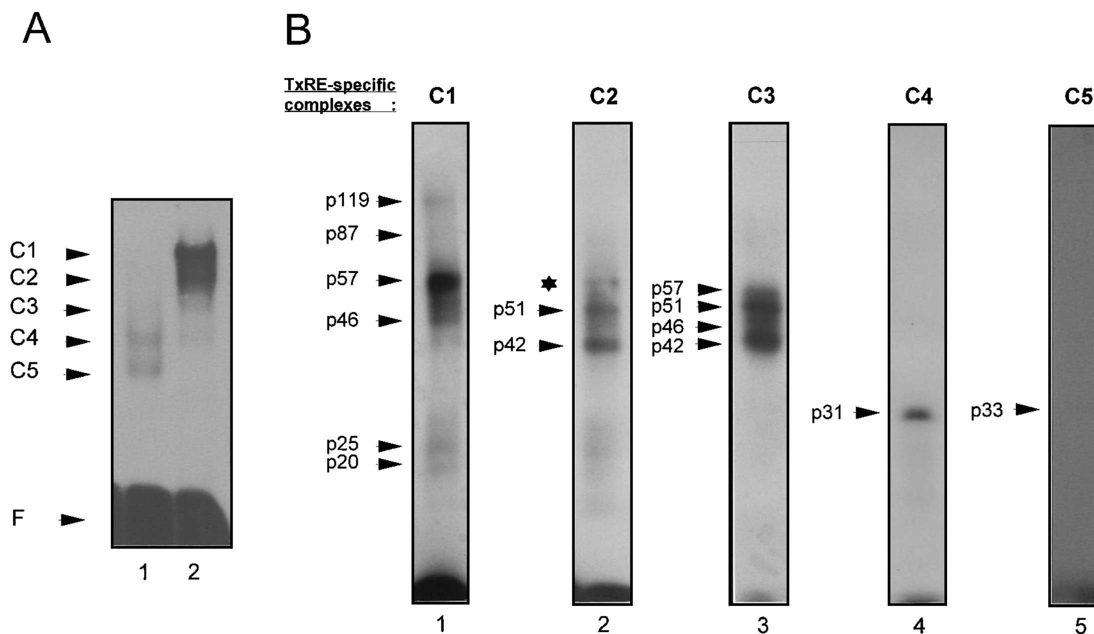


FIG. 1. Characterization of the proteins present in the five TxRE-specific complexes by cross-linking experiments. (A) Gel shift profile with the BrdU-substituted TxRE oligonucleotide. The BrdU-substituted, ^{32}P-labeled 21-bp oligonucleotide (TxRE probe) was incubated with a nuclear extract prepared from noninfected B lymphocytes after 48 h of culture without an inducer (lane 1) or from infected PBMCs (from a cow [B163] with PL) after 24 h of culture in the presence of PHA (1 μg/ml) and PMA (0.1 μM) (lane 2). C1 to C5, specific protein-DNA complexes; F, free probe. (B) The BrdU-substituted ^{32}P-labeled, 21-bp oligonucleotide (TxRE probe) was incubated with nuclear extracts (20 μg of total protein) prepared with PBMCs from an infected cow (B163) with PL (lanes 1, 2, and 3) or with B lymphocytes from an uninfected cow (B78) (lanes 4 and 5). These cells were freshly isolated (lanes 1 and 2) or cultured for 24 h with PHA (1 μg/ml) and PMA (0.1 μM) (lane 3) or for 48 h without an inducer (lanes 4 and 5). The reaction mixtures were irradiated with UV light for 1 h, and free and protein-complexed ^{32}P-labeled DNA species were separated by gel retardation. Polyacrylamide slices containing adducts of the ^{32}P-labeled DNA and protein formed with ^{32}P-labeled TxRE were excised from the retention gels and analyzed directly on denaturing SDS–15% polyacrylamide gels. The nucleoprotein adducts are indicated. The star represents a contaminant protein from the C1 complex.

supernatants were then collected and boiled for 5 min in an equal volume of SDS-PAGE loading buffer, and the samples were resolved on an SDS–15% polyacrylamide gel.

Plasmid constructions. The pLTRCAT reporter contains the BLV LTR *Eco*RI fragment (77) cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in plasmid pGem7Zf (Promega). The plasmid pSGCREB contains the bovine CREB2 cDNA inserted into the pSG5 eukaryotic expression plasmid (driven by the simian virus 40 promoter) and was described previously (77). The plasmids pGem-ATF-1 and pGem-ATF-2 were kindly provided by John J. Keilty and M. Green (University of Massachusetts Medical Center). The ATF-1 eukaryotic expression vector was produced by inserting the ATF-1 *Eco*RI cDNA fragment into a unique *Eco*RI site of a pSG5 vector. The ATF-2 eukaryotic expression vector was obtained by inserting a *Bam*HI fragment which contained all of the coding sequence of ATF-2 into the *Bam*HI site of the pSG5 vector. The eukaryotic expression vectors pPKA and pPKC were gifts from Richard Maurer and I. B. Weinstein, respectively, while the expression plasmids pCaMKII and pCaMKIV were obtained from Anthony Means.

