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Involvement of the Cyclic AMP-Responsive Element Binding Protein in Bovine Leukemia Virus Expression In Vivo

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The TAR element (Tax-responsive element; also called TxRE) is a major determinant of the regulation of bovine leukemia virus (BLV) expression. In order to gain insight into the mechanisms of viral expression, complexes formed between proteins and the TAR enhancer DNA were analyzed by gel retardation assays. We report here that nuclear lysates from ex vivo-isolated B lymphocytes contain proteins that specifically bind to TAR. An antibody directed toward the cyclic AMP-responsive element binding (CREB) protein supershifted a complex (C1) present only in BLV-infected B lymphocytes. The CREB protein thus appears to be a major transcription factor involved in BLV expression in vivo.

Bovine leukemia virus (BLV) is a naturally occurring, exogenous, oncogenic retrovirus associated with persistent lymphocytosis (PL) and lymphosarcoma in cattle (6, 11, 32). PL is characterized by a marked increase in the percentage of BLV-infected B lymphocytes in the blood (22). On the basis of studies of well-characterized cattle populations, less than 10% of BLV-infected cattle develop lymphosarcoma and about 30% develop PL (13). Thus, a large majority of BLV-infected cattle are asymptomatic virus carriers. In cattle, BLV is predominantly or exclusively B-cell tropic, and BLV-infected B lymphocytes partially account for the increased number of peripheral blood mononuclear cells (PBMCs) seen in cattle with PL (12). Infection by BLV is characterized by a long clinical latency period associated with a lack of viral expression. Circulating lymphocytes harbor integrated proviruses but do not produce detectable amounts of virus (26). However, since an antiviral immune response persists throughout the life span of the infected animals, low-level and/or transient BLV expression occurs in vivo.

Expression of BLV is thought to be blocked at the transcriptional level (3, 16, 26, 46). However, the synthesis of readily detectable amounts of BLV RNA, viral proteins, and viral particles can be induced or enhanced by culturing PBMCs for a few hours (3, 15, 24, 43). It should be noted that BLV expression is more readily detected in cultured cells from cattle with PL than in those from asymptomatic animals (8). A likely explanation for this fact is that approximately 30% of the PBMCs in cattle with PL are infected with BLV (23) compared with less than 5% in hematologically normal BLV-infected cattle (25, 26). Several activators which can up-regulate BLV replication in vitro have been identified. These activators include fetal calf serum, lipopolysaccharides, anti-immunoglobulin M (IgM) antibodies (27), lectin mitogen (PHA) (3, 43), and phorbol esters (19). The molecular mechanism by which these inducing agents enhance BLV transcription is not clear. Protein kinase C (PKC) appears to mediate the initiation of BLV expression in short-term PBMC cultures and in phorbol 12-myristate 13-acetate (PMA)-stimulated NBC-13 cells (a persistently BLV-infected B-lymphocyte cell line) (19). On the other hand, the cyclic AMP (cAMP) pathway could also play a role in the regulation of BLV expression in D17 osteosarcoma cells (49). Taken together, these findings suggest that expression of BLV might occur within the infected B cell consequently to activation of one or several transducing signal pathways triggered by different stimuli (interaction with the B-cell membrane receptors, for instance). The fact that an antibody raised against the B-cell surface immunoglobulin M (IgM) can enhance the in vitro expression of BLV corroborates this hypothesis (27). Consequently, the immune activation of a latently infected cell could initiate viral expression within the host. If so, BLV could use the transcription factors normally implicated in the regulation of genes whose expression is induced during proliferation and differentiation of activated B cells.

BLV is closely related to the human retrovirus human T-cell leukemia virus type I (HTLV-I), which is associated with adult T-cell leukemia/lymphoma (39). These two viruses share numerous homologies in their pathologies and in their genomic structures (38). Both viruses contain an X region, which encodes a protein called Tax involved in the transactivation of long terminal repeat (LTR)-directed gene expression (9, 48). Transcriptional activation of the BLV LTR by Tax requires the presence of a 75-bp element in the U3 region (9). Nucleotide sequence data show that this *cis*-acting element is composed of two 21-bp repeats (also called TAR or TxRE for Tax-responsive element) centered at about 148 and 123 bp upstream of the RNA start site. A third related element found at position −48 upstream of the RNA cap site is apparently poorly active in the absence of upstream sequences. These three repeats are very similar to elements found in the LTRs of HTLV-I and -II with respect to their nucleotide sequences and spatial arrays. All these elements have a common 8-bp core sequence, TGACGTCA, known as the cAMP-responsive element (CRE) or the binding site of a cellular transcription factor, ATF (21). There is no evidence for direct binding of Tax to DNA (9). This observation suggests that Tax might exert its effect through the cellular proteins that recognize these enhancer elements. Several different proteins binding to the HTLV LTR have been identified: CREB (the cAMP-responsive element binding protein) (4, 52, 53), CREM (the CREB modulator) (44), ATFs (the activating transcription factors) (51), HEB1

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and HEB2 (the 21-bp binding proteins) (5), and TREBs (Tax-responsive element-binding proteins) (45). To date, only one cellular protein has been shown to bind the BLV enhancer sequences. A cDNA encoding a 21-bp motif binding protein was cloned and characterized (49). Its nucleotide sequence revealed that this clone corresponds to the bovine CREB2 protein. Moreover, CREB2 transactivates the BLV LTR in D17 osteosarcoma cells and does so significantly when a protein kinase A (PKA)-expressing plasmid is cotransfected with the CREB2-expressing construct. CREB2 is thus a cellular factor able to induce BLV LTR-directed gene expression in the absence of Tax and could be involved in the early stages of viral infection. Nothing is known about the induction of BLV expression in infected B lymphocytes, the natural host cells of this retrovirus.

