Nucleic Acids Research, 1993, Vol. 21, No. 16 3677–3682

Member of the CREB/ATF protein family, but not CREBα plays an active role in BLV *tax trans* activation *in vivo*

Endre Kiss-Toth, Saowakon Paca-uccaralertkun, Ildiko Unk and Imre Boros
Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, 6701 Szeged, POB 521, Hungary

Received May 11, 1993; Revised and Accepted July 12, 1993

ABSTRACT

The *trans* activator protein of Bovine Leukaemia Virus (*tax*) increases the rate of transcription from the virus promoter through 21 bp sequences located in three tandem copies in the virus LTR. Based on data obtained by three different experimental approaches we concluded that the central CRE-like motif found in each of the BLV 21 bp repeats plays an important and indispensable role in *tax* mediated *trans* activation. These include (i) *in vivo* analysis of the function of mutant 21 bp sequences in transient transfection, (ii) gel mobility shift assay to show that CREB binds to BLV 21 bp repeats *in vitro* and (iii) the demonstration that the production of antisense CREB mRNA inhibits *tax trans* activation. Further studies with different deletion mutant CREB proteins suggest that although CREBα can interact with factors involved in BLV *trans* activation, it does not promote transcription initiation; consequently some other member/s of the CREB/ATF family must be involved.

INTRODUCTION

Bovine leukaemia virus (BLV) is a retrovirus that causes enzootic bovine leukosis (4). The genome of BLV encodes a protein called p^{34}*tax* which can dramatically increase transcription from the viral long terminal repeat (LTR) (6,25).

Trans activation of the BLV LTR by p^{34}*tax* requires 21-bp tandem repeats present in the U3 region of the LTR (6). Nucleotide sequence data show that similar repeats are found in the LTRs of the closely related human T-cell lymphotropic viruses (HTLV-I and HTLV-II). All these elements have a sequence similar to the cyclic AMP (cAMP) -responsive element (CRE) which is the binding site of cellular transcription factors belonging to the CREB/ATF protein family (14).

The 21-bp repeats are essential for *tax* mediated *trans* activation in both the BLV and HTLV systems. Since *tax* does not bind directly to the promoter region, it is believed to exert its effect through cellular proteins that recognise these sequences. Several proteins binding to the HTLV- and BLV LTRs have been identified (1,8,13,17,18,24). All these contain amino acid sequences similar to those identified as conserved functional domains in members of the CREB/ATF protein family (leucine zipper, multiple potential phosphorylation sites and a basic domain). Moreover, recent studies demonstrated that CREB but not ATF directly interacts with HTLV-I *tax* (28). In these experiments CREBα, the first recognised form of the CREB protein family was used. CREBα is present in a large variety of organisms and in different cell lines (2). This protein can induce transcription of several genes in response to the increased cAMP level (16). In the CREBα protein three functional domains have been identified: an N-terminal *trans* activator domain (containing a glutamine rich Q region and a 14 amino acid long α peptide, which interacts with the phosphorylation motifs to stimulate transcription activation (27)), a basic domain, involved in DNA-binding and a Leu-zipper dimerization domain at the C-terminal part of the protein. CREBα also contains a phosphorylation cluster in which the phosphorylation motifs of the protein kinase A, C, and casein kinase II can be found. Another form of CREB missing the α peptide (CREBΔα) is produced by alternative splicing. The α peptide is believed to modulate the activity of CREBα via conformational changes (27).

In this paper we demonstrate that CREBα binds to the BLV 21-bp repeats *in vitro*. Constructing 'synthetic LTRs' bearing different point mutations in the repeat elements we found, that the CRE-like element was an important target of *trans* activation in BLV. In accordance with these, expression of the antisense CREB mRNA strongly reduced the BLV *tax* activation of the LTR linked CAT reporter gene in transient cotransfection experiments. Surprisingly, expression of the CREBα protein also inhibited BLV *tax trans* activation. Using deletion mutants of CREBα we demonstrated that the N-terminal 197 amino acids were essential for producing this inhibitory effect. As this region did not include the DNA-binding domain of the protein, most probably the inhibition by CREBα was not the result of blocking the target site but was realised via protein-protein interaction. Based on these data we hypothesise that although proteins belonging to the CREB family can interact with activator factors involved in *tax* activation, only specific members can promote the transcription initiation.