Transfection and CAT assays. D17 osteosarcoma cells were grown in minimum essential medium supplemented with 10% fetal calf serum. The cells (3.5 $\times$ 10^5) were transfected by the calcium phosphate coprecipitation procedure. Three micrograms of pLTR-CAT (the reporter construct which contains the BLV LTR promoter sequences cloned upstream of the CAT gene) was cotransfected with 1 μg of each protein kinase expression vector (pPKA, pPKC, pCaMKII, and pCaMKIV) and 1 μg each of pSGCREB, pSGATF-1, and pSGATF-2 (alone or in combination). When a plasmid was omitted in the transfection experiments, the DNA levels were kept constant by using the pSG5 vector. At 48 h posttransfection, cells were harvested and CAT activities were determined as described previously (10). The net CAT values were normalized to the data obtained after cotransfection of the pSGCREB, pPKA, and pLTR-CAT plasmids. The data (in percent) represent the mean values from at least three independent transfections.

RESULTS

Characterization of the proteins binding to the TxRE element ex vivo. To identify the cellular transcription factors involved in the activation of the BLV provirus, we previously performed gel retardation assays with nuclear extracts prepared from latently infected lymphocytes derived from BLV-infected cattle affected with PL (1). By using an oligonucleotide (5′-AAGCTGGTGACGTCAGCTGGT-3′) corresponding to the 21-bp repeat centered around 123 bp upstream of the RNA start site as a probe (called the TxRE probe), five TxRE-specific complexes (C1, C2, C3, C4, and C5) were detected after short-term culture. Now, in order to identify the polypeptide components of these complexes, we have used a UV-induced indirect labeling technique in conjunction with EMSA. For this purpose, a TxRE probe (5′-AAGC-BrdU-GG-BrdU-GACG-BrdU-CAGC-BrdU-GG-BrdU-3′) containing 5-BrdU uniformly incorporated into the sense strand was synthesized. The T residues replaced by 5-BrdU were thus located both in flanking sequences and in the CRE motif (TGACGTCA). This 5-BrdU-containing TxRE probe was then ^{32}P labeled and incubated with nuclear extracts allowing the formation of the five previously identified complexes. After irradiation with UV light to generate covalent linkages between DNA and proximally attached proteins, the mixture was electrophoresed on a nondenaturing gel to separate the nucleoprotein complexes from free DNA (EMSA procedure) (data not shown). The incorporation of 5-BrdU instead of T did not interfere with the formation of the five complexes (Fig. 1A). Each of the five TxRE-specific complexes was excised from the nondenaturing gel. The complexes were then electrophoresed on an SDS-PAGE gel. The binding of the radiolabeled DNA probe allowed the visualization of interacting proteins by autoradiography (Fig. 1B).

In the case of the C1 complex, the protein-DNA adducts included two major species migrating with apparent molecular masses of 57 kDa ($p57^{C1}$) and 46 kDa ($p46^{C1}$) (Fig. 1B, lane 1).

Four minor bands were also detected, at 119, 87, 25, and 20 kDa. A similar analysis of the C2 complex revealed two major species with molecular masses of approximately 51 kDa ($p51^{C2}$) and 42 kDa ($p42^{C2}$) (Fig. 1B, lane 2). Because of the proximity of the C1 and C2 complexes on the EMSA gel, the $p57^{C1}$ polypeptide often contaminated the C2-specific protein preparations (Fig. 1B, lane 2 [star]). The C3 complex contains all of the species present in the C1 and C2 complexes with the exception of $p119^{C1}$ (Fig. 1B, lane 3). In contrast, only one polypeptide seemed to be involved in the formation of the two fast-mobility C4 and C5 complexes. They showed apparent molecular masses of 31 kDa ($p31^{C4}$) and 33 kDa ($p33^{C5}$) (Fig. 1B, lanes 4 and 5, respectively). Nevertheless, it should be noted that, despite the fact that the C5 complex was clearly detected in the gel shift with the BrdU-substituted TxRE probe (Fig. 1A, lane 2), the proteins present in the C5 complex were always weakly observed in our UV cross-linking experiments.

ATF-1 and ATF-2 transcription factors bind to TxRE DNA. Using competition assays, we have previously shown that the CRE motif is essential to the formation of complexes C1 and C2 (1). This CRE consensus sequence is a palindromic octanucleotide TGACGTCA (48, 55). Several CRE binding factors (CREB-1, CREB-2, ATF-1, ATF-2, ATF-3, and ATF-4) have been identified and belong to a unique ATF/CREB family of related proteins (30, 33, 39, 40, 49). By supershift assays, the C1 complex was shown to contain the CREB protein (1). To investigate whether other members of the ATF/CREB family were involved in these two TxRE-specific complexes (C1 and C2), several antibodies directed against these proteins were used in gel retardation assays. Since no ATF/CREB antibodies are available for the bovine system, a series of six human-specific sera was used. They are site specific (antipeptides CREB-2 and ATF-3) or epitope restricted (monoclonal antibodies anti-CREB-1, anti-ATF-1, anti-ATF-2) to the human (but not bovine) CREB-1, CREB-2, ATF-1, ATF-2 (two different sera), and ATF-3 proteins. These antibodies were added after incubation of the TxRE probe (not substituted with 5-BrdU) with the infected PBMC nuclear extract allowing exclusively the formation of complexes C1 and C2 (1) and analyzed by gel retardation assay (Fig. 2). The polyclonal anti-CREB antibody and the anti-CREB-1 and the anti-ATF-2 monoclonal antibodies moved the C1 complex to C1′ but did not supershift complex C2 (Fig. 2, lanes 2, 3, and 6, respectively). In our previous study, no change in the mobilities of C1 and C2 was observed in the presence of a polyclonal anti-ATF-2 antibody (1). This antibody was a rabbit antipeptide directed against human ATF-2 (antipeptide ATF-2 antibody [C-19]). In the present study, we used another anti-ATF-2 monoclonal antibody (F2BR-1) raised against the recombinant human ATF-2 protein. This serum did supershift C1. The anti-ATF-1 antibody induced a different supershift pattern (Fig. 2, lane 5). In this case, two supershifts in the electrophoretic mobility of TxRE-binding proteins were observed, while C1 and C2 seemed unaffected. Thus, it was unclear which complex contained the ATF-1 proteins. In contrast, the anti-human CREB-2, the anti-ATF-3, and the preimmune serum did not supershift complex C1 or C2 (Fig. 2, lanes 4, 7, and 1, respectively).

