JOURNAL OF VIROLOGY, Feb. 1992, p. 766–772
0022-538X/92/020766-07$02.00/0

Vol. 66, No. 2

A Cyclic AMP-Responsive DNA-Binding Protein (CREB2) Is a Cellular Transactivator of the Bovine Leukemia Virus Long Terminal Repeat

LUC WILLEMS,[1]* RICHARD KETTMANN,[1] GAO CHEN,[1] DANIEL PORTETELLE,[1] ARSÈNE BURNY,[1,2] AND DAVID DERSE[3]

Faculty of Agronomy, B5030 Gembloux,[1] and University of Brussels, B1640 Rhode-St-Genèse,[2] Belgium, and National Cancer Institute, Frederick, Maryland 21701[3]

Received 8 July 1991/Accepted 22 October 1991

To gain insight into the cellular regulation of bovine leukemia virus (BLV) *trans* activation, a lambda-gt11 cDNA library was constructed with mRNA isolated from a BLV-induced tumor and the recombinant proteins were screened with an oligonucleotide corresponding to the *tax* activation-responsive element (TAR). Two clones (called TAR-binding protein) were isolated from 750,000 lambda-gt11 plaques. The binding specificity was confirmed by Southwestern (DNA-protein) and gel retardation assays. Nucleotide sequence analysis revealed that TAR-binding protein is very similar to the CREB2 protein. It contains a leucine zipper structure required for dimerization, a basic amino acid domain, and multiple potential phosphorylation sites. A vector expressing CREB2 was transfected into D17 osteosarcoma cells. In the absence of the *tax* transactivator, the CREB2 protein and the cyclic AMP-dependent protein kinase A activate the BLV long terminal repeat at a basal expression level: *trans* activation reached 10% of the values obtained in the presence of *tax* alone. These data demonstrate that CREB2 is a cellular factor able to induce BLV long terminal repeat expression in the absence of *tax* protein and could thus be involved in the early stages of viral infection. In addition, we observed that in vitro *tax*-induced *trans* activation can be activated or inhibited by CREB2 depending on the presence or absence of protein kinase A. These data suggest that the cyclic AMP pathway plays a role in the regulation of viral expression in BLV-infected animals.

Bovine leukemia virus (BLV) is the causative agent of enzootic bovine leukosis, a lymphoproliferative disease of cattle (4). The 3′ end of the BLV provirus encodes a protein called *tax* involved in *trans* activation of long terminal repeat (LTR)-directed gene expression (5, 24). Furthermore, *tax* is able to transform primary cells in cooperation with the *ras* oncogene and can thus be considered as a member of the immortalizing oncogene subgroup (25).

trans activation of the BLV LTR by $p34^{tax}$ requires a 75-bp element present in the U3 region (5). Nucleotide sequence data show that this *cis*-acting element is composed of two 21-bp repeats centered around 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 with respect to their nucleotide sequence and spatial array to elements found in the LTRs of human T-cell lymphotropic virus types I (HTLV-I) and -II. All these elements have a common 8-bp core sequence, TGACGTCA, known as the cyclic AMP (cAMP)-responsive element (CRE) or the binding site of a cellular transcription factor, ATF (15). The mutually exclusive *trans* activation of the BLV and HTLV promoters by their cognate *tax* proteins would suggest that the shared CRE represents a functional center of a larger response element whose specificity is determined by the flanking nucleotides.

In the HTLV system, the 21-bp repeat elements are essential for basal expression and *trans* activation by *taxI*. Since *taxI* does not directly bind to the 21-bp element, it may exert its effect through the cellular proteins that recognize these sequences. Several different proteins binding to the HTLV LTR have been identified (1, 7, 14, 17, 18). Four cDNA clones encoding nuclear proteins that bind to the 21-bp element were recently isolated from an HTLV-I-infected cell line (21, 28). All these proteins contain a leucine zipper and a basic amino acid domain (which are conserved in Fos, Jun, and CREB) and multiple potential phosphorylation sites.

The HTLV-I LTR-directed expression is modulated through the protein kinase A (PKA) and protein kinase C (PKC) pathways. The *taxI*-responsive element (TAR) is different from the sequences required for cAMP and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activation (6). Furthermore, the cAMP and TPA activators can enhance HTLV-I LTR-directed gene expression in the absence of the *taxI* transactivator. In contrast, the BLV LTR does not (or very poorly) respond to activation by cAMP and TPA in experimental conditions known to enhance expression in the HTLV-I system (unpublished data). Moreover, the BLV LTR is silent in the absence of the *tax* protein (5, 24). The apparent lack of basal expression of the BLV LTR and the observed lack of responsiveness to soluble cAMP analogs and TPA thus seem correlated.

These two main differences between BLV and HTLV-I prompted us to study cellular factors binding to the BLV 21-bp element. We isolated and characterized a TAR-binding protein (TBP) which appeared to be the bovine CREB2 protein. We also demonstrated that CREB2 *trans* activates the BLV LTR and does so significantly when a PKA-expressing plasmid is cotransfected with the CREB2-expressing construct.