Consequently, the present study was initiated to identify the cellular transcription factors involved in the activation of the BLV provirus. For this purpose, gel retardation assays were performed with nuclear extracts prepared from BLV-infected lymphocytes.

MATERIALS AND METHODS

Cell isolations and culture conditions. Two BLV-seropositive adult cattle affected with PL (B76 and B163) and one BLV-seronegative cow (B78) were kept at the National Institute for Veterinary Research (Uccle, Belgium). By using EDTA as an anticoagulant, venous blood was collected by jugular venipuncture. 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). All cultures were incubated at 37°C with 5% CO_2. Different substances were used for the stimulation of viral expression: phytohemagglutinin (BACTO-PHA-P) (Difco Laboratories) at a final concentration of 1 μg/ml, PMA (Sigma Chemical Co.) at 0.1 μM, and forskolin (FSK) (Sigma Chemical Co.) at 20 μM.

B-cell isolation by negative selection. T lymphocytes were depleted from bovine PBMCs by antibody-dependent complement-mediated cytolysis. Briefly, washed PBMCs were suspended at a concentration of 10×10^6 cells per ml in sterile cold PBS and incubated for 30 min on ice with four anti-T cell monoclonal antibodies (1,000-fold-diluted ascites fluid). Two of these anti-T cell monoclonal antibodies were provided by J. Naessens (International Laboratory for Research on Animal Diseases, Nairobi, Kenya), IL-A11 (which binds Bo-CD4 and is specific for helper T cells) and IL-A105 (which binds Bo-CD8 surface molecule and is specific for cytotoxic suppressive T lymphocytes). The two other anti-T cell monoclonal antibodies were provided by J.-J. Letesson (Faculté Notre-Dame de la Paix, Namur, Belgium); these were 1H8 (which binds Bo-CD6 and is specific for mature T cells) and 11F5 (which binds non-T, non-B null cells). The PBMCs were then centrifuged ($300 \times g$, 10 min), washed once with PBS, resuspended at 10×10^6 cells per ml in RPMI 1640 (with 100 U of penicillin per ml and 100 μg of streptomycin per ml) containing 10% baby rabbit complement (Séra-Lab), and incubated at 37°C for 1 h with gentle shaking. The selected B cells were then isolated by centrifugation, washed three times with sterile PBS, and resuspended at 2×10^6 cells per ml in culture medium.

Surface Ig-positive cells in the selected B-cell populations were quantitated by direct immunofluorescence using fluorescein isothiocyanate-conjugated rabbit anti-bovine IgM (1H4) (supplied by J.-J. Letesson, Faculté Notre-Dame de la Paix, Namur, Belgium) (29). The percentage of T cells remaining in the negatively selected B-cell populations was determined by indirect immunofluorescence using both an anti-bovine CD2 monoclonal antibody (CH128A) (VMRD Inc., Pullman, Wash.) and an anti-bovine CD6 monoclonal antibody (BAQ91A) (VMRD Inc.) and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Amersham). PBS was used instead of CH128A and BAQ91A as a control.

BLV p24 titration. Cells (2×10^6) were centrifuged at $150 \times g$ for 15 min. The pellet was resuspended in 100 μl of PBS, and cells were lysed by vigorous agitation in a 1% final concentration of Triton X-100, incubated at 37°C for 1 h with occasional agitation, and left at 4°C for 1 h. The BLV p24 antigen was titrated by an immunoenzymatic capture technique as described by Portetelle et al. (36). To determine the amounts of BLV p24 in the pellet and in the cell culture supernatant, twofold serial dilutions of a BLV antigen (culture supernatant of FLK cells containing 2 mg of p24 per ml) were assayed in quadruplicate in the BLV p24 antigen capture enzyme-linked immunosorbent assays (ELISAs). A standard curve of relative antigen concentration versus optical density was developed for BLV p24. Optical density values obtained with the pellet and the culture supernatant were subsequently converted to relative amounts of BLV p24 by using the standard curve line equations.

Preparation of nuclear extracts. Nuclear extracts were prepared from bovine B cells (or PBMCs) as described previously (35). 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). 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. 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 phenylmethylsulfonyl fluoride) 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 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 phenylmethylsulfonyl fluoride) for 5 h. 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 use.

Oligonucleotides for gel retardation assays (electrophoretic mobility shift assays) and competition experiments. The TAR oligonucleotides CRE, 5′-AAGCTGGTGACGTCAGCTG GT-3′, TRE, 5′-AAGCTGGTGACTCAGCTGGT-3′, Δ1-2, 5′-AAGCTGGGCTGGT-3′, Δ3-4, 5′-AAGCTGGTGACGT CA-3′, and Δ5-6, 5′-TGACGTCAGCTGGT-3′, were previously described (49).

The wild-type TAR oligonucleotide was end labelled with polynucleotide kinase and [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and then purified by Sephadex G-25 chromatography (Pharmacia).