These serologic analyses indicate that, in addition to CREB, the ATF-1 and ATF-2 transcription factors could bind to the TxRE element in ex vivo-BLV-infected cells. It should be stressed here that the six human-specific sera did not supershift the C4 and C5 complexes (data not shown). The identities of the $p31^{C4}$ and $p33^{C5}$ polypeptides are thus presently unknown.

Characterization of the bovine transcription factors CREB, ATF-1, and ATF-2 in ex vivo-BLV-infected cells. The gel retardation assays indicate that the transcription factors CREB, ATF-1, and ATF-2 could be present in complexes C1 and C2. Furthermore, we have shown by UV cross-linking experiments that both the C1 and C2 complexes contain two major TxRE-binding polypeptides ($p57^{C1}$ and $p46^{C1}$ for C1 and $p51^{C2}$ and $p42^{C2}$ for C2). In order to identify which polypeptides correspond to these transcription factors, we have used the UV indirect labeling technique in combination with immunoprecipitation. For this purpose, the 5-BrdU-substituted TxRE probe labeled with ^{32}P was incubated with the nuclear extracts (under conditions identical to those used in the EMSA procedure described above), and the binding reaction mixtures were irradiated with UV light. The covalently linked protein-DNA complexes were then immunoprecipitated with six specific antisera and analyzed by electrophoresis on SDS-polyacrylamide gels and autoradiography (Fig. 3A).

The anti-CREB antiserum specifically precipitated two major proteins, $p57^{C1}$ and $p119^{C1}$ (both present in the C1 complex) (Fig. 3A, lane 1). These two proteins were also observed when the monoclonal anti-CREB-1 antibody was used instead of the polyclonal anti-CREB antibody (Fig. 3A, lane 2). Two major proteins were precipitated by the anti-ATF-1 antibody (Fig. 3A, lane 4). They showed apparent molecular masses of 46 ($p46^{C1}$) and 51 ($p51^{C2}$) kDa. As shown in Fig. 1B (lane 1), they were associated with complexes C1 and C2, respectively. The anti-ATF-2 monoclonal antibody (F2BR-1) precipitated a polypeptide with an apparent molecular mass of 87 kDa (Fig. 3A, lane 5). This protein, which belongs to the C1 complex (Fig. 1B, lane 1), was also detected with the ATF-2 antipeptide

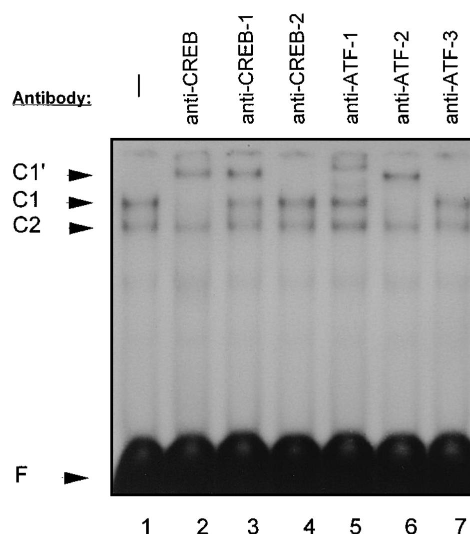
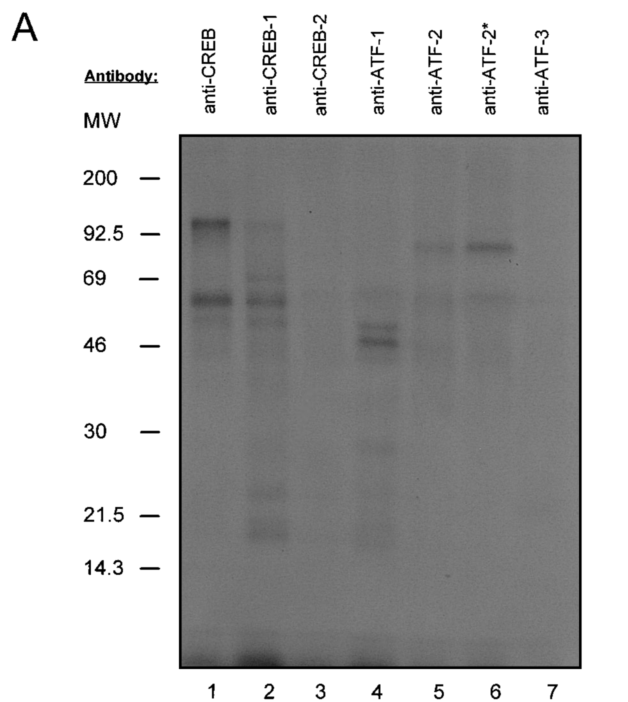


