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# CREB-2, a Cellular CRE-Dependent Transcription Repressor, Functions in Association with Tax as an Activator of the Human T-Cell Leukemia Virus Type 1 Promoter

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**The Tax protein of the human T-cell leukemia virus type 1 (HTLV-1) has been implicated in human T-cell immortalization. The primary function of Tax is to transcriptionally activate the HTLV-1 promoter, but Tax is also known to stimulate expression of cellular genes. It has been reported to associate with several transcription factors, as well as proteins not involved in transcription. To better characterize potential cellular targets of Tax present in infected cells, a *Saccharomyces cerevisiae* two-hybrid screening was performed with a cDNA library constructed from the HTLV-1-infected MT2 cell line. From this study, we found 158 positive clones representing seven different cDNAs. We focused our attention on the cDNA encoding the transcription factor CREB-2. CREB-2 is an unconventional member of the ATF/CREB family in that it lacks a protein kinase A (PKA) phosphorylation site and has been reported to negatively regulate transcription from the cyclic AMP response element of the human enkephalin promoter. In this study, we demonstrate that CREB-2 cooperates with Tax to enhance viral transcription and that its basic-leucine zipper C-terminal domain is required for both in vitro and in vivo interactions with Tax. Our results confirm that the activation of the HTLV-1 promoter through Tax and factors of the ATF/CREB family is PKA independent.**

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL). The pathogenesis of ATL is still not understood, but it has been postulated that the viral Tax protein is involved in the proliferation and transformation of T cells in ATL. Tax is a 40-kDa regulatory protein which stimulates viral transcription through three imperfect cyclic AMP (cAMP) response element (CRE)-containing 21-bp regulatory sequences in the long terminal repeat (LTR) (12, 33, 39). Tax has also been shown to stimulate the transcription of several cellular genes. However, Tax does not interact directly with DNA but rather stimulates transcription through protein-protein interactions with different host factors including activating transcription factors/CRE-binding proteins (ATF/CREB) (1, 2, 10, 29, 36, 40, 42, 50, 52), NF-κB–I-κB complex (15, 43, 44), $p67^{SRF}$ (11, 43), Ets1 (8), NF-Y (34), and Sp1 (47). In addition, Tax binds to the basal transcription factors TFIIA (6) and TFIID through TATA-binding protein (TBP) (4) and TBP-associated factor $TAF_{II}28$ (3). Moreover, Tax interacts with CREB-binding protein (CBP) (13, 22), a cofactor facilitating transcriptional activation by CREB.

Tax has also been reported to interact with proteins that are not part of the transcription machinery including $p16^{INK4A}$ (30, 45), protein kinase C (28), proteasome subunits (37), cytokeratin (48), *trans*-activation response RNA-binding protein (TRBP) (9), human homologue of the *Drosophila* discs large tumor suppressor (hDlg) (25), G-protein pathway suppressor 2 (GPS2) (20), mitotic checkpoint protein MAD1 (19), α-internexin (35), PDZ domain of cellular proteins (38), and Int-6 (7). However, because Neuveut et al. (32) recently showed that Tax and Int-6 have different localizations within cells, Neuveut et al. suggested that it may be necessary to reconsider the biological importance of the Tax–Int-6 interaction. Last, Tax forms homodimers (17, 46). Altogether, more than 20 different polypeptides have been reported to bind to Tax, but the functional relevance of these interactions on the viral cycle and the progression of the disease still remains unclear. Several of the proteins listed above have been characterized by *Saccharomyces cerevisiae* two-hybrid approaches (7, 9, 19, 20, 34–38, 48) by screening cDNA libraries derived from noninfected cells. To directly isolate proteins that can potentially interact with Tax in infected T cells, we conducted a yeast two-hybrid screen using a cDNA library synthesized from mRNA of the MT2 cell line, a T-cell line persistently infected by HTLV-1. MT2 cells express the ATL-associated antigens, produce viral particles, and have been widely used to study Tax biology (31).