* Corresponding author.

MATERIALS AND METHODS

Construction of lambda-gt11 cDNA library. RNA was prepared from a BLV-induced tumor from bovine 3894. cDNAs were prepared by using oligo(dT)$_{12-18}$ as primers. The cDNAs were cloned at the unique *Eco*RI site of lambda-gt11 vector by the method of Huynh et al. (13). Recombinant lambda bacteriophages from an amplified library were used to infect the Y1090 strain of *Escherichia coli* and induced to produce β-galactosidase fusion proteins as described by Huynh et al. (13).

Screening of protein replica filters with DNA binding site probes (20). Protein replica filters were prepared by the method of Huynh et al. (13). Infected Y1090 cells were plated in 0.7% agarose. After induction at 42°C for 3 h, the isopropyl-β-D-thiogalactopyranoside-saturated nitrocellulose filter overlays were incubated overnight at 37°C. Plates were cooled at 4°C for 5 to 10 min before lifting the filters. After the positions of the filters were marked on the plates, the filters were lifted and immediately immersed in aliquots of BLOTTO (5% nonfat milk powder, 50 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT]). Filters were incubated in BLOTTO for 60 min at room temperature with gentle shaking and washed twice (1 to 5 min for each wash) with aliquots of TNE-50 (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 M DTT). For screening, the filters were incubated in aliquots of TNE-50 containing [^{32}P]DNA binding site probe (1×10^6 to 2×10^6 cpm/ml) and salmon sperm DNA (10 μg/ml). After 60 min at room temperature with gentle agitation, the filters were washed four times (a total of 30 min) with aliquots of TNE-50. They were patted dry and exposed to Kodak X-OmatAR film (12 to 24 h) with an intensifying screen at −70°C.

Oligonucleotides TAR(CRE), 5'AAGCTGGTGACGTCA GCTGGT3', and TAR(TRE) (TRE, TPA-responsive element), 5'AAGCTGGTGACTCAGCTGGT3', were synthesized by using the Gene Assembler Plus (Pharmacia).

Analysis of β-galactosidase recombinant proteins by Western (immunoblot) transfers. Y1089 lysogens harboring lambda-gt11 phage were isolated (13) and induced to express high levels of their respective β-galactosidase fusion proteins. Cells from 1.25-ml aliquots of the induced lysogen cultures were pelleted and resuspended with 100-μl aliquots of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After heating at 100°C for 5 min, proteins in each sample (25 μl) were resolved by 10% SDS-PAGE. The separation gel was then soaked in 25 mM Tris–190 mM glycine (pH 8.3)–20% (vol/vol) methanol for 30 to 60 min. Proteins were electrophoretically transferred onto a nitrocellulose membrane using the same buffer. After transfer, the filters were blocked with BLOTTO (60 min at room temperature) and then washed twice with TNE-50 (1 to 5 min for each wash). The transfers were screened with ^{32}P-labeled probe.

Alternatively, transferred proteins were revealed with a rabbit anti-β-galactosidase serum or a monoclonal antibody mix directed against *tax* (25) and subsequent coloration by the alkaline phosphatase procedure (Promega).

Gel electrophoresis DNA binding assays. Extracts for assaying the DNA-binding activities of the β-galactosidase fusion proteins were prepared as follows. Cells from 1.25-ml aliquots of the induced lysogen cultures were rapidly pelleted and resuspended in 100-μl aliquots of buffer A (50 mM Tris [pH 7.5], 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride).

Cell suspensions were subjected to a rapid freeze-thaw cycle and incubated with lysozyme (0.5 mg/ml) for 15 min on ice. NaCl was then added to 1 M, and the mixtures were incubated on a rotator for 15 min at 4°C. After a 30-min spin in a microcentrifuge, the supernatants were dialyzed with Millipore filters (type VS, 0.025-μm pore size) against buffer A (60 min at 4°C). The dialyzed extracts were quick-frozen and stored at −70°C.

Binding reactions and gel electrophoresis were performed as previously described (20) with a modified running buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA [pH 8.3]). Complexes were inhibited by competition with a 100-fold molar excess of unlabeled oligonucleotides.

Plasmid constructs and nucleotide sequence. The 1,093-bp *Eco*RI insert was excised from the recombinant lambda-gt11 DNA and inserted into the pGem7Zf vector (Promega) or the pSG5 eukaryotic expression plasmid (pSGCREB). Nucleotide sequences were determined by using the Erase-a-base kit (Promega) and the Sequenase DNA sequencing kit (United States Biochemical). The pLTRCAT reporter contains the BLV LTR *Eco*RI fragment (25) cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in plasmid pGEM7Zf (Promega).

In vitro transcription and translation. Complementary RNA was synthesized with SP6 RNA polymerase and was translated into a rabbit reticulocyte lysate (Stratagene). Gel retardation assays were performed as described by Hai et al. (11).