FIG. 2. The transcription factors CREB, ATF-1, and ATF-2 bind the TxRE DNA. The ^{32}P-end-labeled 21-bp oligonucleotide (TxRE probe) was incubated with a nuclear extract prepared from infected PBMCs from a cow (B163) with PL that were cultured for 48 h in the presence of PHA (1 μg/ml) and PMA (0.1 μM). These culture conditions lead exclusively to C1 and C2 complex formation (1). Before being loaded on the gel, the binding reaction mixtures were preincubated with a preimmune serum (lane 1), a polyclonal antibody directed against CREB (lane 2), a monoclonal anti-CREB-1 antibody (raised against the recombinant human CREB-1 protein) (lane 3), a polyclonal anti-CREB-2 antibody (raised against a synthetic peptide corresponding to amino acid residues 330 to 350 mapping to the carboxy terminus of human CREB-2) (lane 4), a monoclonal anti-ATF-1 antibody (raised against the recombinant human ATF-1 protein) (lane 5), a monoclonal anti-ATF-2 antibody (raised against the recombinant human ATF-2 protein) (lane 6), or a polyclonal anti-ATF-3 antibody (raised against a synthetic peptide corresponding to amino acid residues 194 to 212 mapping to the carboxy terminus of human ATF-3) (lane 7). C1′, supershifted complex; F, free probe.



B

Protein-DNA adducts	Predicted Size	Immunoreactivity	Complex
p119	108 kD	anti-CREB	C1
p87	76 kD	anti-ATF-2	C1
p57	46 kD	anti-CREB	C1 (C3)
p51	40 kD	anti-ATF-1	C2 (C3)
p46	35 kD	anti-ATF-1	C1 (C3)
p42	31 kD	?	C2 (C3)
p33	22 kD	?	C5
p31	20 kD	?	C4

FIG. 3. Identification of CREB, ATF-1, and ATF-2 in BLV-infected bovine cells. (A) Immunoprecipitation experiments with the TxRE cross-linked proteins. Nuclear extracts (20 μg of total protein) prepared with freshly isolated infected PBMCs from a cow (B163) with PL were incubated with the BrdU-substituted, ^{32}P-labeled TxRE probe. The reaction mixtures were irradiated with UV light for 1 h, and the cross-linked proteins were immunoprecipitated with a polyclonal antibody directed against CREB (lane 1), a monoclonal anti-CREB-1 antibody (raised against the recombinant human CREB-1 protein) (lane 2), a polyclonal anti-CREB-2 antibody (raised against a synthetic peptide corresponding to amino acid residues 330 to 350 mapping to the carboxy terminus of human CREB-2) (lane 3), a monoclonal anti-ATF-1 antibody (raised against the recombinant human ATF-1 protein) (lane 4), a monoclonal anti-ATF-2 antibody (raised against the recombinant human ATF-2 protein) (lane 5), the polyclonal anti-ATF-2* (raised against a synthetic peptide corresponding to amino acid residues 487 to 505 mapping to the carboxy terminus of human ATF-2) (lane 6), or a polyclonal anti-ATF-3 antibody (raised against a synthetic peptide corresponding to amino acid residues 194 to 212 mapping to the carboxy terminus of human ATF-3) (lane 7). After immunoprecipitation, the proteins were analyzed on denaturing SDS–10% polyacrylamide gels. Molecular masses (MW) in kilodaltons are indicated. (B) Summary of the sizes and immunoreactivities of the TxRE cross-linked proteins and the complexes in which they are found. The predicted size is the molecular mass of the protein after subtraction of the predicted oligonucleotide molecular mass.

(C-19), which did not induce a supershift in EMSA (1) (Fig. 3A, lane 6). In contrast, no detectable protein was immunoprecipitated by the anti-human CREB-2 and anti-ATF-3 antibodies (Fig. 3A, lanes 3 and 7, respectively). A summary of the sizes and immunoreactivities of the different proteins and the complexes in which they are found is presented in Fig. 3B.

In order to verify the specificities of the different antisera, the same UV cross-linking and immunoprecipitation experiments were performed with in vitro-synthesized bovine CREB2 and human ATF-1 and ATF-2 proteins (Fig. 4). After UV cross-linking to the TxRE element and immunoprecipitation, the in vitro-translated CREB2, ATF-1, and ATF-2 proteins migrated at exactly the same positions as their TxRE cross-linked counterparts present in the nuclear extracts of bovine infected cells (Fig. 4; compare lanes 1 and 2 for CREB, lanes 3 and 4 for ATF-1, and lanes 5, 6, and 7 for ATF-2). On the other hand, direct analysis by SDS-PAGE of these three ^{35}S-labeled proteins produced by using reticulocyte lysates revealed a major band at 46 kDa and a faint one at 90 kDa for CREB2, two bands at 37 and 40 kDa for ATF-1, and one band at 77 kDa for ATF-2 (data not shown). As a result, the mass contribution of the TxRE probe can be assessed to be about 11 kDa. Subtraction of this predicted oligonucleotide molecular mass from those of the cross-linked complexes reveals that the bovine B-cell transcription factors CREB, ATF-1, and ATF-2 show molecular masses that fit with those of their human counterparts (33–35, 65). Together, these data establish the specificities of the antibodies used in the supershift experiments shown in Fig. 2.