MATERIALS AND METHODS

Plasmid constructs

Plasmids pBLH2CAT, pU3RCAT, pXB-RS and pET11 have been described previously (5,20,6,22). For the bacterial

expression of CREBα a full length CREBα cDNA was cloned into the pET11 plasmid (This plasmid was kindly provided by C. Giam). For the construction of a BLV minimal promoter a 474 bp *Hin*dIII-*Hin*dIII fragment containing the BLV LTR was cloned from pBLH2CAT into pHC624 (3). The resulting plasmid was digested with *Pvu*II (cleaves at −123 and −148 in the U3 region), and after *Bal*31 treatment (for various times) ligated with *Bgl*II linkers. The ends of the created deletions were determined by nucleotide sequencing and a plasmid carrying the BLV promoter region from −64 to +294 was selected. From this the BLV promoter was inserted in front of the CAT coding sequence as a *Bgl*II-*Hin*dIII fragment, resulting in plasmid pBΔ16.

Cell culture, transfection and CAT assay

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum. Transfections were performed by the calcium phosphate procedure using 1μg of BLV *tax* expression plasmid DNA (pXB-RS), 3 μg of the reporter constructs (full length or the mutant BLV LTR sequences cloned upstream of the CAT gene), and 2−4μg of CREB expression plasmid (either the full length or mutant CREB cDNA under the control of the Rous sarcoma virus promoter). When a plasmid was omitted in the transfection experiments, DNA levels were kept constant by using supercoiled plasmid DNA as carrier (pHC624) (3). Cells were harvested 40 hours after transfection and CAT activities were determined as have been described previously (11). CAT activity was quantitated by cutting the appropriate spots and determining their radioactivity by LSC. In the experiments with CREB the reaction containing the pBLH2CAT reporter plasmid and the pXB-RS *tax* expressing construct was used as a reference and its CAT activity was taken to be 100%.

Overproduction of CREBα and preparation of HeLa nuclear extracts

Cultures of BL21(DE3) *E.coli* cells harbouring the pET11CREB plasmid were induced by addition of 1mM IPTG in logarithmic phase. Three hours later cells were harvested, suspended in bufferA (50mM Tris−HCl pH=8.0, 50mM NaCl, 1mM EDTA, 0.5mM DTT and 0.5mM PMSF), and sonicated. Supernatant obtained after high speed centrifugation (10.000 rpm 20 min in Sorvall GSA rotor) was used in gel-shift experiments. In extracts prepared from uninduced culture we could not detect any proteins which interacted with the labelled oligonucleotides under the same conditions. HeLa cell nuclear extract was prepared as described by Dignam et al. (7)

Gel mobility shift assay

The determination of DNA-protein complexes by gel-electrophoresis was carried out as have been described previously (20).

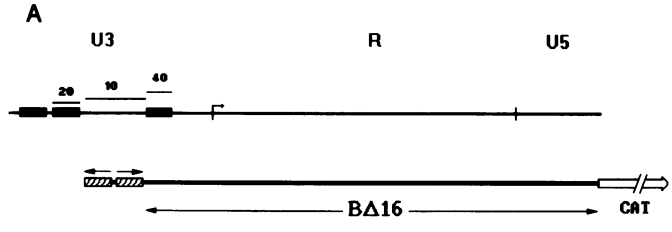
In vitro mutagenesis of CREBα

The N-terminal 197 amino acid coding sequence of the CREBα protein was cloned into the M13 bacteriophage as an *Hin*dIII-*Kpn*I fragment. Site directed mutagenesis was performed as described by Kunkel et al. (15), using the following synthetic oligonucleotides: CREBo1 (GCAGACAACCCCGGGAGTGG-AGAT), CREBo2 (GTTCAAGCCCCCGGGCAGATTGCC), CREBo3 (ACATTAGCCCCCCGGGGTATCCATG), CREBo4 (CCCAATGGGCCCGGGGTCCAGGTC), CREBSer (TCAA-GGAGGGCATGCTACAGGAAA).