With a Stratagene Poly(A) Quick mRNA isolation kit, polyadenylated RNA was purified from total MT2 RNA, extracted as previously described (27). cDNA was synthesized by the protocol of the Clontech two-hybrid cDNA library construction kit. MT2 cDNA was fused to the GAL4 activation domain of the pGAD10 vector (for more details, see the description of the library in Table 1). The MT2 cDNA library was screened by using the entire Tax protein as bait fused to the GAL4 DNA binding domain of the pGBT9 vector. Briefly, pGBT-Tax and the fusion cDNA library were cointroduced into the *Saccharomyces cerevisiae* HF7c strain by the lithium acetate method (16). The HF7c yeast strain possesses the His synthase gene (*His3*) and the *lacZ* gene under the control of GAL4 binding sites. From approximately $10^7$ clones screened, we selected robust colonies growing to >2-mm diameter on agar medium lacking Trp, Leu, and His for those colonies that contained both types of plasmids ($Leu^+$ and $Trp^+$) and that also ex-

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TABLE 1. Construction of MT2 cDNA library[a]

| Characteristic | Value |
|---|---|
| mRNA source | MT2, HTLV-1-producing cell line |
| Cloning vector | pGAD10 |
| Cloning site | *Eco*RI |
| Priming method | oligo(dT) + random primed |
| Host strain | Easypores *E. coli* (Eurogentec) |
| No. of clones | $1.5 \times 10^6$ |
| Average insert size | 0.8 kb |
| Insert size range | 0.4–2.2 kb |
| Titer | $4 \times 10^9$ CFU/ml |

[a] Poly(A)$^+$ RNA (5 μg) was either oligo(dT)- or randomly primed, and first strand-synthesis was carried out with murine Moloney leukemia virus reverse transcriptase. Second-strand cDNA synthesis was performed with an enzyme cocktail (*Escherichia coli* DNA polymerase I, Rnase H, and DNA ligase) and treated with T4 DNA polymerase to create blunt ends. After ligation of *Eco*RI-*Not*I-*Sal*I adaptors, cDNA molecules were fractionated on a column to remove molecules smaller than 0.4 kb, ligated into *Eco*RI-digested pGAD10, and introduced in bacteria by electroporation.

pressed interacting hybrid proteins (His$^+$). A total of 538 transformants were obtained and assayed for the expression of *lacZ* by the β-galactosidase filter assay as described in the Clontech protocol. At this step, 158 clones were strongly positive for β-galactosidase activity. Plasmid DNA was extracted and analyzed by digestion with restriction enzymes *Acc*I, *Eco*RI, and *Sau*3AI, partial sequencing, and hybridization on dot blots (data not shown). As summarized in Table 2, seven families of clones were identified from this study. Among this collection, several clones encoded proteins previously characterized for their ability to bind to Tax, including Tax itself (31 clones) (17, 46), the cellular factor hDlg (28 clones) (25), and Tax-binding protein TXBP151 (24 clones) already isolated from a HeLa cDNA library by a two-hybrid approach (18). We also isolated cDNA coding for a protein of unknown function (myeloblast KIAA0147 gene; 27 clones) or a sequence (data not shown) with no GenBank match (8 clones). In addition, 11 cDNA clones coding for a hexapeptide, TSDGLC, were selected. Amino acid sequence comparisons indicated that this hexapeptide possesses four amino acids in common with the Tax sequence 31-ISGGLC-36 containing a cysteine involved in Tax dimerization (17). This observation suggests that Tax and the hexapeptide could interact via their cysteines. The last family of cDNA (29 clones) contained sequence encoding CREB-2, an unconventional member of the ATF/CREB family of transcription factors.

Because factors of the ATF/CREB family are known to be involved in HTLV-1 transcription (1, 2, 10, 29, 36, 40, 42, 50, 52) and because the CREB-2 cDNA was one of the most common positive clones, we concentrated our investigations on this molecule. CREB-2 is also known as human ATF-4 or TAXREB67; a partial cDNA clone of human ATF-4 was originally isolated by its ability to bind to the CRE motif (14), and