In conclusion, these UV cross-linking and immunoprecipitation experiments allowed us to identify the different proteins

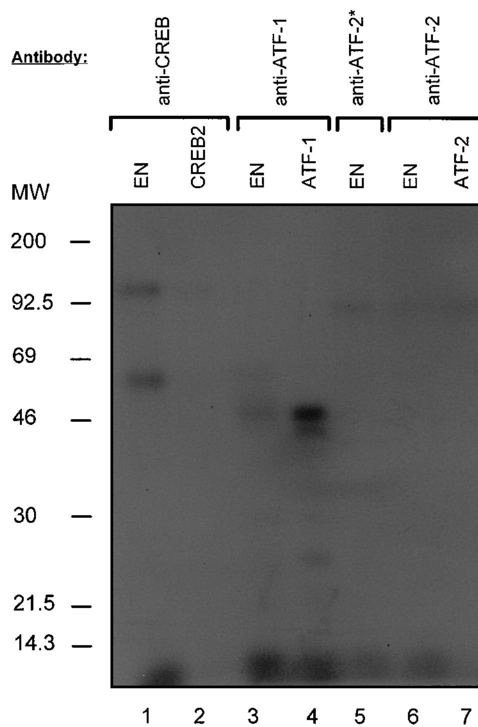


FIG. 4. Comparison of in vivo and in vitro CREB2, ATF-1, and ATF-2. The BrdU-substituted, ^{32}P-labeled 21-bp oligonucleotide (TxRE probe) was incubated with a nuclear extract (EN) (20 μg of total protein) prepared with 48-h-cultured infected PBMCs from a cow (B163) with PL (lanes 1, 3, 5, and 6), in vitro-synthesized bovine CREB2 (lane 2), human ATF-1 (lane 4), or human ATF-2 (lane 7). The reaction mixtures were irradiated with UV light for 1 h, and the cross-linked proteins were immunoprecipitated with a polyclonal antibody directed against CREB (lanes 1 and 2), a monoclonal anti-ATF-1 antibody (raised against the recombinant human ATF-1 protein) (lanes 3 and 4), a polyclonal anti-ATF-2 antibody (raised against a synthetic peptide corresponding to amino acid residues 487 to 505 mapping to the carboxy terminus of human ATF-2) (anti-ATF-2*) (lane 5), and a monoclonal anti-ATF-2 antibody (raised against the recombinant human ATF-2 protein) (lanes 6 and 7). After immunoprecipitation, the proteins were analyzed on denaturing SDS–10% polyacrylamide gels. Molecular masses (MW) in kilodaltons are indicated.

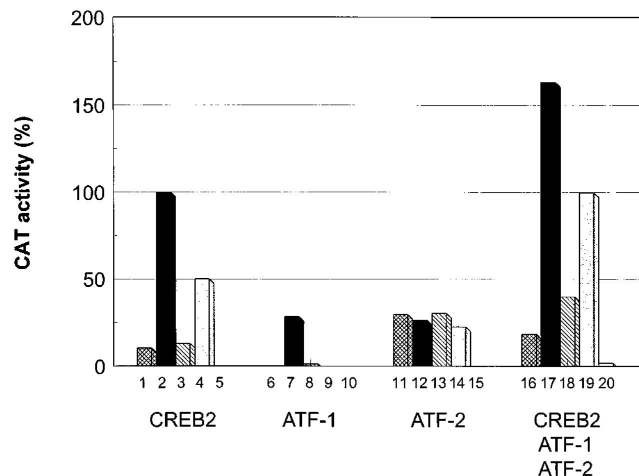


FIG. 5. Regulation of BLV LTR-directed transcription. D17 osteosarcoma cells were transfected with 3 μg of pLTR-CAT reporter and with 1 μg of the three transcription factors expression vectors (pSGCREB2, pSGATF-1, and pSGATF-2) alone (▩) or in combination with 1 μg of the different kinase-expressing plasmids (pPKA [■], pPKC [▧], pCaMII [□], and pCaMIV [▨]). DNA concentrations were kept constant by using the pSG5 plasmid DNA. At 48 h posttransfection, the cells were recovered and the CAT activities were determined from the lysates. The bars represent the means from at least three independent transfections. The values are expressed as percentages of the CAT activities elicited by the cotransfection of pSGCREB2 and pPKA-expressing vectors. This value represents about 7% of the CAT activity elicited by cotransfection of the Tax protein (data not shown).

present in the C1 complex: CREB, ATF-1, and ATF-2. We should mention here that C1 is the main complex that is specifically observed in uncultured BLV-infected B lymphocytes.