RESULTS

The CRE-like sequences in the 21-bp repeats are involved in BLV *tax trans*-activation

Plasmid pBΔ16 contained only the TATA-box and the first 21 bp repeat of the BLV LTR. As this promoter did not respond to *tax* activation (Fig. 2. lane 1), we used it as a minimal promoter to determine the *tax* responses of different 21 bp motifs. Three 21 bp repeats were synthesised: B20 contained the wild type sequence of the second 21 bp repeat, B21 is identical to B20 except that the CRE-like motif was destroyed by three base substitutions, similarly B25 contains a single base substitution which creates a palindromic CRE (Fig. 1.). Two copies of these oligonucleotides were inserted upstream of the pBΔ16 minimal promoter, resulting in a 'synthetic LTR' with a structure similar to the repeats found in the wild type promoter. Transient expression system was used to examine the *tax* responsivity of the obtained constructs (Fig. 2. lane 2−4). As anticipated, insertion of two copies of the wild type or consensus CRE containing 21 bp repeat rendered the pBΔ16 minimal promoter



B

```
B 40        agctTGAGCTGCTGACCTCACCTGCTGAT
                ACGCGACGACTGGAGTGGACGACTAgc

B 20        tcgaAAAGCTGGTGACGGCAGCTGGTGGCTAG
                TTTCGACCACTGCCGTCGACCACCGATCgatc

B 21        tcgaAAAGCTGGTGACACGAGCTGGTGGCTAG
                TTTCGACCACTGTGCTCGACCACCGATCgatc

B 25        tcgaAAAGCTGGTGACGTCAGCTGGTGGCTAG
                TTTCGACCACTGCAGTCGACCACCGATCgatc

CRE consensus           TGACGTCA

B 10        ctagACCTCCCCAACTTCCCCTTTCCCGAAAAATCCACACCCTA
                TGGAGGGGTTGAAGGGGAAAGGGCTTTTTAGGTGTGGGATtcga
```

Figure 1. Structure of the BLV LTR **A**: The BLV LTR consists of three regions. All the promoter elements can be found in the U3. This region includes three 21 bp long repeats (black boxes) which are the major targets of *tax trans* activation. The R and U5 are the 5′ non-translated regions of the virus RNA. BΔ16 contains the LTR from the 5′ end of the proximal 21 bp repeat and CAT as a reporter gene. Hatched boxes represent the positions of the oligonucleotides B20, 21, 25, which were cloned upstream of the LTR resulting in constructs pBΔ1620−25d. **B**: Sequences of the synthetic oligonucleotides: B 40: wild type sequence of the first BLV 21bp repeat; B 20: wild type sequence of the second 21 bp repeat; B 21: sequence of the second repeat with a destroyed CRE-like element; B 25: a point mutation in the second repeat, resulting in a consensus CRE sequence. (The nucleotides in boldface show the mutations affecting the CRE-like element. The lower case letters represent the flanking nucleotides used to clone the oligonucleotides as dimers into the *Xho*I site of the pBΔ16 plasmid.) B 10: TRE2 sequence of BLV (present between the first and second repeat elements).

tax responsive while the mutation which destroyed the CRE-like sequence abolished *tax* activation.

In vitro interaction of CREB and the BLV 21-bp repeats

The experiments described above demonstrated that the CRE-like elements at the centre of the 21 bp repeats are involved in mediating *tax* activation. To determine whether these motifs are recognised by the cellular CRE binding factor (CREB) we carried out gel shift experiments. The rat CREBα protein was overexpressed in bacteria using the pET11CREB expression plasmid. As labelled probe, the three oligonucleotides described above, a synthetic copy of the first 21 bp repeat (B40), or the region between the first and the second 21 bp repeats (B10) were used. CREBα formed complexes with B20, B40 and B25. Complexes with identical mobility were observed when HeLa nuclear extract was used instead of bacterially expressed CREBα (Fig. 3.). Consistent with the notion that CREB is involved in mediating the *tax* response, oligonucleotides B21 and B10 failed to interact with CREB. These results are in agreement with the *in vivo* data: the oligonucleotides which showed *tax* responses interacted with CREB while those which were inactive in *trans* activation did not form any complexes.