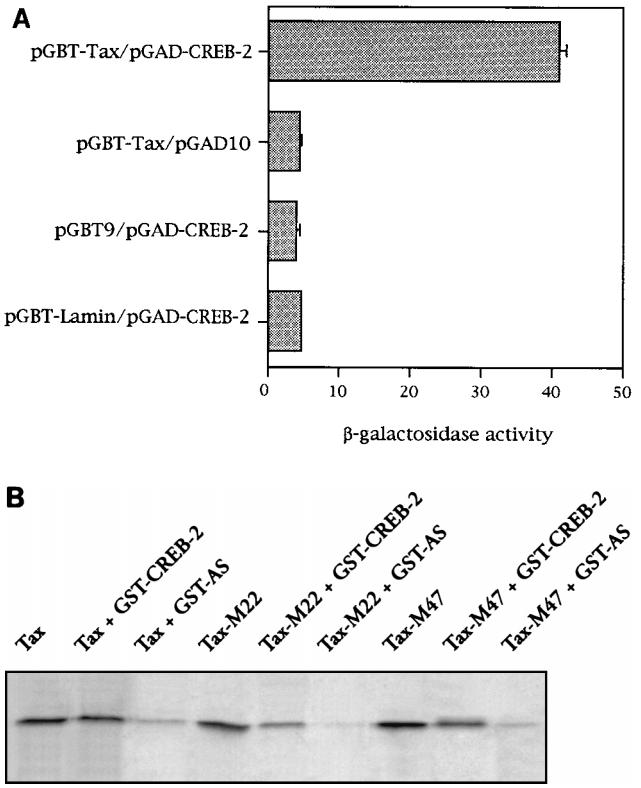


FIG. 1. Specific association of Tax with CREB-2. (A) Analysis of the interaction between Tax and CREB-2 by β-galactosidase assay. The HF7c yeast cells were transformed with an expression vector containing the CREB-2 cDNA clone fused to the GAL4 activation domain (pGAD-CREB-2) together with a plasmid expressing either the GAL4 DNA binding domain alone (pGBT9) or with plasmid pGBT9 fused to Tax (pGBT-Tax) or fused to Lamin (pGBT-Lamin). pGBT-Tax was also cotransformed with a plasmid expressing the GAL4 activation domain alone (pGAD10). The β-galactosidase assay with *O*-nitrophenyl-β-D-galactoside (ONPG) as the substrate was performed on three independent colonies per transformation as described in the Clontech protocol. The mean values expressed in Miller units are shown. (B) In vitro binding assays of wild-type Tax, Tax-M22, and Tax-M47 to CREB-2. The GST-CREB-2 and GST-AS fixed to glutathione Sepharose beads were mixed with either in vitro-translated wild-type $^{35}$S-Tax, $^{35}$S-Tax-M22, or $^{35}$S-Tax-M47 (lanes Tax, Tax-M22, and Tax-M47). Bound Tax was analyzed by SDS-PAGE (see the lanes corresponding to Tax + GST-CREB-2, Tax + GST-AS, Tax-M22 + GST-CREB-2, Tax-M22 + GST-AS, Tax-M47 + GST-CREB-2, and Tax-M47 + GST-AS).

TABLE 2. MT2 cDNA library clones promoting β-galactosidase activity in yeast cotransformed with pGBT-Tax

| GenBank match (accession no.) | No. of positive clones | β-Galactosidase filter assay result[a] | | |
|---|---|---|---|---|
| | | pGBT Tax | pGBT9 | pGBT Lamin |
| Tax protein (S67443) | 31 | +++ | − | − |
| Human CREB-2 mRNA (M86842) | 29 | +++ | − | − |
| Human homolog of *Drosophila* discs large tumor suppressor (U13897) | 28 | +++ | − | − |
| Human myeloblast KIAA0147 gene (D63481) | 27 | +++ | − | − |
| Human Tax-binding protein TXBP151 mRNA (U33821) | 24 | +++ | − | − |
| Hexapeptide TSDGLC | 11 | +++ | − | − |
| No match | 8 | +++ | − | − |

[a] Following plasmid isolation in HB101 bacteria, pGAD cDNA was reintroduced into the HF7c yeast strain with the indicated plasmids. Four days after transformation, the transformants were subjected to galactosidase filter assay. Symbols: +++, dark blue colonies that turned blue after 0.5 h; −, colonies that were still white after 8 h.