Preparation of nuclear extracts. Nuclear extracts were prepared from FLK cells (BLV-infected fetal lamb kidney) as described previously (16). Briefly, cells were washed in phosphate-buffered saline 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 DTT, 0.5 mM phenylmethylsulfonyl fluoride) for 1 h at 4°C with gentle shaking. Nuclear extract was centrifuged at 126,300 × *g* for 30 min, and the supernatant was 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 × *g* for 30 min. The supernatant was dispensed in aliquots and stored at −80°C until use.

Cell transfection and CAT assays. D17 osteosarcoma cells (3×10^5) were transfected by the calcium phosphate procedure with 1 μg of the pSGtax effector plasmid DNA (which expresses a functional *trans* activation protein), 3 μg of the reporter construct (which contains the LTR promoter sequences cloned upstream of the CAT gene), the pPKA expression vector (1 μg), and 1 μg of pSGTBP (which express the TBP cDNA under the control of the simian virus 40 promoter). When a plasmid was omitted in the transfection experiments, 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 (3). The data (in counts per minute) represent the mean values of at least four independent transfections. As a control for transfection efficiencies, a β-galactosidase assay was performed with the pRSVβgal plasmid (kindly provided by J. Ghysdael). The PKA inhibitors H7 [1-(5-isoquinoline-

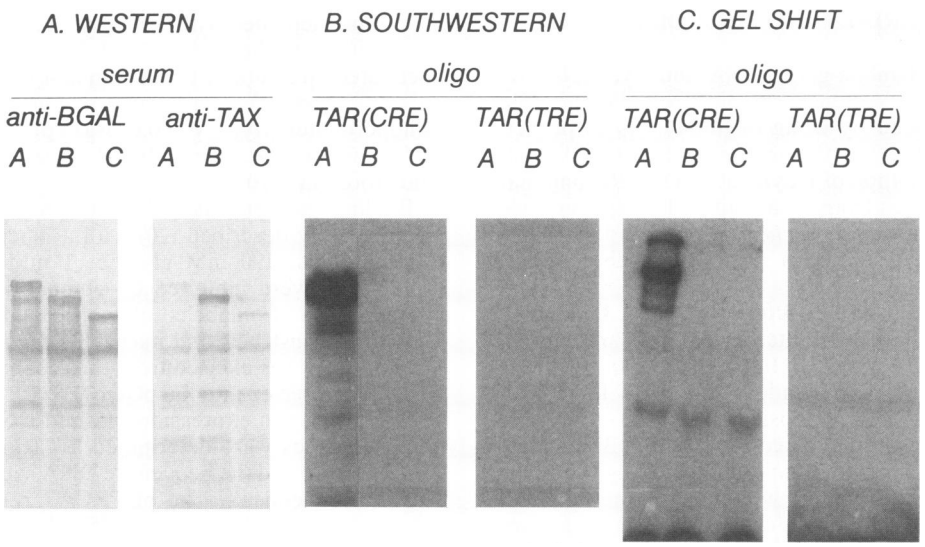

FIG. 1. Binding activity of the TBP to the 21-bp enhancer element. (A) Western blot analysis of the CREB2 (lanes A)-, *tax* (lanes B)-, and β-galactosidase (wild type; lanes C)-containing lysates. Sera used are a rabbit anti-β-galactosidase serum (anti-BGAL) and a mix of monoclonal antibodies directed against *tax* (anti-TAX). (B) Southwestern blot with oligonucleotides TAR(CRE) and TAR(TRE) and TBP lysates (lanes A), *tax* (lanes B), or β-galactosidase (lanes C). (C) Gel retardation assay corresponding to the Southwestern blot.

sulfonyl)-2-methylpiperazine dihydrochloride] and H8 [*N*-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide dihydrochloride] were provided by Seikagaku. The PKA activities were assayed with a kit provided by GIBCO.

RESULTS

Isolation of cDNA encoding a TBP. To gain insight into the regulation of BLV expression, we decided to identify and characterize cellular proteins involved in the *trans* activation complex. The 21-bp TAR element appears to be a major determinant in LTR responsiveness to the $p34^{tax}$ transactivator protein. Therefore, a lambda-gt11 cDNA expression library was prepared from mRNA extracted from a bovine tumor (T3894), and the recombinant proteins were screened with a ^{32}P-labeled oligonucleotide corresponding to the TAR sequence (20). Two clones (called lambda-TBP1 and -2) from 750,000 phage plaques were identified and purified. Southern hybridization and restriction map analysis showed that the two cDNAs corresponded to the same transcript (data not shown).

The specificity of the DNA-binding activity of the protein expressed by one of the cDNAs (lambda-TBP1) was confirmed by Southwestern (DNA-protein) and gel shift assays. Y1089 lysogens were obtained after infection with recombinant lambda-TBP1, and a lysate was analyzed by Western blotting (immunoblotting) with a rabbit anti-β-galactosidase serum. A fusion protein of approximately 150 kDa was observed (Fig. 1A, anti-BGAL, lane A). Lanes B and C correspond to control lysates containing β-galactosidase–Tax (138 kDa) or β-galactosidase (114 kDa) protein, respectively (23). Only the β-galactosidase–Tax product was revealed by a mixture of monoclonal antibodies directed against *tax* (25) (Fig. 1A, anti-TAX, lane B).