Inhibition of CREB expression abolishes *trans* activation

The experiments described above established that the CRE-like sequences are essential for the *trans* activation and that the CREB protein *in vitro* interacts with these elements. To determine, if this interaction takes place *in vivo* as well, we attempted to inhibit the expression of CREB in HeLa cells. For this purpose, a plasmid which directs the transcription of the N-terminal segment of CREBα in antisense orientation was constructed. (Fig. 4A.). The fragment of CREB encoded by this region is conserved in all known members of this protein family, consequently the antisense RNA product could inhibit the expression of all cellular CREB forms. When this construct was cotransfected with the pBLH2CAT indicator and pXB-RS, *tax* expression plasmids, the

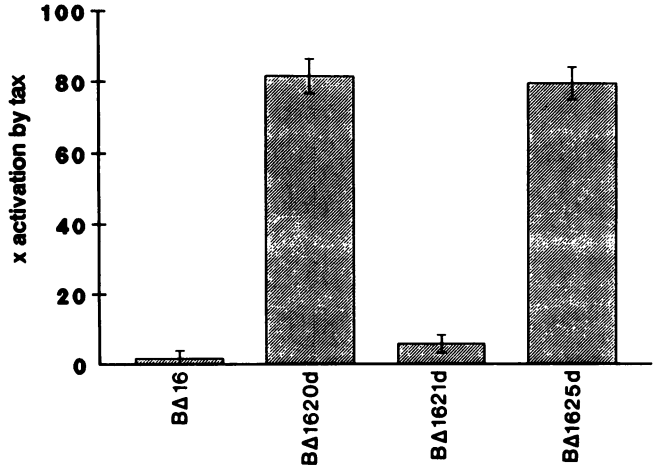


Figure 2. Effects of the synthetic oligonucleotides on *tax* dependent *trans* activation. HeLa cells were transiently transfected with 3μg of the reporter constructs and 1μg pXB-RS. The reporter constructs were: lane 1: pBΔ16; lane 2: pBΔ1620d; lane 3: pBΔ1621d; lane 4: pBΔ1625d.

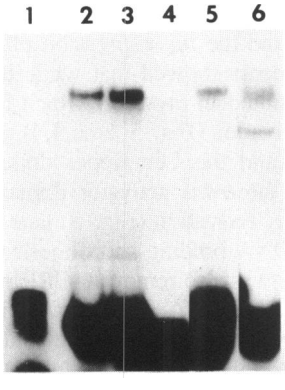


Figure 3. Interaction between CREBα and the BLV 21bp repeats. Gel mobility shift assay was carried out with ^{32}P -labelled DNA fragments, containing the B10 (lane 1), B20 (lanes 2, 6), B40 (lane 3), B21 (lane 4), B25 (lane 5) BLV repeats (see Fig. 1.), the crude extract, containing the overexpressed CREBα (lanes 1–5) or HeLa (lane 6) cell nuclear extracts and 1μg of poly dI-dC.