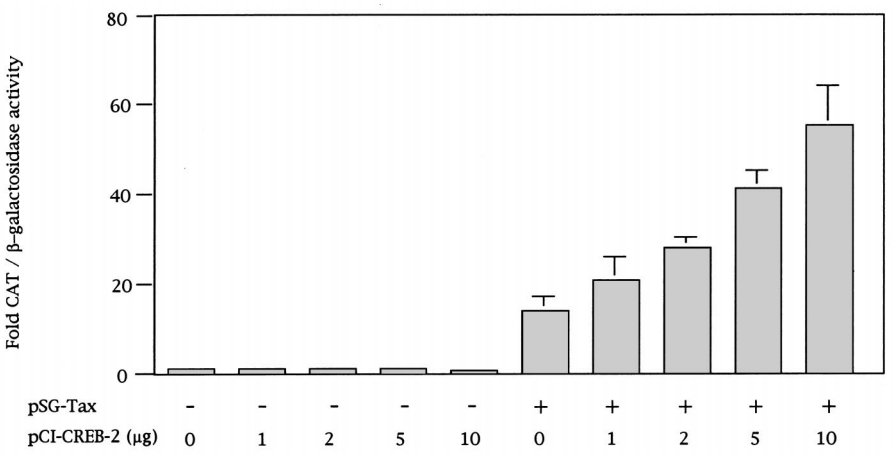


FIG. 2. Activation of the HTLV-1 promoter by CREB-2. CEM cells were cotransfected with 2 μg of HTLV-1 LTR-CAT, 5 μg of pACβ1 (β-galactosidase-containing reference plasmid), 1 μg of pSG-Tax (+) or empty pSG-5 vector (−), and 0 to 10 μg of pCI-CREB-2 or empty pCI-neo. CAT values were normalized for β-galactosidase activity and are expressed as fold increase relative to that of cells cotransfected with pSG-5, pCI-neo, and HTLV-1 LTR CAT. CAT assays were conducted by using a CAT antigen capture assay kit (Boehringer Mannheim). Values are the means ± standard deviations ($n = 4$).

the full-length clone was later isolated and named TAXREB67 (49) or CREB-2 (21). Members of the ATF/CREB family are characterized by basic-leucine zipper (bZIP) C-terminal domains required for DNA recognition and binding and for protein dimerization. They bind to CRE sequences, and their transcriptional activity is dependent upon phosphorylation by cAMP-dependent protein kinase A (PKA). PKA phosphorylation of CREB/ATF is a necessary step to recruit the coactivator CBP involved in transcriptional stimulation of cAMP-responsive genes (5, 23). Unlike CREB, CREB-2 lacks a potential PKA phosphorylation site and the α-helical transcriptional activator domain (21). CREB-2 has been shown to negatively regulate transcription from the human enkephalin promoter CRE (21) and has therefore been postulated to function as a specific repressor of CRE-dependent transcription. Tax has been reported to interact with several members of ATF/CREB family, including CREB (1, 2, 42, 50), CREM (2, 42), ATF-1 (2, 40), ATF-2 (10), and ATF-3 (29), but these interactions have not been characterized by two-hybrid approaches. It has been claimed that the inability to detect Tax-CREB interaction in yeast cells may be due to the masking of the Tax N terminus by the GAL4 DNA binding domain (40). However, as shown in Fig. 1A, in a liquid β-galactosidase assay, although Tax was fused with GAL4 DNA binding domain at its N terminus, it interacted specifically and strongly with CREB-2 in yeast. Recently, CREB-2 has also been isolated by two-hybrid approach from a peripheral blood lymphocyte cDNA library screened with complete Tax as a bait (36). This result together with ours suggest that CREB-2 could interact with Tax in a different way than that of the other members of the ATF/CREB family.