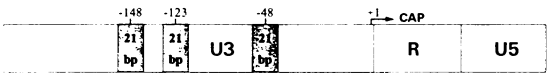
Gel retardation assays. The assays were carried out as previously described (42) with a modified running buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA [pH 8.3]). The nuclear extract (1.2 μg of total protein) was preincubated with 0.5 μg of poly(dI-dC) in 20 μl of binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) at room temperature for 30 min before addition of the labelled

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. Complexes were inhibited by competition with a 100-fold molar excess of unlabelled oligonucleotides added 15 min prior to addition of the probe. Binding reactions using the CREB2 protein contained 2 μl of in vitro-translated protein, the radiolabelled oligonucleotide probe (30,000 cpm), and 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). The plasmid pSGCREB contains the CREB2 cDNA cloned downstream of the bacteriophage T7 promoter (49). The CREB protein was obtained after transcription and translation performed by using the TNT kit (Promega). All gels were dried and subjected to autoradiography with intensifying screens.

In the supershift experiments, the appropriate antibody (2 μl per sample) was added to the reaction mixtures 30 min after addition of the probe to the sample and incubated for an additional 15 min. The polyclonal anti-ATF2 antibody (raised against a synthetic peptide corresponding to amino acid residues 487 to 505 mapping at the carboxy terminus of human ATF-2) and the polyclonal anti-Jun antibody (raised against a synthetic peptide corresponding to amino acid residues 247 to 263 within the C-terminal DNA binding domain of the mouse c-*jun*-encoded protein) were purchased from Santa Cruz Biotechnology, Inc. The polyclonal anti-CREB antibody was kindly provided by G. Schutz and W. Schmid.

RESULTS

Several nuclear factors bind to the 21-bp TAR motif. To date, the molecular mechanisms of BLV latency and its reversal upon in vitro cultivation of infected cells are not established. However, several observations suggest that BLV transcription must result from the binding of one or more cellular *trans*-acting proteins to the retroviral enhancer elements (49). The first aim of this study is to demonstrate the existence of a cellular protein(s) specifically binding to the TAR within the BLV LTR. Gel retardation assays were performed to identify TAR DNA-binding proteins. For this purpose, an oligonucleotide (5′-AAGCTGGTGACGTCAGC TGGT-3′) corresponding to the 21-bp repeat centered at about 123 bp upstream of the RNA start site was used as a probe (Fig. 1). This sequence was selected because an oligonucleotide containing a multimer of the corresponding motif is sufficient to confer Tax responsiveness to a heterologous promoter (9, 21). This probe was end labelled and incubated with nuclear extracts from latently infected B cells derived from two BLV-infected cattle with PL. To prepare these extracts, T cells were selectively depleted from PBMCs by antibody-dependent complement-mediated T-cell cytolysis (see Materials and Methods). The remaining B-cell-enriched populations were then cultured for 48 h in the presence of PMA (0.1 μM) in combination with PHA (1 μg/ml) (the most effective inducers of BLV expression in our possession). In D17 cells, CREB transactivation of the BLV LTR requires a PKA expression vector, suggesting that the cAMP pathway could play a role in the regulation of BLV expression (49). Consequently, these bovine B cells (infected or not) were also treated with FSK, an agent that increases the concentration of cAMP by directly binding to adenylate cyclase (41). In order to correlate the gel shifts with viral expression, the p24 major core protein levels in cell extracts and in cell culture supernatants



-148	CAGACAG	AGACGTC	AGCTGCC
-123	AAGCTGG	TGACGTC	AGCTGGT
-48	GAGCTGC	TGACCTC	ACCTGCT
	NAGNQPG		AGCTGNQ
Consensus		TGACGTC	

FIG. 1. Locations and sequences of the TAR repeats of the BLV LTR. (Upper part) The three 21-bp TAR repeats are located in the U3 region of the BLV LTR (shaded boxes). (Lower part) The three TAR elements have been aligned around a common 7-bp core sequence. The position of each repeat within the LTR, shown at the left, corresponds to its location relative to the RNA start site. A consensus sequence based on these data is shown at the bottom. N, any nucleotide; P, purine; Q, pyrimidine (10).

were measured by using an immunoenzymatic capture technique (ELISA) (36).

The nuclear proteins extracted from the B cells (infected or not) cultured for 48 h were incubated with a double-stranded ^{32}P-end-labelled 21-bp TAR probe and migrated on a nondenaturing gel (electrophoretic mobility shift assay procedure). Incubation of this probe with nuclear extracts from either BLV-infected B cells or noninfected B cells yielded several electrophoretically retarded DNA-protein complexes (Fig. 2). When a nuclear extract was prepared from unstimulated B cells from a noninfected cow, two discrete complexes (designated C4 and C5) appeared (Fig. 2, lane 1). These two complexes were also observed when nuclear extracts derived from the same cells were cultured in the presence of FSK (Fig. 2, lane 4) or PMA combined with PHA (Fig. 2, lane 7). In contrast, nuclear extracts made from BLV-infected B cells from two cattle with PL (B76 [extract BLV$^+$A] and B163 [extract BLV$^+$B]) resulted in the formation of three additional complexes, C1, C2, and C3 (Fig. 2, lanes 2, 3, 5, 6, 8, and 9). Complex C3 (appearing as a smear) was detected only when the nuclear extracts derived from infected B cells were treated with PMA and PHA (Fig. 2, lanes 8 and 9). The amounts of the C1 and C2 complexes in gel shifts appeared to parallel the BLV expression (as measured by p24 levels), but the intensities of these complexes did not necessarily increase with the p24 level. The complexes were hardly detectable in uninfected cells (Fig. 2, lanes 1, 4, and 7), appeared in BLV-infected cells (stimulated with FSK or not) (Fig. 2, lanes 2, 3, 5, and 6), and increased in the presence of an inductor of expression (Fig. 2, lanes 8 and 9). A similar observation was made with nuclear extracts prepared from infected PBMCs treated for 48 h with several other agents known to induce BLV expression, including an anti-IgM antibody (data not shown). In conclusion, these gel shift assays allowed the detection of five TAR-specific complexes. Three of them (C1, C2, and C3) were specifically observed in gel shift experiments using BLV-infected cells but were not observed (or were only weakly observed) in experiments using normal cells. A positive correlation between the amounts of these complexes and the level of BLV expression could exist.