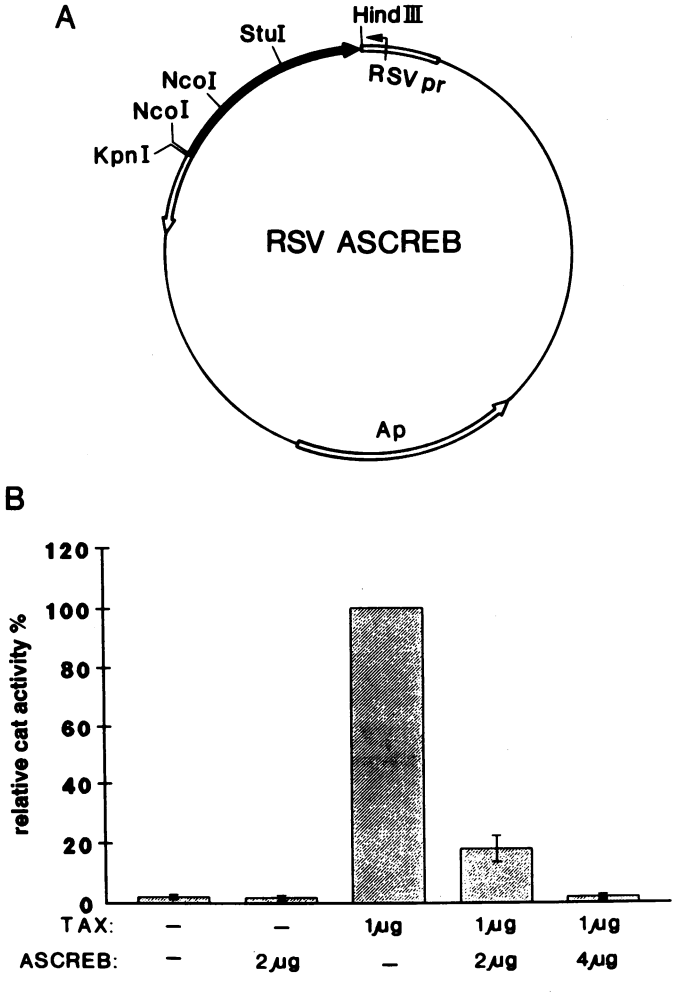


Figure 4. Structure and effect of the antisense CREB construct on *trans* activation. **A**: The *Hind* III– *Kpn* I fragment of the plasmid pRSV ASCREB contains the Q region and the phosphorylation motifs for protein kinases of the CREBα in antisense orientation, and it is transcribed from the point indicated by an arrow. This plasmid was constructed from pRSVcat (4). **B**: HeLa cells were transfected with 3μg of the pBLH2CAT reporter construct and the appropriate other plasmids.

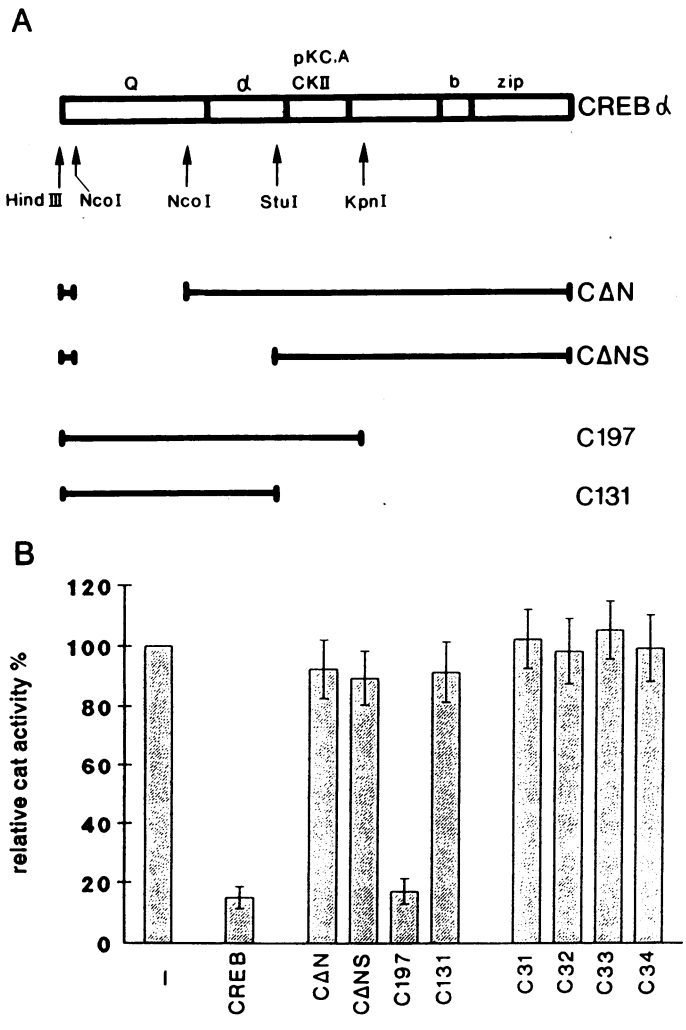


Figure 5. Structure and effect of the wild type and mutant CREBα proteins. **A**: All of the deletion constructs were cloned into pRSVCREBα instead of CREBα. CΔN was constructed with *Nco*I digestion and T4 polymerase reaction. In CΔNS, an *Nco*I−*Stu*I fragment was deleted. For the C-terminal deletions we used *Kpn*I and *Stu*I sites to insert a nonsense linker containing a *Xba*I site. **B**: 6 mg of total DNA was used for transfections. Each lane contained 3μg of pBLH2CAT and 1μg pXB-RS *tax* expressing plasmid. The lanes 3−10 show the effect of the different mutant CREB proteins.