From the results described above, the possible explanation that the interaction between Tax and CREB-2 was not direct but rather required additional components could not be ruled out. To eliminate this possibility, we analyzed the interaction between Tax and CREB-2 in vitro by using recombinant proteins. CREB-2 cDNA was subcloned into pGEX (Pharmacia) in both orientations to obtain pGEX-CREB-2, a plasmid producing a glutathione *S*-transferase (GST)-CREB-2 fusion protein, and pGEX-AS containing CREB-2 cDNA in the antisense orientation and used as a negative control. Tax cDNA cloned into pSG-5 (37) was transcribed and translated in the presence of [$^{35}$S]methionine by using the TNT T7 coupled transcription-translation reticulocyte lysate system of Promega, and incubated at 4°C with equal amounts of GST-CREB-2 or GST-AS immobilized on glutathione Sepharose beads (Bulk GST Purification Module of Pharmacia) in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, and 10 mg of bovine serum albumin per ml. After a 0.5-h incubation, the beads were washed five times with incubation buffer and the bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 1B, $^{35}$S-labeled Tax bound to GST-CREB-2. This result demonstrated that Tax interacted directly with CREB-2. To further explore the significance of the interaction between Tax and CREB-2, the same in vitro binding assay was carried out with Tax mutants M22 and M47 that fail to activate NF-κB and ATF/CREB-dependent promoters, respectively (41). As shown in Fig. 1B, both mutants interacted with CREB-2. This result suggests that the inability of M47 to transactivate the HTLV-1 LTR (data not shown) is not due to a defect in Tax–CREB-2 interaction. Indeed, previously published results show that M47 binds to CREB but has impaired interactions with basal transcriptional factors (1, 46). Some Tax, although in smaller amounts, is retained by GST-AS (Fig. 1B). It probably corresponds to a weak interaction between Tax and the polypeptide encoded by the antisense cDNA of CREB-2. We noted that this polypeptide presents some similarities with amino acid domains reported to interact with Tax.

In order to test the functionality of the CREB-2–Tax interaction on the HTLV-1 promoter, the complete CREB-2 coding region was amplified from cDNA prepared from MT2 cells and cloned into a eukaryotic expression vector, pCI-neo (Promega). CEM cells were cotransfected as already described (26) with an HTLV-1 LTR CAT reporter plasmid and increasing amounts of pCI-CREB-2 in the presence or absence of the Tax expression vector pSG-Tax (37). As shown in Fig. 2, Tax alone was found to activate expression of the chloramphenicol acetyltransferase (CAT) reporter gene by about 15-fold. Moreover, CAT activity was stimulated about 60-fold in the presence of CREB-2. Control transfections indicated that in the absence of Tax, no activation could be detected. These results showed that CREB-2 associated with Tax was able to activate the HTLV-1 LTR, in contrast to its repressive effect on CRE-