Regulation of BLV LTR-directed transcription in D17 cells. In vitro, the bovine CREB2 is able to transactivate the BLV LTR but does so significantly when PKA-expressing plasmid is cotransfected with the CREB2-expressing construct. However, several agents known to activate the PKA pathway (forskolin and db-cAMP) failed to induce viral protein synthesis in BLV-infected B lymphocytes (1). Although CREB was originally identified as mediating responses to cAMP (28, 33, 54, 79), this transcription factor can also activate transcription in response to an increased intracellular Ca^{2+} concentration through calmodulin-dependent protein kinases (CaMKII and -IV) (15, 62, 63). To study the role of CaMKII and CaMKIV in the activation of CREB, we have used expression vectors encoding truncated forms of these enzymes which are constitutively active and no longer require Ca^{2+}/calmodulin for activation. Since BLV expression can be induced by phorbol esters (e.g., PMA) (1, 37), we have also tested the ability of PKC (pPKC vector) to induce BLV LTR-directed transcription. As a control, we used a pPKA vector (pPKA) previously shown to enhance BLV expression in D17 cells (77). All of these kinase-expressing plasmids were cotransfected with the pLTR-CAT reporter and with the CREB2 (pSGCREB2), ATF-1 (pSGATF-1), and ATF-2 (pSGATF-2) expression vectors.

As shown in Fig. 5, the bovine CREB2 protein alone only marginally stimulated LTR-directed CAT expression in the absence of protein kinase (Fig. 5, bar 1). However, when a vector encoding the α isoform of the catalytic subunit of the cAMP-dependent PKA (pPKA) was cotransfected with the pLTR-CAT and pSGCREB2 plasmids, LTR-directed expression was greatly enhanced, in agreement with our previous results (77) (Fig. 5, bar 2). In contrast, the PKC-expressing vector had little effect on the ability of CREB2 to increase LTR-directed CAT gene expression (Fig. 5, bar 3). The pCaMKIV vector substantially increased the ability of CREB2 to stimulate expression of the CAT gene (Fig. 5, bar 4). In these experiments performed with D17 cells, the CaMKIV kinase was, however, less active than PKA: only about half of the activity was obtained. On the other hand, no enhancement of CAT activity was observed when CREB2 was cotransfected with the pCaMKII vector (Fig. 5, bar 5).

The ATF-1 and ATF-2 transcription factors are involved in the formation of C1 and C2 complexes in vivo. They could also be functionally implicated in the regulation of BLV transcription. When the pSGATF-1 plasmid was cotransfected with pLTR-CAT, no transactivation of the reporter gene was observed in the absence of kinase (Fig. 5, bar 6). In the presence of PKA, ATF-1 activated LTR-directed CAT expression (Fig. 5, bar 7). This transactivation was, however, 3.5-fold lower than that of CREB2 and PKA. No enhancement of CAT activity was observed in the presence of three other kinases, i.e., PKC, CaMKII, and CaMKIV (Fig. 5, bars 8 to 10).

ATF-2 had a basal activity threefold higher than that of CREB2 (Fig. 5; compare bars 11 and 1). Cotransfection of pSGATF-2 and the PKA, PKC, or CaMKIV expression vector had a marginal stimulatory effect on CAT basal activity (Fig. 5, bars 12 to 14). A reproducible inhibitory effect on the basal activity induced by ATF-2 was even induced by CaMKII (Fig. 5, bar 15).

In order to analyze the synergistic action of CREB2, ATF-1, and ATF-2, these transcription factors were analyzed in D17 cells, either alone or in combination with the different protein kinases. In the presence of either PKA or CaMKIV, CREB2, ATF-1, and ATF-2 had additive effects on BLV expression (Fig. 5, bars 17 and 19, respectively). In contrast, no additive effect was observed in presence of PKC or CaMKII (Fig. 5, bars 18 and 20, respectively).

In summary, these data confirm our previous observations on the transactivation potential of CREB2 in the presence of PKA. In addition, our results show that PKA can also activate viral transcription through ATF-1. Furthermore, we now demonstrate that the CaMKIV, in contrast to PKC and CaMKII, is another kinase able to activate BLV LTR-directed expression through CREB2 in D17 cells. Interestingly, the ability of CaMKIV to mediate BLV LTR-directed expression is increased in the presence of a combination of the CREB2, ATF-1, and ATF-2 transcription factors. It is noteworthy that these transcription factors belong to the C1 complex specifically observed in the early steps of BLV proviral expression.

DISCUSSION

Host cell transcription factors seem to play a central role in the regulation of BLV expression (77). Recently, using nuclear extracts from ex vivo-isolated bovine B lymphocytes, we have identified five complexes (C1, C2, C3, C4, and C5) of cellular proteins and the TxRE enhancer sequence of the BLV LTR (1). C1 is the major complex identified in freshly isolated BLV-infected lymphocytes from cattle with PL. A CREB protein is present in this complex (1). Here, using gel shift assays in conjunction with UV cross-linking and immunoprecipitation experiments, we showed that C1 is composed of two others members of the ATF/CREB family of transcription factors, i.e., ATF-1 (35 kDa) and ATF-2 (76 kDa). These cellular proteins in conjunction with CREB (46 kDa) could thus be the major transcription factors involved in the initiation of BLV genome transcription.