Southwestern experiments demonstrated that the 21-bp oligonucleotide AAGCTGGTGACGTCAGCTGGT [called TAR(CRE)] specifically bound to the β-galactosidase–TBP fusion protein [Fig. 1B, TAR(CRE), lane A] but not to β-galactosidase–Tax (lane B) or to β-galactosidase (lane C). When the CRE was mutated into a TRE (TGACTCA), no binding occurred [Fig. 1B, TAR(TRE), lanes A, B, and C], demonstrating that the CRE is a major determinant in the DNA-protein association complex.

These Southwestern experiments were confirmed by DNA gel shift assays with the two oligonucleotides mentioned above and partly purified Y1089 lysates (Fig. 1C). It appeared that efficient binding between the TBP and the ^{32}P-end-labeled TAR oligonucleotide indeed requires the CRE.

Nucleotide sequence of lambda-TBP1 cDNA. After subcloning into plasmid pGem7Zf, the lambda-TBP1 *Eco*RI insert was completely sequenced (26). This analysis revealed that the 1,093-bp fragment corresponds to the bovine homolog of the CREB2 cDNA. The amino acid sequence revealed that TBP contains a leucine zipper structure (residues 297 to 319), a basic amino acid domain (residues 271 to 294), and several potential phosphorylation sites. Seven point mutations in 315 amino acids were identified at the amino terminus in comparison with the human sequence (12, 26). None of them affected the above-mentioned domains.

It is important to mention here that the bovine CREB2 differs from the proteins recently identified in the HTLV-I system (21, 28). However, all these TBPs are structurally related and belong to the CREB/ATF family.

CREB-like nuclear factor binds to TAR(CRE) sequence. As a control, cRNA to the CREB2 cDNA was synthesized by using SP6 RNA polymerase and translated in a rabbit reticulocyte lysate. ^{32}P-labeled TAR(CRE) oligonucleotides of 21 bp were incubated with the in vitro-synthesized CREB2 proteins. After binding, the DNA-protein complexes were separated on a nondenaturing gel. Several complexes were observed (Fig. 2, lane 1). They were blocked by a TAR(CRE) unlabeled probe (Fig. 2, lane 3) but not by the TAR(TRE) oligonucleotide (Fig. 2, lane 2).

When a nuclear extract prepared from BLV-infected FLK cells was incubated with a ^{32}P-labeled TAR(CRE) probe, a specific complex was observed (Fig. 2, lane 4). This complex was blocked when the unlabeled TAR(CRE) sequence was

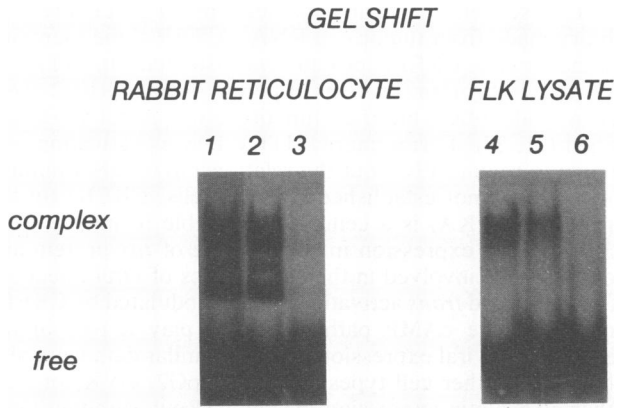

FIG. 2. CREB-like nuclear factor binds to the TAR(CRE) sequence. Gel shift assay of the ^{32}P-labeled TAR(CRE) oligonucleotide incubated with a CREB2 cRNA-programmed reticulocyte lysate (lanes 1, 2, and 3) or with a nuclear extract of BLV-infected fetal lamb kidney (FLK) cells (lanes 4, 5, and 6). In lanes 2 and 4, unlabeled TAR(TRE) was added to the binding reaction mixtures. In lanes 3 and 6, specific complexes were blocked with the TAR(CRE) probe.

added to the reaction mixture (Fig. 2, lane 6); no competition occurred with the TAR(TRE) oligonucleotide (Fig. 2, lane 5). This specific complex containing nuclear proteins comigrated with the largest in vitro-synthesized CREB2-TAR-(CRE) complex.

CREB2 is a ubiquitous protein synthesized in many cell lines. No difference in CREB2 expression could be correlated with the level of BLV transcription (data not shown; see Discussion).

CREB2 protein is involved in in vitro BLV LTR basal expression. The BLV and HTLV-I systems share many common properties in genome organization and gene regulation. However, a main difference concerns the basal expression of their respective LTRs. The BLV LTR, in contrast to the HTLV-I LTR, is silent in the absence of viral transactivator protein. The mechanisms involved in the early stages of viral expression are thus unknown in the BLV system.