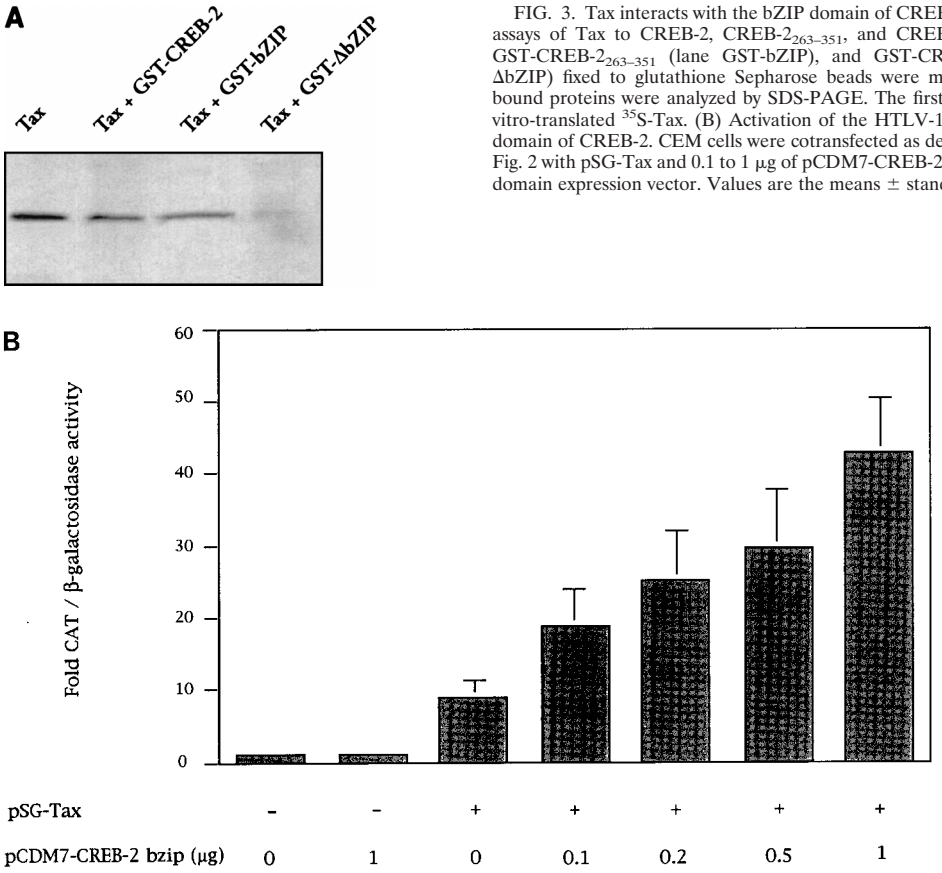


FIG. 3. Tax interacts with the bZIP domain of CREB-2. (A) In vitro binding assays of Tax to CREB-2, CREB-$2_{263–351}$, and CREB-$2_{1–262}$. GST-CREB-2, GST-CREB-$2_{263–351}$ (lane GST-bZIP), and GST-CREB-$2_{1–262}$ (lane GST-ΔbZIP) fixed to glutathione Sepharose beads were mixed with $^{35}$S-Tax, and bound proteins were analyzed by SDS-PAGE. The first lane corresponds to in vitro-translated $^{35}$S-Tax. (B) Activation of the HTLV-1 promoter by the bZIP domain of CREB-2. CEM cells were cotransfected as described in the legend to Fig. 2 with pSG-Tax and 0.1 to 1 μg of pCDM7-CREB-$2_{262–351}$, a CREB-2 bZIP domain expression vector. Values are the means ± standard deviations ($n$ = 4).

dependent transcription of the human enkephalin promoter (21).

Sequencing revealed that the CREB-2 cDNA isolated here by the two-hybrid approach coded for the complete sequence of CREB-2 except for the N-terminal 40 amino acids (data not shown) and therefore contained the bZIP domain, the region of ATF/CREB reported to be required for interaction with Tax (36, 50, 51). To determine whether the effect of Tax might be mediated through interactions with the CREB-2 bZIP domain, we performed in vitro binding assays and CAT reporter assays by using the CREB-2 bZIP domain only. Reverse transcription-PCR was used to prepare CREB-2 deletion mutants containing either the entire coding sequence except for the bZIP domain (amino acids 1 to 262) or only the C-terminal 89 amino acids including the entire bZIP domain (amino acids 263 to 351). As shown in Fig. 3A, $^{35}$S-labeled Tax bound to GST-CREB-$2_{263–351}$ (GST-bZIP) but not to GST-CREB-$2_{1–262}$ (GST-ΔbZIP), confirming that CREB-2 interaction with Tax is dependent on the bZIP domain. For CAT reporter assays, CEM cells were cotransfected with the HTLV-1 LTR CAT reporter plasmid pSG-Tax and increasing amounts of the CREB-2 bZIP domain expression vector pCDM7-CREB-$2_{262–351}$ (21). The bZIP domain of CREB-2 activated the HTLV-1 promoter by about 45-fold in the presence of Tax (Fig. 3B). Control transfections indicated that in the absence of Tax, no activation could be detected. In contrast to a previous study indicating that the CREB-2 C-terminal region including the bZIP domain acts as a repressor of transcription (21), our results show that this region of CREB-2 enhances Tax-stimulated transcription from the HTLV-1 promoter. Similarly, by utilizing a repressor isoform of CREM, CREMΔ(C-G), which contained the bZIP domain but not the PKA phosphorylation site or the transcriptional activator domain, Laurance et al. (24) demonstrated that the CREM activation domains are not essential for viral promoter stimulation by CREM associated with Tax. They suggested that Tax could provide a bridge between the CREM protein bound to HTLV-1 promoter and the coactivator CBP through a mechanism that is PKA independent. Our results confirm this model, since Tax is able to interact with the minimal bZIP domain of CREB-2 to activate HTLV-1 LTR-dependent transcription.