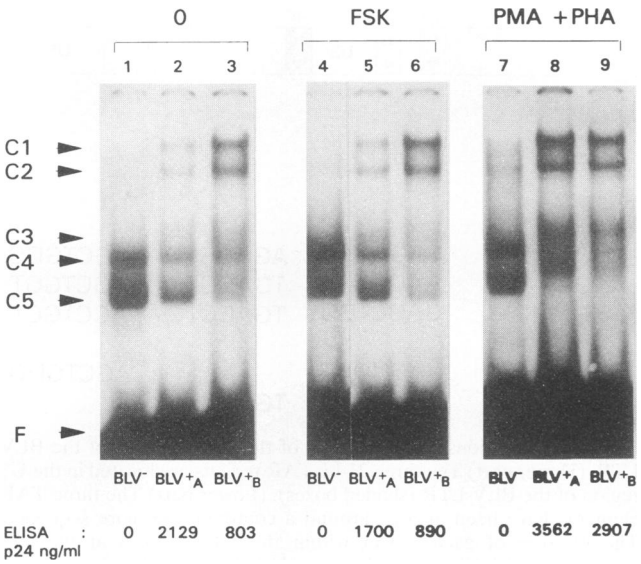


FIG. 2. Nuclear factors from bovine B cells bind the TAR motif. The ^{32}P-end-labelled 21-bp oligonucleotide (TAR probe) was incubated with nuclear extract (1.2 μg of total protein) prepared from B lymphocytes of an uninfected cow (B78 [extract BLV$^-$]) (lanes 1, 4, and 7) or two infected cattle with PL (B76 [extract BLV$^+$A] and B163 [extract BLV$^+$B]) (lanes 2, 5, and 8 and 3, 6, and 9, respectively). These B lymphocytes were cultured for 48 h without treatment (lanes 1 through 3), in the presence of FSK (20 μM) (lanes 4 through 6), or with PHA (1 μg/ml) in combination with PMA (0.1 μM) (lanes 7 through 9). Complexes were separated on a 6% nondenaturing polyacrylamide gel. C1 to C5 are the specific protein-DNA complexes. F, free probe. The BLV p24 antigen was titrated in both cell extracts and cell culture supernatants by an immunoenzymatic capture technique (ELISA).

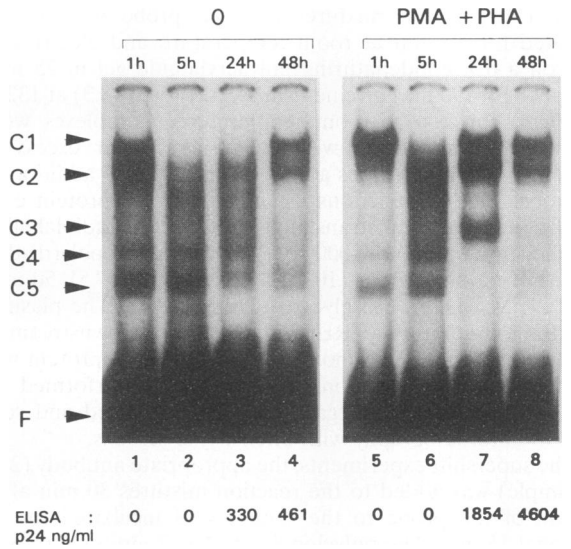


FIG. 3. Kinetics of induction of TAR-specific complexes. The ^{32}P-end-labelled 21-bp oligonucleotide (TAR probe) was incubated with nuclear extract (1.2 μg of total protein) prepared with PBMCs from an infected cow with PL (B163). These PBMCs were cultured for various periods (1, 5, 24, and 48 h) without treatment (lanes 1 through 4) or in the presence of PHA (1 μg/ml) in combination with PMA (0.1 μM) (lanes 5 through 8). Complexes were separated on a 6% nondenaturing polyacrylamide gel. C1 to C5 are the specific protein-DNA complexes. F, free probe. The BLV p24 antigen was titrated in both cell extracts and cell culture supernatants by an immunoenzymatic capture technique (ELISA).