The CRE motif within the TxRE element (at position −123) is essential for the formation of C1 (1). Since CREB, ATF-1,

and ATF-2 belong to the complex C1, they could bind the TxRE DNA as a trimer. This appears unlikely, however. Alternatively, these transcription factors could also bind as dimers, since they contain a leucine zipper that is required for dimerization and an adjacent basic region that make direct contact with DNA (30). In this case, the formation of C1 may involve binding by each factor alone in a homodimer or in a heterodimer with each other. C1 would thus be composed of different TxRE-binding complexes. The immunoprecipitation of the p119 species by the anti-CREB antibodies both in nuclear extracts and with in vitro-translated-CREB2 argues for a certain proportion of CREB homodimers. On the other hand, the transcription factors ATF-1 and CREB (along with CREM, the cAMP response element modulator) constitute a subgroup within the ATF/CREB family defined by the ability to form heterodimers with each other but not with members of other subfamilies of CRE-binding proteins such as ATF-2 (32). If so, ATF-2 would be present in C1 as a component of TxRE-specific complexes which would comigrate with heterodimers and/or homodimers of CREB and ATF-1 proteins. It should be mentioned here that the $p87^{C1}$ protein corresponding to ATF-2 is weakly observed in complex C1 (Fig. 1B, lane 1) despite being correctly immunoprecipitated (Fig. 3A, lanes 5 and 6). The ATF-2 transcription factor could thus be a minor protein in vivo. The functional role of these protein-protein interactions is presently unknown. We can speculate that the different regulatory responses that occur through the ATF/CREB sites would be mediated by different homo- or heterodimer complexes. Since ATF-1 has distinct homodimeric and heterodimeric conformations (34), it could differentially interact with other parts of the transcriptional machinery, depending on heterodimerization with CREB. In this respect, Ellis et al. (18) reported that ATF-1 could act as a transdominant negative regulator of CREB. CREB homodimers are thought to be responsible for cAMP-inducible transcription (18). However, the ability of CREB to mediate a cAMP response appears to be down-regulated by heterodimer formation with ATF-1 (18). The ability to respond to a particular regulatory signal could thus depend on the presence of a particular complex within the cell.

The signaling pathways that regulate CREB activation in cells have been extensively studied. CREB can activate transcription in response to both increased intracellular cAMP and Ca^{2+} concentration (15, 27, 63). Studies of CREB indicate that its Ser-133 residue and the flanking amino acids constitute a phosphorylation site for cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinases II and IV (15, 27, 63). Phosphorylation of this site is required for CREB to mediate both cAMP and Ca^{2+} inducibility (15, 26, 27, 47, 63). Previously we showed with D17 cells that the bovine CREB2 protein in the presence of the cAMP-dependent PKA is able to induce BLV LTR-directed expression in the absence of the Tax protein (77). We now show that a constitutively active form of CaMKIV can phosphorylate CREB2 to induce BLV LTR-mediated transcription. Furthermore, this transcriptional activity is enhanced by cotransfection of ATF-1 and ATF-2, the two other major components of C1. The efficiency of this CaMKIV-mediated transcriptional activation was, however, lower than that of PKA under similar conditions. Partial exclusion of CaMKIV from the nucleus, because of a limited diffusion of CaMKIV into the nucleus or a rate-limiting step in nuclear transport, could explain the reduced activity of CaMKIV as a transcriptional activator (51). Another possibility would be related to the fact that CaMKIV needs to be phosphorylated by the newly discovered CaM kinase IV kinase to be fully active (20). Under similar conditions, CaMKII is ineffective at stimulating BLV LTR-directed transcription through the CRE/CREB system. This is surprising, since CaMKII is a good catalyst for phosphorylation of CREB on Ser-133, the site required for transactivation by CREB (15, 19, 63). However, recently Enslen et al. (19) reported that CaMKII equally phosphorylates a second site within the transcriptional activation domain of CREB. This site (Ser-142) functions as a negative determinant of CREB-mediated transcriptional activation (19, 67). This negative regulation could account for the lack of BLV LTR-mediated transcriptional activation by CaMKII. It should be noted that an identical consensus CaMKII site (Asn–Asp–Leu–Ser-142–Ser–Asp) is present in the bovine CREB2 at position 126 (Asn–Asp–Leu–Ser-126–Ser–Asp) (76). Moreover, in D17 cells, cotransfection of CaMKII with PKA reduced the BLV LTR-directed transcriptional activation that was observed with PKA alone (data not shown). The existence of phosphorylation sites which are negative regulators of transcription has been identified in other transcription factors, such as c-Jun (9), c-Fos (58, 70), and, recently, another CRE-binding protein, CREMτ (16).