Last, to confirm the in vivo relevance of the interaction between Tax and CREB-2, we studied the localization of both proteins inside cells. Because the bZIP domain of CREB-2 is directly involved in in vitro interaction with Tax and in vivo activation of the HTLV-1 LTR, the fragment encoding CREB-$2_{263–351}$ was subcloned into the pEGFP-C1 vector (Clontech) to produce in vivo a GFP (green fluorescent protein)-CREB-$2_{263–351}$ fusion protein. Cos7 cells were cotransfected with pEGFP-CREB-$2_{263–351}$ and pSG-Tax. Cells were examined under the confocal microscope (Fig. 4). Colocalization of CREB-2 and Tax in the nucleus was visualized by the yellow color (Fig. 4C), which corresponds to the merging of the red staining of Tax detected by indirect immunofluorescence (rhodamine) (Fig. 4A) and the green fluorescence of the GFP-CREB-$2_{263–351}$ fusion protein (Fig. 4B).

PKA phosphorylation of CREB/ATF is a necessary step to recruit the coactivator CBP involved in transcriptional stimulation of cAMP-responsive genes (5, 23). However, it has been shown that activation of the HTLV-1 promoter through CREB/ATF and Tax is PKA independent (24), with CBP being recruited through interactions with Tax (13, 22). Our results

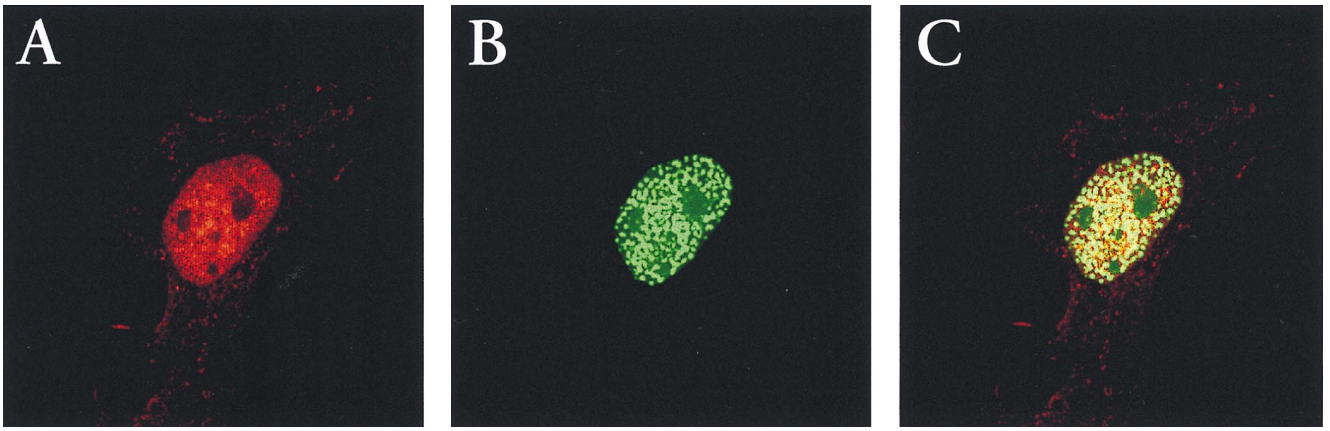


FIG. 4. Confocal microscopy analysis of the colocalization of Tax and CREB-2 in vivo. Cos7 cells were cotransfected with pSG-Tax and pEGFP-CREB-$2_{263–351}$. Analysis of the red (A), green (B), and merged (C) fluorescence was performed with a Bio-Rad MRC 1024 confocal microscope. The Tax protein was detected by using culture supernatant of the anti-Tax 168A51-42 hybridoma and goat anti-mouse immunoglobulin G antibody coupled to rhodamine (Pierce).

confirm this model since unlike CREB, CREB-2 lacks a PKA phosphorylation site (21). The possibility that Tax uses CREB-2 as an adapter to allow its own localization in an appropriate molecular environment that favors its interaction with other members of the cellular transcriptional machinery should be investigated further. In this context, it will be of great interest to study the effects of Tax on transcription of cellular genes controlled by CREB-2. The stimulation by Tax of transcription of a cellular gene repressed by CREB-2 may provide a molecular basis by which HTLV-1 could induce T-cell transformation.

F.G. and A.P. contributed equally to this work.

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