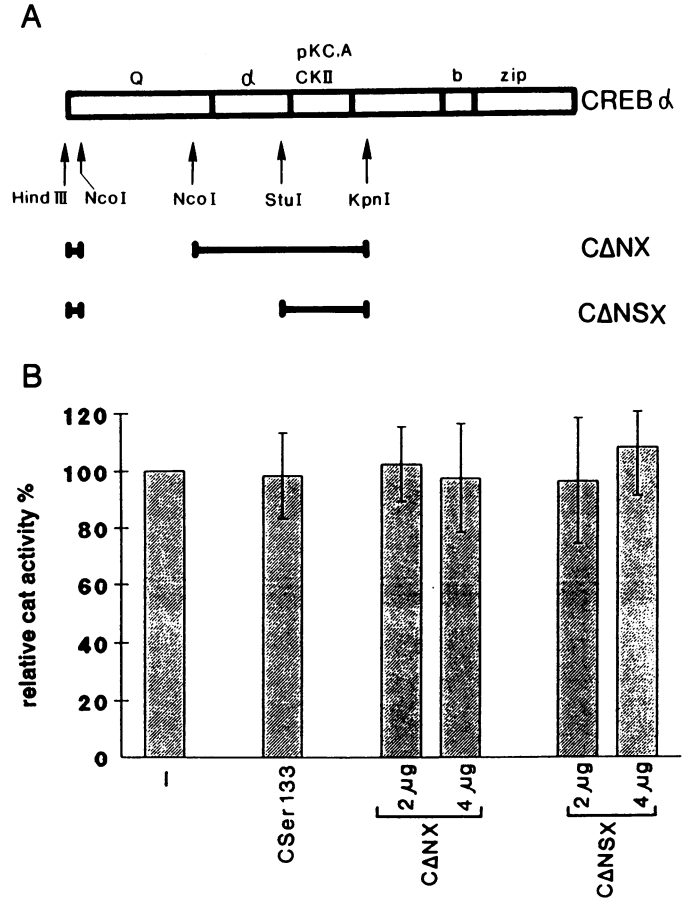


Figure 6. Structure and effect of the overexpressed CREB phosphorylation cluster and the CSer133 mutant on *tax* activation. **A.** CΔNX and CΔNSX were constructed form CΔN and CΔNS by insertion of a nonsense linker into the *Kpn* I site. In the CSer133 mutant the Pro132Ser133 motif was changed into Ala132Cys133. **B.** The cells were transfected with 3μg reporter, and 1μg effector plasmids. In lane 2 2μg CSer133 was cotransfected. In lanes 3−6 the appropriate other constructs were added.

level of *trans* activation was strongly reduced (Fig. 4B.). These data further supported the notion that a member of the CREB protein family plays an important role in the *trans* activation of BLV.

Overexpression of CREBα inhibits the *tax* activation

The observed inhibition of *tax* activation with antisense CREB mRNA prompted us to test if overexpression of CREB would give rise to a higher level of *trans* activation. To test this, a plasmid, directing the expression of CREBα was cotransfected with pBLH2CAT and pXB-RS into HeLa cells. Contrary to our expectation, CREBα inhibited the *tax trans* activation (Fig. 5. lane 2), resulting in only 10−20 percent of the CAT activity compared to the level obtained in its absence (Fig. 5. lane 1). Similar level of inhibition was detected if the homologous mouse protein was used (data not shown). To analyse which functional domain of CREBα might cause this surprising effect we constructed several plasmids to express different deletion mutants of the CREBα protein (Fig. 5. lane 3−6). The effect of these mutations on the *trans* activation was tested by cotransfecting with pBLH2CAT and the *tax* expression plasmid. The results from these experiments showed that while deletions at the N-terminal region resulted in proteins, which lost their ability to inhibit the *trans* activation (Fig. 5. lane 3,4), a mutant, in which the DNA binding and the Leu-zipper domains were deleted (CREB197) while the *trans* activator domain was preserved, strongly reduced *tax* activation (Fig. 5. lane 5). If, in addition to the C-terminal DNA-binding and dimerization domains, the phosphorylation site was also removed (CREB131), no inhibition was observed (Fig. 5. lane 6). These results indicated that only the N-terminal *trans* activator region (including the Q region, the α peptide and the phosphorylation cluster) but not the DNA-binding domain of CREBα was essential for the inhibition of the *tax trans* activation. This domain of CREB contains several glutamine residues which were suggested to be involved in CREB mediated *trans* activation (9). We changed some of these glutamines to ProGly in mutations CREB31, -32, -33, -34 (Gln10Gln11, Gln30Pro31, Gln38Val39, Gln62Thr63). None of these proteins inhibited *tax trans* activation (Fig. 5. lane 7−10).

The mouse CREBΔα protein, in which the α peptide was not present did not inhibit the *trans* activation either (not shown).