Kinetics of induction of TAR-specific complexes. To examine the kinetics of the complex formation, nuclear extracts were prepared from infected PBMCs (cow B163) after different culture periods (1, 5, 24, and 48 h). Gel retardation assays using the TAR probe were performed as described above (Fig. 3). After 1 h of culture, four complexes were observed, C1 and C2 as smeary complexes and C4 and C5 as very intense ones (Fig. 3, lane 1). At 5 h, only two complexes (C4 and C5) were clearly detected. Subsequent culture induced the formation of the complexes C1 and C2, while C4 and C5 gradually disappeared (Fig. 3, lanes 3 and 4). Similar profiles were obtained by using PHA- and PMA-stimulated PBMCs. However, the different complexes appeared more intense (Fig. 3, lanes 5 through 8). Moreover, after 24 h of culture, the complexes C1, C2, and C3 were already detected, while the mobility shift profile was still smeary in the case of the noninduced cells (Fig. 3; compare lanes 7 and 3). After 48 h of treatment with the expression inducers PHA and PMA, the C3 complex appeared less distinctly, although C1 and C2 were still present. Complexes C4 and C5 disappeared after 24 h of culture. Altogether, these gel shifts revealed a pattern of five specific complexes depending on the culture period. Moreover, two complexes (C1 and C2) seemed to be associated with the highest expression levels (Fig. 3, lanes 4 and 8).

Competition experiment. The TAR repeats can be divided into three imperfectly conserved domains (designated A, B, and C) (Fig. 4B). Domain B (5′-TGACGTCA-3′) is a binding site for the ATF and CREB transcription factors (17, 33, 50). This sequence motif (named the CRE) is present in a number of cellular and viral promoters and notably mediates the induction of the promoter activity in response to cAMP (18, 30, 37). To investigate the contribution of each of the three domains (A, B, and C) in the formation of the five TAR-specific complexes (C1, C2, C3, C4, and C5), competition experiments with double-stranded oligonucleotides were performed. A series of oligonucleotides used as unlabelled competitors were synthesized and added to the binding reaction together with the wild-type ^{32}P-labelled TAR (Fig. 4A). The oligonucleotides Δ1-2, Δ3-4, and Δ5-6 correspond to the TAR sequences but lack domains B, C, and A, respectively (figure 4B). The TRE oligonucleotide lacks a single G nucleotide in the B domain, transforming the CRE site into a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (5′-TGACTCA-3′) which is specifically recognized by the transcription factor AP-1 (1, 28) (Fig. 4B).

The competition experiments were performed with three different nuclear extracts prepared from (i) infected PBMCs (cow B163) cultured for 1 h without inducer (Fig. 4A, left panel), (ii) infected PBMCs (cow B163) cultivated for 24 h in the presence of PMA in combination with PHA (Fig. 4A, middle panel), and (iii) noninfected B cells treated for 48 h with FSK (Fig. 4A, right panel). These particular nuclear lysates were chosen because the five specific complexes C1 to C5 could be distinctly observed. However, the competition assays were confirmed with other lysates (data not shown). The formation of complexes C1 and C2 is completely blocked when the competitor oligonucleotide contains an intact CRE sequence (oligonucleotides Δ5-6 and Δ3-4) (Fig. 4A, left and middle panels, lanes 4 and 5) but not when this motif is partially or completely deleted (oligonucleotides TRE and Δ1-2, respectively) (Fig. 4A, left and middle panels, lanes 2 and