In vivo, immune activation of BLV-infected B cells could sporadically lead to induction of BLV expression within a few cells. In vitro, up-regulation of BLV replication by polyclonal activators such as anti-immunoglobulin M supports this hypothesis (46). The biochemical events that follow immunoglobulin receptor ligation result, notably, in the hydrolysis of phosphatidylinositol (8), leading to the generation of inositol triphosphates and diacylglycerol (6, 57). Diacylglycerol subsequently triggers the activation of PKC (57). Inositol triphosphate increases the levels of intracellular calcium by releasing Ca^{2+} from intracellular stores (7). Pharmacologic mimicry of these molecular events by the addition of PMA (an activator of PKC) and A23187 (a Ca^{2+} ionophore) to BLV-infected PBMC cultures is sufficient to induce a substantial enhancement of BLV expression as well as a significant increase in the binding of the two major TxRE-specific complexes C1 and C2 (reference 1 and data not shown). Together, these data lead to the hypothesis that, in vivo, PKC- and Ca^{2+}-mediated signaling pathways could trigger the initiation of BLV transcription by activation of CREB, ATF-1, and ATF-2 that bind to the TxRE element. However, in D17 cells, despite the presence of consensus phosphorylation sites, PKC did not stimulate CREB2, ATF-1, or ATF-2 activity. To our knowledge, PKC has not yet been implicated in the phosphorylation of these factors in vivo. We cannot exclude the possibility that the PKC isoform used in this study may not be the correct candidate. Alternatively, PKC would act indirectly on BLV, e.g., by activation of another intermediate kinase responsible for CREB, ATF-1, and/or ATF-2 phosphorylation. This kinase could be, for example, the recently discovered Ras-dependent CREB kinase (25). Interestingly, in murine B lymphocytes, Xie and Rothstein (78) observed that a PKC is implicated in the phosphorylation of CREB Ser-133 (and possibly ATF-1 as well) induced by antigen receptor triggering. Moreover, agents that act to raise the level of cAMP, such as forskolin or db-cAMP, do not induce this phosphorylation (78). In BLV-infected B cells, although PKA enhanced BLV LTR-directed transcription through CREB2, ATF-1, and ATF-2 in D17 cells, the cAMP-dependent PKA pathway does not seem to be involved in the induction of BLV expression in vivo. Indeed, db-cAMP did not enhance viral synthesis in ex vivo-infected lymphocytes (reference 1 and data not shown). In contrast, our data suggest that, in vivo, BLV gene transcription could be regulated by a Ca^{2+}-dependent signaling pathway. Furthermore, our transcription studies argue that CaMKIV may be a likely mediator of this Ca^{2+}-mediated viral expression by phosphorylation of the

TxRE-bound transcription factors (CREB, ATF-1, and ATF-2), thereby increasing their transcriptional activities. It should be stressed, however, that CaMKIV is expressed in a number of tissues, but it is not ubiquitous (38, 52). For instance, CaMKIV is expressed in human T lymphocytes but apparently not in human B lymphocytes or monocytes (31). However, Mosialos et al. (56) reported that this kinase can be induced by the Epstein-Barr virus oncogene LMP1 in Epstein-Barr virus-transformed B lymphoblastoid cell lines. The expression of CaMKIV can thus be up-regulated by a viral gene product. CaMKII could be another candidate for mediating the Ca^{2+}-induced BLV expression in vivo. Indeed, although CaMKII phosphorylates a second site (Ser-142) in CREB that acts as a negative determinant for transactivation, the presence of this kinase in GH3 cells does not block the ability of Ca^{2+} influx to activate CREB (36). In this case, selective dephosphorylation of Ser-142 by a protein phosphatase could enhance the ability of CaMKII to activate transcription through CREB phosphorylation. CaMKI has been found to phosphorylate CREB on Ser-133 (63). This kinase can also phosphorylate ATF-1 on Ser-63 and mediate Ca^{2+}-inducible gene expression in vivo (51).

In conclusion, the present study was focused on the understanding of the initial steps involved in the induction of BLV expression in vivo. The CREB, ATF-1, and ATF-2 proteins appear to be the major transcription factors implicated in this process. Consequently, they may initiate a low level of transcription from the LTR promoter and lead to the synthesis of small amounts of the Tax transactivator, which could then amplify transcription. In the human T-cell leukemia virus type 1 (HTLV-1) system, CREB and ATF-2 are the main T-cell proteins that directly bind the HTLV-1 21-bp repeats and activate transcription in vitro (21). Moreover, the ATF/CREB proteins appear to mediate the transcriptional stimulation by HTLV-1 Tax. According to several recent reports, Tax could stimulate viral transcription through enhancement of the DNA binding of CREB and ATF-2 to the 21-bp repeat (2, 3, 5, 21, 50, 61, 68, 73, 81). Others studies suggest that Tax could act through direct anchoring to the promoter via protein-protein interactions with the bound ATF/CREB proteins (14, 22, 23, 71). Finally, Brauweiler et al. (11) have recently provided evidence that Tax could stabilize the binding of CREB (but not ATF-2) to the 21-bp repeats. In the BLV system, the mechanisms by which Tax transactivates the BLV transcription are presently still unknown. However, it is likely that Tax interacts with the CREB, ATF-1, and ATF-2 cellular factors, which specifically bind to the 21-bp elements.

ACKNOWLEDGMENTS

We thank G. Schutz and W. Schmid for the anti-CREB polyclonal antibody. We are also grateful to R. Maurer, I. B. Weinstein, and A. Means for providing eukaryotic expression kinase vectors (pPKA, pPKC, and pCaMKII and pCaMKIV, respectively) and to J. J. Keitlty and M. Green for plasmids pGem-ATF-1 and pGem-ATF-2.

This work was financially supported by the Fonds Cancérologique de la Caisse Générale d'Epargne et de la Retraite, the Belgian Service Fédéraux des Affaires Scientifiques, Techniques et Culturelles (SSTC, PAI15), the Belgian Fonds National de la Recherche Scientifique (FNRS), and the Belgian Association contre le Cancer. E.A., R.K., and L.W. are, respectively, Télévie Fellow, Directeur de Recherches, and Maître de Recherches of the FNRS.

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