CREBα activates the target promoters as a phosphoprotein (26). The major phosphorylation site is Ser133 (12). To determine if phosphorylation is needed for inhibiting *trans* activation, we changed the Pro132Ser133 in CREB into Ala132Cys133 residues to generate mutation CREB133. Cotransfection of this plasmid with *tax* expressing plasmid had no inhibitory effect (Fig. 6B. lane 2). These data showed, that phosphorylation was essential for the inhibition by CREBα. To test, whether the overexpression of the CREBα phosphorylation cluster has any effect on the *tax* activation, we constructed the plasmids CΔNX and CΔNSX (Fig. 6A.), and cotransfected them with the reporter pBLH2CAT and the *tax*-expressing pXB-RS. These constructs were unable to inhibit the BLV promoter activation (Fig. 6B. lanes 3–6). These results, in accordance with the effects of the different CREB constructs (CREBΔα, CΔN, CΔNS, C31–34), which might also be the *in vivo* targets of the endogenous protein kinases, suggested, that the down-regulation of the BLV promoter *tax* activation is not the result of titration out the pool of protein kinases.

DISCUSSION

Each member of the HTLV/BLV retrovirus group contains three similar 21 bp repeats in the U3 region. The most conserved part of these is a 7 bp sequence motif, similar to the cAMP responsive element found in many cellular promoters. Detailed studies on the HTLV-I *tax trans* activation indicate that these motifs serve as indispensable part of a larger *tax* responsive element (8,13,19,23). Moreover, the 21 bp repeats of HTLV-I have been shown to form complexes with members of the cellular CREB/ATF protein family (28). To examine the role of CRE-like motifs present in the centre of the three BLV 21-bp long repeats we synthetised oligonucleotides with mutations that modified the CRE elements. Different 'synthetic LTRs' were constructed inserting these oligonucleotides upstream of a minimal BLV promoter. Cotransfections with these plasmids and a *tax* expression plasmid showed that destroying the CRE abolished *tax* responsiveness. These experiments, in accordance with the findings of Katoh at al.(14), strongly suggested that the CRE-like motifs are essential for mediating BLV *tax trans* activation. In good correlation with the data obtained in transient expression, we found that the elements which responded to *tax* activation *in vivo*, formed complexes *in vitro* with CREBα, the best known member of the CREB/ATF protein family. Cotransfection of a plasmid, from which the conservative coding region of the CREB family was transcribed in antisense orientation strongly inhibited *tax* activation. Taken together, these results suggested that a member of the CREB family binds to the BLV repeats and plays an active role in mediating *tax trans* activation.

To study the possible mechanism by which CREB might exert its role in *tax* action a plasmid, directing the expression of CREBα was cotransfected with a reporter construct—containing the BLV LTR linked to the CAT gene—and the *tax* expression plasmid. Surprisingly, in this case the *trans* activation was strongly inhibited. The reason of this could be a competition effect either for the DNA target sequence or for other transcription factor(s) involved in the activation. To distinguish between these possibilities, we constructed different mutations in CREBα and determined the effect of these in cotransfection. Our results showed, that the N-terminal 197 amino acids were essential for the inhibitory effect of CREBα, while deleting the DNA binding and Leu-zipper domains from CREBα, the suppression was preserved. These data indicated that the reason of the inhibition was not the competition for the CRE-like sequence. CREB mutants, in which glutamines in the Q activating domain were changed or the major phosphorylation site was eliminated lost their ability to inhibit the *tax trans* activation. These results suggested, that the inhibition took place via protein-protein interaction and a relatively large part of CREBα was needed to exert this effect (Q-region; α-domain; phosphorylation site). An other possible explanation for the observed CREB inhibition could be, that the overproduction of CREB protein titrated out an important protein kinase, and the unphosphorylated form of CREB although interacted with the target sequence, was functionally inactive. A similar observation was reported by Willems at al. studying the effect of CREB2 on BLV *tax trans* activation (24). However, in our system this explanation seems less likely, since we found that overexpression of only the phosphorylation site of CREBα did not change the efficiency of the *trans* activation. In accordance with these results, some of the protein constructs did not inhibit the *tax*, although they might also be the targets of protein kinases in the cells. Based on this, we believe that the reason of the suppressing effect could not be the accumulation of the unphosphorylated protein.

In conclusion, our data indicate that a member of the CREB protein family, very similar to CREBα, plays a role in BLV *tax trans* activation. This protein through the *trans* activator domain—which include the glutamine rich Q region, the α peptide and the phosphorylation site—can compete with CREBα for interaction with *tax* or some other factors. Experiments to identify this protein and to demonstrate its interaction with *tax* are in progress.