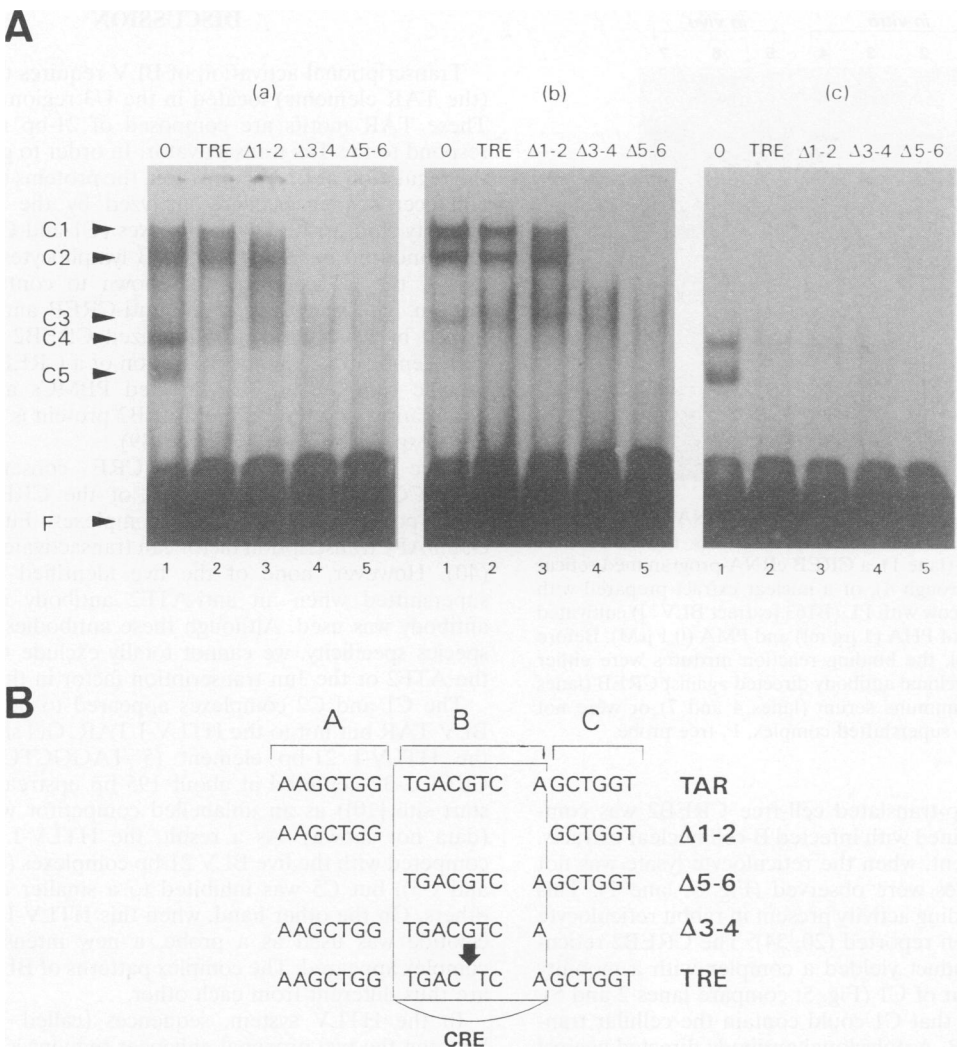


FIG. 4. Competition experiments with several mutated 21-bp oligonucleotides. (A) Gel shift assays of the TAR element. The ^{32}P-end-labelled 21-bp oligonucleotide (TAR probe) was incubated with nuclear extracts (1.2 μg of total protein) prepared from (a) infected PBMCs (from a cow with PL [B163]) after 1 h of culture without inducer, (b) infected PBMCs (from the same cow, B163) after 48 h of culture in the presence of PHA (1 μg/ml) and PMA (0.1 μM), or (c) noninfected B lymphocytes after 48 h of culture in the presence of FSK (20 μM). The competitors (oligonucleotides TRE [lane 2], Δ1-2 [lane 3], Δ3-4 [lane 4], and Δ5-6 [lane 5]) were used at a 100-fold molar excess. No competitor was added in lane 1. Complexes were separated on a 6% nondenaturing polyacrylamide gel. C1 to C5 are the specific protein-DNA complexes. F, free probe. (B) Sequences of the TAR mutants used as specific competitors. The Δ1-2 oligonucleotide has a deletion of the CRE motif (domain B), the Δ5-6 oligonucleotide has a deletion of the 5′ extremity (domain A), the Δ3-4 oligonucleotide has a deletion of the 3′ extremity (domain C), and the TRE oligonucleotide has a deletion of one nucleotide (G), transforming the CRE site into a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element.

3). These results imply that the CRE sequence within the TAR oligonucleotide is essential for C1 and C2 complex formation (present in lanes 1). The C3 complex, however, could not be completely blocked with any oligonucleotide (Fig. 4A, middle panel, lanes 1 through 5) except the wild-type TAR motif (data not shown). Complexes C4 and C5 were efficiently inhibited by the oligonucleotides TRE, Δ1-2, Δ3-4, and Δ5-6 (Fig. 4A, left and right panels, lanes 1 through 5).

To define the nucleotide determinants for complexes C4 and C5, gel shift assays using oligonucleotides Δ1-2 (5′-AAGC TGGGCTGGT-3′), Δ3-4 (5′-AAGCTGGTGACGTCA-3′), and Δ5-6 (5′-TGACGTCAGCTGGT-3′), which contain the sequence AGCTGGT/G, were performed. The Δ5-6 motif (and to a lesser extent the Δ3-4 motif) permitted C4 and C5 complex formation (data not shown). However, these two complexes did not appear when the Δ1-2 motif was used as a probe (data not shown). From these results, we conclude that, although required, the AGCTGGT/G sequence is not sufficient for C4 and C5 complex formation.

Altogether, these results show that the C1 and C2 complexes require the CRE motif and that the integrity of C4 and C5 relies on the flanking sequences and part of the CRE motif. The C3 complexes exhibit an intermediate phenotype.

CREB protein binds to TAR DNA. As suggested by the competition assays, the CRE motif is essential for formation of complexes C1 and C2. It seems likely that these complexes contain members of the CREB-ATF family. We have previously shown that the CREB2 protein can bind and transactivate the BLV LTR at a basal expression level in D17 osteosarcoma cells (49). Therefore, the mobility shift profile

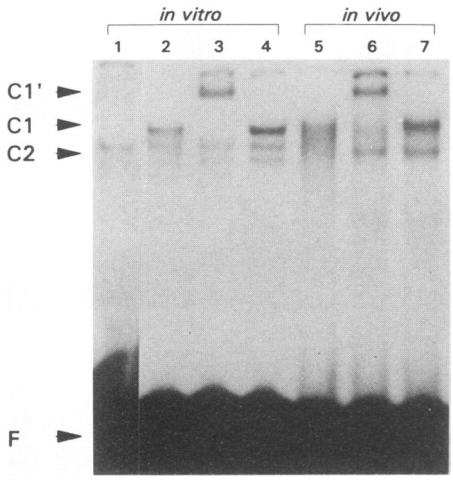


FIG. 5. The CREB protein binds the TAR DNA. The ^{32}P-end-labelled 21-bp oligonucleotide (TAR probe) was incubated with reticulocyte lysate alone (lane 1), a CREB cRNA-programmed reticulocyte lysate (lanes 2 through 4), or a nuclear extract prepared with infected PBMCs from a cow with PL (B163 [extract BLV$^+$]) cultivated for 48 h in the presence of PHA (1 μg/ml) and PMA (0.1 μM). Before being loaded on the gel, the binding reaction mixtures were either preincubated with a polyclonal antibody directed against CREB (lanes 3 and 6) or with a preimmune serum (lanes 4 and 7) or were not preincubated. C1′ is the supershifted complex. F, free probe.