To gain insight into a possible involvement of the cAMP pathway in this process, we introduced the 1,093-bp *Eco*RI insert of the bovine CREB2 cDNA into a eukaryotic expression vector (pSGCREB) and transfected it into D17 osteosarcoma cells, in which high activation by *tax* has been demonstrated (5) (Table 1). As an internal transfection control, we used the pRSVβGal plasmid, which contains the Rous sarcoma virus LTR cloned upstream of the *lacZ* gene, and performed a β-galactosidase assay. The transfection efficiencies were very reproducible (less than 5% variation).

When the pLTRCAT plasmid (which contains the BLV LTR cloned upstream of the CAT gene) was transfected into D17 cells, no CAT activities were detected above background level, confirming that the BLV LTR is silent in uninfected cells (5, 24). Cotransfection of plasmids pLTRCAT and pSGCREB was also not able to induce *trans* activation of the BLV LTR-directed CAT gene expression. In contrast, when a cAMP-dependent PK expression vector (pPKA, kindly provided by R. Maurer) was cotransfected with plasmids pLTRCAT and pSGCREB, detectable CAT activities were obtained (Table 1; net CAT activity = 742 cpm). These values represent 10% of the CAT activities obtained after cotransfection of plasmids pSGtax (which expresses a functional transactivator protein) and pLTRCAT (Table 1; net CAT activity = 7,257 cpm). Low, but detectable, levels of CAT activity were measured after cotransfection of plasmids pLTRCAT and pPKA (Table 1; net CAT activity = 131 cpm). These values represent only 1.8% of the CAT activity obtained after cotransfection of plasmids pSGtax and pLTR-CAT. Transfection of the pPKA plasmid into D17 cells induced a twofold increase of the basal PKA mean activity (470 instead of 255 pmol of peptide-incorporated phosphate per min as measured by the GIBCO PKA assay system). This corresponds in fact to a 200-fold increase of PKA activity assuming 1% transfection efficiency.

These experiments confirm that the *tax* protein is required to obtain efficient *trans* activation. They also demonstrate

TABLE 1. *trans* activation of the BLV LTR in D17 cells by the *tax*, CREB2, and PKA proteins[a]

Inhibitor	Tax	CREB	PKA	Net CAT activity (cpm)[b]	Relative CAT activity	
					% versus Tax$^+$ CREB$^-$ PKA$^-$	% versus Tax$^+$ CREB$^-$ PKA$^-$ in the absence of PK inhibitor
None	−	−	−	1	<1	
	−	+	−	1	<1	
	−	−	+	131 ± 36	1.8	
	−	+	+	742 ± 145	10.2	
	+	−	−	7,257 ± 580	100.0	
	+	+	−	2,903 ± 174	40.0	
	+	−	+	4,921 ± 490	67.8	
	+	+	+	21,702 ± 2,604	299.0	
H8 (5×10^{-5} M)	−	−	−	1		<1
	−	+	−	1		<1
	−	−	+	1		<1
	−	+	+	1		<1
	+	−	−	3,931 ± 690		54.2
	+	+	−	3,136 ± 720		43.2
	+	−	+	3,799 ± 305		52.3
	+	+	+	6,723 ± 530		92.6

[a] D17 osteosarcoma cells were transfected with 3 μg of pLTRCAT reporter and, when indicated (+), with 1 μg of pSGCREB (which expresses a functional bovine CREB2 protein), 1 μg of pSGtax (synthesizing the BLV *tax* protein), or 1 μg of the pPKA plasmid (which expresses the catalytic subunit of PKA).

[b] The data represent the mean value of at least four independent transfections. These CAT activities were normalized to values obtained after cotransfection of 1 μg of pSGtax effector and 3 μg of pLTRCAT reporter. DNA concentrations were kept constant by using plasmid pSG5 DNA.

that significant LTR-directed expression levels can be obtained in the absence of *tax* provided that the CREB2 and PKA proteins are provided in *trans*. It thus appeared that the bovine CREB2 protein may be considered to be a cellular transactivator of the BLV LTR-directed gene expression.

TBP and PKA regulate *tax*-induced *trans* activation of the BLV LTR. To evaluate the role of the CREB2 protein in *tax*-induced *trans* activation, the pSGtax, pSGCREB, and pLTRCAT plasmids were cotransfected in D17 cells; the net CAT activity decreased to 40% in comparison with values obtained after cotransfection of pSGtax and pLTRCAT (Table 1; compare net CAT activities of 2,903 and 7,257 cpm). It appeared that the BLV *tax*-induced *trans* activation was significantly inhibited by the CREB2 protein.

tax-induced *trans* activation inhibition of the BLV LTR may result from inefficient phosphorylation of the TBP (see Discussion). The CREB DNA-binding protein is phosphorylated at Ser-133 by the catalytic subunit of the cAMP-dependent PKA (8). Therefore, the cAMP-dependent PKA expression vector (pPKA) was cotransfected in D17 cells with plasmids pSGCREB, pSGtax, and the pLTRCAT reporter. A threefold increase in *trans* activation was observed (Table 1; compare net CAT activity of 21,702 cpm with 7,257 cpm) and is called superinduction of *trans* activation.