ACKNOWLEDGEMENTS

The authors warmly thank Dr Chou-Zen Giam for providing plasmid pET11CREB, Dr Timothy J.Cole for mouse CREBα and CREBΔα cDNA clones, and Dr Andor Udvardy for helpful discussions. We also thank Ökrös Gyuláné and Galló Rita Katalin for skilful technical assistance.

REFERENCES

1. Altman,R., Harrick,D., Garcia,J. and Gaynor,R., (1988) *J.Virol.* **62**, 1339–1346.
2. Berkowitz,L.,A. and Gilman,M.,Z. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5258–5262.
3. Boros,I., Posfai,G. and Venetianer,P. (1984) *Gene* 30, 257–260.
4. Burny,A., Cleuter,Y., Kettmann,R., Mammerickx,M., Marbaix,G., Portetelle,D., Van den Broeke,A., Willems,L. and Thomas,R. (1987) *J. Virol.* **61**, 2462–2471.
5. Derse,D. (1985) *Nature* **227**, 317–320.
6. Derse,D. (1987) *J. Virol.* **61**, 2462–2471.
7. Dignam,J.,P., Lebovitz,R.,M. and Roeder,R.,G. (1983) *Nucleic Acid Res.* **11**, 1475–1488.
8. Giam,C. and Xu,Y., (1989) *J. Biol. Chem.* **264**, 15236–15241.
9. Gonzalez, G.,A., Menzel, P., Leonard, J., Fischer, W.,H. and Montminy, M.,R., (1991) *Mol. Cell. Biol.*, 11 1306–1312.
10. Gorman, C.,M., Merlino, G.,T., Willingham,M.,C., Pastan,I. and Howard, B., H. (1982) *Proc. Natl. Acad. Sci.USA* **79**, 1044–1048.
11. Gorman,C.,M., Moffat,L.,F. and Howard,B.,H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
12. Gustavo,A., Gonzalez,G.,A. and Montminy,M.,R. (1989) *Cell* **59**, 675–680.
13. Jeang,Q.-T., Boros,I., Brady,J., Radonovich,M. and Khoury,G. (1988) *J. Virol.* **62**, 4499–4509.
14. Katoh,I., Yoshinaka,Y. and Ikawa,Y. (1989) *EMBO J.* **8**, 497–503.

15. Kunkel,T.,A., Roberts,J.,D. and Zakour,R.,A. (1987) *Methods in Enzymology* 154, 367–382.
16. Montminy,M.,R. and Bilezikjian,L.,M. (1987) *Nature* **328**,175–178.
17. Nyborg,J. and Dynan,W. (1990) *J. Biol. Chem.* **265**, 8230–8236.
18. Nyborg,J., Dynan,W., Chen,I. and Wachsman,W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1457–1461.
19. Potetat,H.,T., Kadison,P., McGuire,K., Park,L., Park,R.,E., Sodorski,J.,G. and Haseltine,W.,A. (1989) *J. Virol.* **63**, 1604–1611.
20. Singh,H., Lebowitz,J., Baldwin,A. and Sharp,P. (1988) *Cell* **52**, 415–423.
21. Sodorski,J.,G., Rosen,C.,A. and Haseltine,W.,A. (1984) *Science* 225, 381–385.
22. Studier,F.,W., Rosenberg,A.,H., Dunn,John.,J. and Rubendeorff,J.,W. (1990) *Methods in Enzymology* **185**, 60–89.
23. Tan,T.-H., Jia,R. and Roeder,R.,G. (1989) *J. Virol.* **63**, 3761–3768.
24. Willems,L., Kettmann,R., Chen,G., Portetelle,D., Burny,A. and Derse,D. (1992) *J. Virol.* 66, 766–772.
25. Willems,L., Gegonne,A., Chen,G., Burny,A., Kettmann,R. and Ghysdael,J. (1987) *EMBO J.* **6**, 3385–3389.
26. Yamamoto,K.,K., Gonzalez,G.,A., Biggs,W.,H. and Montminy,M.,R. (1988) *Nature* 334, 494–498.
27. Yamamoto,K.,K., Gonzalez,G.,A., Menzel,P., Rivier,J. and Montminy,M.,R. (1990) *Cell* 60, 611–617.
28. Zhao,L.-J. and Giam,C.-Z. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 7070–7074.