obtained with in vitro-translated cell-free CREB2 was compared with those obtained with infected B-cell nuclear extracts. In a control experiment, when the reticulocyte lysate was not primed, two complexes were observed (Fig. 5, lane 1). This endogenous CRE binding activity present in rabbit reticulocyte lysate has already been reported (20, 34). The CREB2 reticulocyte translation product yielded a complex with a mobility nearly identical to that of C1 (Fig. 5; compare lanes 2 and 5). This result suggested that C1 could contain the cellular transcription factor CREB. A polyclonal antibody directed against CREB was added after incubation of the TAR probe with the infected B-cell nuclear extract (Fig. 5, lane 6). This antibody moved the C1 complex to position C1′ but did not supershift the C2 complex (Fig. 5; compare lanes 5 and 6) or the other complexes (data not shown). When the same experiment was performed with in vitro-synthesized CREB2, the CREB2 complex was also displaced to a position similar to C1′ (Fig. 5; compare lanes 3 and 6). These supershifts were not observed with a preimmune serum (Fig. 5, lanes 4 and 7). Thus, we conclude that complex C1 (but not C2) contains the CREB protein.

ATF2 is a distinct member of the CREB-ATF family that shows a high degree of sequence similarity to CREB in the bZIP domains. Furthermore, the c-Jun/AP-1 transcription factor can also bind to the CRE sequence (40). To determine whether these transcription factors were involved in the formation of the five complexes, two additional antibodies directed against these proteins were tested in the gel shift experiments. No complex was ever specifically shifted, suggesting that neither ATF2 nor Jun exhibits TAR binding activity ex vivo (data not shown).

Taken together, these experiments demonstrated that the CREB protein, present in infected B-cell nuclear extracts, specifically binds to the BLV TAR enhancer. Furthermore, the amount of CREB-TAR complexes correlates with levels of BLV expression.

DISCUSSION

Transcriptional activation of BLV requires three enhancers (the TAR elements) located in the U3 region of the 5′ LTR. These TAR motifs are composed of 21-bp repeats able to respond to the Tax transactivator. In order to gain insight into the regulation of BLV expression, the proteins binding to these enhancer sequences were analyzed by the electrophoretic mobility shift assay. Two complexes (C1 and C2) were specifically induced in BLV-infected B lymphocytes. By supershift assays, the C1 complex was shown to contain the CREB protein. The specificity of the anti-CREB antibody was confirmed by using in vitro-synthesized CREB2 protein. These data demonstrate the incorporation of a CREB protein into a specific complex in BLV-infected PBMCs and extend our observation that the bovine CREB2 protein is able to activate BLV expression in cell culture (49).

Since TAR contains the CRE consensus sequence (TGACGTCA), other members of the CREB-ATF family could possibly belong to the complexes. Furthermore, the c-Jun/AP1 transcription factor can transactivate CRE elements (40). However, none of the five identified complexes was supershifted when an anti-ATF2 antibody or an anti-Jun antibody was used. Although these antibodies were of broad species specificity, we cannot totally exclude the presence of the ATF2 or the Jun transcription factor in the complexes.

The C1 and C2 complexes appeared to be specific to the BLV TAR but not to the HTLV-I TAR. Gel shift assays using the HTLV-I 21-bp element (5′-TAGGCTCTGACGTCTC CCCCC-3′; centered at about 195 bp upstream of the RNA start site [10]) as an unlabelled competitor were performed (data not shown). As a result, the HTLV-I 21-bp element competed with the five BLV 21-bp complexes (C1, C2, C3, C4, and C5), but C5 was inhibited to a smaller extent than the others. On the other hand, when this HTLV-I 21-bp oligonucleotide was used as a probe, a new intense intermediate complex appeared. The complex patterns of BLV and HTLV-I are thus different from each other.

In the HTLV system, sequences (called TRE2) located between the two proximal enhancer sequences appear to bind to a 36-kDa protein (31). This factor bridges the TRE2 motif and the Tax1 transactivator. In the BLV system, the homologous sequences located between the two proximal TAR motifs appear to enhance Tax responsiveness (9, 21). Therefore, a probe (called i21) corresponding to the sequence between the two 21-bp motifs located at positions −123 and −48 was also used in gel shift assays. This oligonucleotide did not form a specific complex under our experimental conditions (data not shown). It is still possible that weak interactions involving specific proteins do exist. Alternatively, a smaller spectrum of cellular proteins binding to BLV LTR could reflect the lack of basal expression in the absence of the Tax transactivator (49).