Transfection of plasmid pPKA without pSGCREB inhibited *tax*-induced *trans* activation; the pPKA expression vector decreased *trans* activation levels obtained after cotransfection of plasmids pSGtax and pLTRCAT (Table 1, 67.8%). Super-*trans* activation thus requires the presence of both TBP and PKA.

Similar results were obtained with another *tax* expression vector, p38RSPA (5), which contains the Rous sarcoma virus promoter (data not shown). Furthermore, the levels of *tax* protein remained fairly constant as revealed by radioimmunoprecipitation (data not shown).

These data demonstrate that the TBP is able to increase *tax*-induced *trans* activation in the BLV system. However, in similar conditions, the HTLV-I LTR was not responsive to the bovine TBP (data not shown). The reason for this is still unknown but could be due to specific differences between the bovine and human CREB proteins (26) or between the target sequences in their respective LTRs.

***trans* activation of BLV LTR in presence of PK inhibitors.** The BLV LTR is efficiently transactivated by CREB2 in the presence of PKA. We tested the effect of PK inhibitors (Table 1). The inhibitor H8 efficiently inhibits the activities of the cyclic nucleotide-dependent PKs. In fact, when pSGCREB and pPKA were cotransfected with the pLTRCAT reporter in the presence of 5×10^{-5} M H8, the basal *trans* activation of the BLV LTR was completely inhibited (Table 1; relative CAT activity of <1 cpm). The *trans* activation of the LTR by *tax* was also reduced (relative CAT activity of 54.2%) as well as the super-*trans* activation obtained after cotransfection of pSGtax, pSGCREB, and pPKA (relative CAT activity of 92.6%). The observed effect of the H8 compound was not due to a direct inhibition of the CAT enzyme since expression of the pRSVCAT vector (which contains the Rous sarcoma virus LTR cloned upstream of the CAT gene) was not affected (data not shown). Similar effects were also obtained with the PK inhibitor H7 (data not shown).

DISCUSSION

The BLV LTR contains three 21-bp repeats (called here TAR) involved in *trans* activation by *tax*. To understand the *trans* activation complex, we screened proteins binding to the TAR sequence from an amplified lambda-gt11 expression library made from tumor cell RNA. A TBP clone identified as the bovine CREB2 protein was isolated. In HTLV-I-infected cells, four proteins different from CREB2 were recently identified, but their functional role is not yet established (21, 28). Furthermore, HTLV-I *trans* activation was shown to require PKA but the relationship to DNA-binding proteins was not established. In D17 cells, CREB2 (in the presence of PKA) is a cellular factor able to induce BLV LTR-directed expression in the absence of *tax* protein and could thus be involved in the early stages of viral infection. As *tax*-induced *trans* activation can be modulated by CREB2 and PKA, the cAMP pathway could play a role in the regulation of viral expression in vivo. Similar data were also obtained in other cell types (HeLa, HepG2, OVK) or with other eukaryotic expression plasmids (Rous sarcoma virus promoter) (data not shown).

The CREB2 protein is a member of a very large family of CRE-binding proteins (11). Some of these proteins have been shown to be involved in gene regulation via specific binding to the CRE, TGACGTCA; i.e., CREB isolated from rat brain stimulates the cAMP-responsive somatostatin gene (27). Transcriptional efficacy of the CREB proteins is regulated through phosphorylation by PKs and dimerization. Biochemical and in vitro mutagenesis experiments revealed that CREB is activated by phosphorylation at a single PKA phosphoacceptor site, Ser-133 (8). Since CREB phosphorylation at Ser-133 activates transcription without changing its DNA-binding affinity, it appears that phosphorylation directly modulates the efficacy of the *trans* activation domain. PKA may regulate CREB activity by altering the tertiary structure of the protein, thereby rendering other regions accessible for the interaction with proteins in the RNA polymerase II complex.

CREB can also be phosphorylated in vitro by PKC and casein kinase (9). Casein kinase is a ubiquitous enzyme which is thought to regulate the expression of a number of growth factors and oncogenes such as the simian virus 40 large T antigen, adenovirus E1A, and *myc*. Phosphorylation by casein kinase could inhibit the PKA or PKC pathway and consequently cAMP-responsive gene transcription. At the cellular level, casein kinase seems to be associated with serum-stimulated cell growth. In contrast, PKA enhances cellular differentiation and loss of proliferation (9).

It is of particular interest that transcription regulation through the cAMP pathway can also be involved in the regulation of BLV viral information. After BLV infects its target cell and integrates into the host genome, the *tax* protein is probably not involved in the early steps of viral replication since no *tax* seems to be present (19). More convincingly, when a plasmid containing an entire BLV provirus is transfected into mammalian cells, the viral LTR is activated even in the absence of the *tax* product (22). The PKA pathway thus appears to be a suitable explanation for the mechanism involved in the initiation of the replicative cycle of the virus. However, we cannot exclude at present the participation of other pathways such as that of PKC.