Our gel shift experiments reveal a complex pattern of proteins binding to the TAR motif. Kinetic data during short-term culture reveal two main groups of complexes. The C1 and C2 complexes appear and are amplified during the culture period, while the C4 and C5 complexes decrease. Furthermore, C1 and C2 appear to be associated with high expression levels in infected cells. The C4 and C5 complexes are also present in uninfected lymphocytes. Finally, the targets in the TAR motif are different (the C1 and C2 complexes requiring the CRE motif and the C4 and C5 complexes requiring both flanking domain A or C and part of this CRE motif). Altogether, these data suggest that C4 and C5 are associated with a latent state which evolves into an activation state corresponding to that of the C1 and C2 complexes. The

C3 complex appears to be an intermediate stage, since it essentially appears at 24 h, between the latent and activated stages. It should be mentioned here that the C1 and C2 complexes can also be observed in uninfected B lymphocytes when the gel shift reactions are overloaded with proteins (data not shown). This suggests that these complexes contain cellular proteins that are activated by the virus. Alternatively, these proteins could also be induced during the spontaneous cell proliferation, resulting in activation of virus expression. The kinetic experiments reveal that four complexes (C1, C2, C3, and C4) are present after 1 h of culture. It thus appears that the preparation of the B lymphocytes allows the formation of the C1 and C2 complexes in infected cells. Alternatively, the infected B lymphocytes contain sufficient protein levels in vivo to form the C1 and C2 complexes. Subsequent culture shows that the initial C1 and C2 complexes disappear (at 5 h). This suggests that the proteins present in these complexes are degraded or modified (by phosphorylation, for instance), leading to a resting stage. De novo synthesis or translational modification should then be required to produce the C1 and C2 complexes again.

The present study is consistent with previous reports concerning the stimulation of BLV expression after in vitro cultivation (19, 27). Several agents, including the phorbol esters (e.g., PMA), activate viral protein synthesis probably through the PKC pathway (7, 19). The levels of the C1 and C2 complexes appear to parallel the amounts of viral protein synthesis (as demonstrated by p24 ELISA). This suggests that PKC could activate cellular transcription factors specific for TAR. Since the CREB protein is present in complex C1, PKC could directly phosphorylate this transcription factor and initiate BLV transcription. Several activators were tested for their ability to enhance BLV expression. These include PMA, PHA, FSK, Ca^{2+} ionophore (A23187), and anti-IgM antibody (alone or in combination). The gel shift patterns were independent of the activators but correlated to the levels of expression (data not shown). The best activators induced more C1 and C2 complex formation. In vitro, the CREB2 transactivator activity requires cotransfection of a PKA vector in D17 osteosarcoma cells (49). Ex vivo, however, activation of the PKA pathway by using either FSK or dibutyryl-cAMP did not enhance viral synthesis in B lymphocytes (unpublished data). This suggests that both activation mechanisms coexist but that their relative potentials differ in vitro and in vivo. Since PKC stimulates the dimer formation of CREB protein purified from rat brain cells, we can speculate concerning the existence of a similar mechanism in the BLV system (50). PKA is required for the transcriptional activity of CREB through the phosphorylation of serine-133 (14). Therefore, we can hypothesize that two limiting steps are required for the initiation of BLV transcription: formation of CREB dimers by PKC and CREB transcriptional activation induced by PKA. The initiation of BLV expression would occur consequently to activation of PKC, when infected B lymphocytes undergo antigen-induced activation, for instance. Moreover, Tax protein could also function cooperatively with the CREB protein and the other CRE-binding factors to enhance BLV transcription during B-cell activation.

Efficient transcriptional activation of the BLV LTR requires the action of the Tax protein through the TAR enhancers (9, 21). Since Tax is unable to bind DNA, the transactivator might indirectly interact with cellular proteins to stimulate transcription. In our experiments, none of the complexes was supershifted by using either a mixture of monoclonal antibodies or a rabbit serum directed towards Tax. This indicates that Tax is not present within the complexes. On the other hand, it is also possible that the anti-Tax antibodies dissociate the Tax-cellular protein-TAR complexes. Nuclear extracts from uninfected B cells cultured for 48 h lacked the complexes C1 and C2 observed in infected B cells at equal protein concentrations (1.2 μg). However, these two complexes could be observed at higher protein concentrations (fivefold, i.e., 1.2 μg) or after stimulation with PMA and PHA (during 48 h of culture). These results indicate that the complexes C1 and C2 preexist in uninfected B cells and could be induced by mitogenic stimulation. Since the electrophoretic mobility shift assay profiles are similar when the reactions are overloaded with uninfected B-lymphocyte lysates, it seems likely that the interactions between Tax and the cellular transcription factors are dissociated during gel migration. Alternatively, Tax binding requires a dimer of TAR as reported for the HTLV system (44). In vitro, the HTLV Tax1 transactivator enhances the dimerization and facilitates the DNA binding activity of several cellular proteins, including CREB and ATF2 (2, 47). Similarly, BLV Tax activation of transcription could be mediated (at least in part) through increased binding of the CREB protein (and other factors yet to be identified) to the TAR elements. The initial rate of BLV transcription would be independent of Tax and could be correlated with the activation of a preexisting CREB protein (and the other components of the complexes). After transcription and translation of the viral genes, the Tax protein could amplify gene expression in combination with these new CRE-binding factors. In agreement with this hypothesis, the amounts of the C1 and C2 complexes seem to correlate with the magnitude of the BLV expression. This suggests that these CRE-specific complexes mediate the activation of the BLV LTR-directed gene transcription.

In conclusion, the CREB transcription factor is present in a specific complex in ex vivo-infected B lymphocytes. In vivo, BLV expression could thus result (at least in part) from the binding of the CREB protein to the TAR element.

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