To gain insight into the role of the TBP in proviral expression in vivo, we analyzed the total levels of CREB2 transcripts by the polymerase chain reaction technique. No significant difference could be observed between high and low (or even negative) BLV producers, suggesting that the expression of TBP is not correlated with BLV expression at the transcriptional level (data not shown). Since transcriptional efficacy of the CREB proteins is regulated through phosphorylation by PKs and dimerization, $^{32}P_i$-labeled cell

lysates were analyzed by immunoprecipitation. No difference in CREB phosphorylation could be observed between cells expressing or not expressing BLV (data not shown). The expression of the BLV provirus in vivo thus seems not to be correlated with the total transcription level or the phosphorylation state of the TBP. Consequently, regulation of BLV expression in vivo seems to involve a very complex array of pathways in which CREB2 and/or other members of the CREB family and flanking sequences of the CRE target elements could play a major role (11, 21, 28).

Phosphorylation of the CREB protein by the catalytic subunit of PKA is required to enhance *tax*-induced *trans* activation. In the absence of PKA, *trans* activation is inhibited by the CREB2 protein in the D17 cells. This suggests that the inhibition of *trans* activation by CREB2 results from the overexpression of an underphosphorylated form of the CREB protein that could titrate out essential cellular factors required for transcription. This mechanism may thus be considered as squelching.

Perhaps the most striking point of this report is that the BLV LTR (silent in uninfected cells) can be transactivated by CREB2 and PKA. The precise mechanism of this basal activation of LTR-directed gene expression is still unknown. In light of experiments performed in the tyrosine aminotransferase gene regulation system, we can hypothesize that the TBP/PKA pathway could relieve a cellular inhibition complex. The regulation of this gene is mediated through the CRE repressed by a tissue-specific extinguisher locus, Tse-I (2). Cotransfection of a vector expressing PKA allows us to overcome this inhibition. Boshart et al. (2) propose a model for the regulation of the limited onset of expression based on the antagonism between Tse-I and the cAMP pathway. A similar mechanism could well be involved in the BLV system. We can hypothesize that a cellular inhibitor is responsible for the lack of LTR-directed gene expression. The cAMP pathway and *trans* activation by *tax* (or another unknown mechanism) should compete with this inhibitor and subsequently allow the LTR-directed gene expression. Such a hypothesis is consistent with the observation that some BLV-infected cells (1 of 5,000) express viral information in vivo, suggesting that the inhibition is not absolute. The plasma protein identified by Gupta and Ferrer (10) which blocks BLV expression could act through cellular receptors and repress transcription via a similar mechanism.

In conclusion, CREB2 might be one of the key elements involved in BLV proviral regulation of expression, playing a role in derepression of proviral expression in the absence of *tax* and in regulation of *tax*-induced *trans* activation during the replicative cycle.

ACKNOWLEDGMENTS

R.K. and L.W. are, respectively, Directeur de Recherches and Chercheur qualifié of the Fonds National Belge de la Recherche Scientifique. We thank the Caisse Générale d'Epargne et de Retraite for financial support and the UICC organization for a ICRETT fellowship.

We are also grateful to R. Martin and Y. Cleuter for skillful technical help, to J. Ghysdael for helpful discussions, and to G. Buchet for photographic illustrations. The protein kinase expression plasmids were kindly provided by R. Maurer and S. McKnight.

REFERENCES

1. **Altman, R., D. Harrick, J. Garcia, and R. Gaynor.** 1988. Human T-cell leukemia virus types I and II exhibit different DNase I protection patterns. J. Virol. **62**:1339–1346.
2. **Boshart, M., F. Weih, A. Schmidt, K. Fournier, and G. Schutz.** 1990. A cyclic AMP response element mediates aminotransferase gene transcription by the tissue specific extinguisher locus TSE-1. Cell **61**:905–916.
3. **Brash, D., R. Reddel, M. Quanrud, K. Yang, M. Farrell, and C. Harris.** 1987. Strontium phosphate transfection of human cells in primary culture: stable expression of the simian virus 40 large T-antigen gene in primary human bronchial epithelial cells. Mol. Cell. Biol. **7**:2031–2034.
4. **Burny, A., Y. Cleuter, R. Kettmann, M. Mammerickx, G. Marbaix, D. Portetelle, A. Van den Broeke, L. Willems, and R. Thomas.** 1987. Bovine leukaemia: facts and hypotheses derived from the study of an infectious cancer. Cancer Surv. **6**:139–159.
5. **Derse, D.** 1987. Bovine leukemia virus transcription is controlled by a virus-encoded *trans*-acting factor and by *cis*-acting response elements. J. Virol. **61**:2462–2471.
6. **Fujisawa, J., M. Toita, and M. Yoshida.** 1989. A unique enhancer element for the *trans* activator ($p40^{tax}$) of human T-cell leukemia virus type I that is distinct from cyclic AMP- and 12-*O*-tetradecanoylphorbol-13-acetate-responsive elements. J. Virol. **63**:3234–3239.
7. **Giam, C., and Y. Xu.** 1989. HTLV-I tax gene product activates transcription via preexisting cellular factors and cAMP responsive element. J. Biol. Chem. **264**:15236–15241.
8. **Gonzalez, G., and M. Montminy.** 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell **59**:675–680.
9. **Gonzalez, G., K. Yamamoto, W. Fisher, D. Karr, P. Menzel, W. Biggs, W. Vale, and M. Montminy.** 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature (London) **337**:749–752.
10. **Gupta, P., and J. Ferrer.** 1982. Expression of bovine leukemia virus genome is blocked by a nonimmunoglobulin protein in plasma from infected cattle. Science **215**:405–406.
11. **Hai, T., F. Liu, W. Coulo, and M. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **3**:2083–2090.
12. **Hoeffler, J., T. Meyer, Y. Yun, L. Jameson, J. Habener.** 1988. Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. Science **242**:1430–1433.
13. **Huynh, T., R. Young, and R. Davis.** 1985. Constructing and screening cDNA libraries in lambda-gt11, p. 49–78. *In* D. L. Glover (ed.), DNA cloning. A practical approach, vol. 1. IRL Press, Oxford.
14. **Jeang, Q.-T., I. Boros, J. Brady, M. Radonovich, and G. Khoury.** 1988. Characterization of cellular factors that interact with the human T-cell leukemia virus type I $p40^{x}$-responsive 21-base-pair sequence. J. Virol. **62**:4499–4509.
15. **Katoh, I., Y. Yoshinaka, and Y. Ikawa.** 1989. Bovine leukemia virus trans-activator $p38^{tax}$ activates heterologous promoters with a common sequence known as a cAMP-responsive element or the binding site of a cellular transcription factor ATF. EMBO J. **8**:497–503.
16. **Nakamura, M., M. Niki, K. Ohtani, and K. Sugamura.** 1989. Differential activation of the 21 bp enhancer element of human T-cell leukemia virus type I by its own trans-activator and cyclic AMP. Nucleic Acids Res. **17**:5207–5221.
17. **Nyborg, J., and W. Dynan.** 1990. Interaction of cellular proteins with the human T-cell leukemia virus type I transcriptional control region. J. Biol. Chem. **265**:8230–8236.
18. **Nyborg, J., W. Dynan, I. Chen, and W. Wachsman.** 1988. Binding of host-cell factor to DNA sequences in the long terminal repeat of T-cell leukemia virus type I: implications for viral gene expression. Proc. Natl. Acad. Sci. USA **85**:1457–1461.
19. **Sagata, N., J. Tsuzuku-Kawamura, M. Nagayoshi-Aida, F. Shimizu, K. Imagawa, and Y. Ikawa.** 1985. Identification and some biochemical properties of the major XBL gene product of bovine leukemia virus. Proc. Natl. Acad. Sci. USA **82**:7879–7883.
20. **Singh, H., J. Lebowitz, A. Baldwin, and P. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell **52**:415–423.

21. **Tsujimoto, A., H. Nyunoya, T. Morita, T. Sato, and K. Shimotohno.** 1991. Isolation of cDNAs for DNA-binding proteins which specifically bind to the *tax*-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I. J. Virol. **65**:1420–1426.
22. **Van den Broeke, A., Y. Cleuter, G. Chen, D. Portetelle, M. Mammerickx, D. Zagury, M. Fouchard, L. Coulombel, R. Kettmann, and A. Burny.** 1988. Even transcriptionally competent proviruses are silent in bovine leukemia virus-induced sheep tumor cells. Proc. Natl. Acad. Sci. USA **85**:9263–9267.
23. **Willems, L., G. Chen, A. Burny, and R. Kettmann.** 1988. Expression in bacteria of beta-galactosidase fusion proteins carrying antigenic determinants of the two X gene products of bovine leukemia virus. Leukemia **2**:1–5.
24. **Willems, L., A. Gegonne, G. Chen, A. Burny, R. Kettmann, and J. Ghysdael.** 1987. The bovine leukemia virus p34 is a transactivator protein. EMBO J. **6**:3385–3389.
25. **Willems, L., H. Heremans, G. Chen, D. Portetelle, A. Billiau, A. Burny, and R. Kettmann.** 1990. Cooperation between bovine leukaemia virus transactivator protein and Ha-ras oncogene product in cellular transformation. EMBO J. **9**:1577–1581.
26. **Willems, L., R. Kettmann, G. Chen, D. Portetelle, A. Burny, and D. Derse.** 1991. Nucleotide sequence of the bovine cyclic-AMP responsive binding protein (CREB2) cDNA. DNA Sequence **1**:415–417.
27. **Yamamoto, K., G. Gonzalez, W. Biggs, and M. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334**:494–498.
28. **Yoshimura, T., J.-I. Fujisawa, and M. Yoshida.** 1990. Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. EMBO J. **9**:2537–2542.