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Protein Domains Involved in both In Vivo and In Vitro Interactions between Human T-Cell Leukemia Virus Type I Tax and CREB

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Gene expression from the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) is mediated by three *cis*-acting regulatory elements known as 21-bp repeats and the transactivator protein Tax. The 21-bp repeats can be subdivided into three motifs known as A, B, and C, each of which is important for maximal gene expression in response to Tax. The B motif contains nucleotide sequences known as a cyclic AMP response element (CRE) or *tax*-response element which binds members of the ATF/CREB family of transcription factors. Though mutations of this element in the HTLV-I LTR eliminate *tax* activation, Tax will not activate most other promoters containing these CRE sites. In this study, we investigated the mechanism by which Tax activates gene expression in conjunction with members of the ATF/CREB family. We found that Tax enhanced the binding of one member of the ATF/CREB family, CREB 1, to each of the three HTLV-I LTR 21-bp repeats but not another member designated CRE-BP1 or CREB2. Tax enhanced the binding of CREB1 to nonpalindromic CRE binding sites such as those found in the HTLV-I LTR, but Tax did not enhance the binding of CREB1 to palindromic CRE binding sites such as found in the somatostatin promoter. This finding may help explain the failure of Tax to activate promoters containing consensus CRE sites. These studies were extended by use of the mammalian two-hybrid system. Tax was demonstrated to interact directly with CREB1 but not with other bZIP proteins, including CREB2 and Jun. Site-directed mutagenesis of both Tax and CREB1 demonstrated that the amino terminus of Tax and both the basic and the leucine zipper regions of CREB1 were required for direct interactions between these proteins both in vivo and in vitro. This interaction occurred in vivo and thus did not require the presence of the HTLV-I 21-bp repeats, as previously suggested. These results define the domains required for interaction between Tax and CREB that are likely critical for the activation of HTLV-I gene expression.

Human T-cell leukemia virus type I (HTLV-I) is a human retrovirus which is the etiologic agent of adult T-cell leukemia/lymphoma (34, 55, 74) and a degenerative neurologic syndrome designated tropical spastic paraparesis (26, 52). The HTLV-I genome encodes a number of regulatory proteins, including Tax and Rex, which are involved in controlling viral gene expression and pathogenesis (11). The 40-kDa transactivator protein Tax is critical for modulating the level of HTLV-I gene expression (7, 15, 22, 66) and is also involved in the cellular transformation of human T lymphocytes (32, 70). In addition to regulating HTLV-I gene expression, Tax has also been demonstrated to activate gene expression from other viral and cellular genes, including those from the interleukin-2 (IL-2), IL-2 receptor α, c-*fos*, and human immunodeficiency virus type 1 (HIV-1) promoters (18–20, 38, 43), and it also represses gene expression from the DNA polymerase-β promoter (41). Thus, Tax is an important modulator of both viral and cellular gene expression.

The HTLV-I long terminal repeat (LTR) contains three relatively conserved elements, designated 21-bp repeats, which are necessary for transactivation by Tax (6, 23, 54, 61). The 21-bp repeats have been further subdivided into three motifs designated A, B, and C (24, 49). The B motif in each of the 21-bp repeats contains the sequence TGACG, which is designated as a cyclic AMP response element (CRE) or the *tax* response element. This element is capable of serving as the binding site for members of the ATF/CREB family of transcription factors (2, 3, 68, 72, 75–77) in addition to other factors such as HEB1 and HEB2 (4, 49). Though the A and C motifs within the 21-bp repeat are important for *tax* activation, mutation of the B motif which eliminates *tax* activation appears to be key (24, 27, 49, 58, 69). In addition, the B motif can mediate activation of HTLV-I gene expression in response to increases in cyclic AMP, indicating that factors normally regulated by cyclic AMP such as CREB may bind to the 21-bp repeat (40, 56). However, *tax* activation of most other viral and cellular promoters is mediated by the binding sites of transcription factors other than ATF/CREB. For example, *tax* activation of the IL-2 receptor α promoter (10, 38, 43) and the HIV-1 LTR (5, 60, 62, 66) is mediated by NF-κB sites. Tax activation of the c-*fos* (18, 19) or the parathyroid hormone-related protein gene (73) is modulated through upstream factor binding sites for proteins, including SRF and AP-2. Thus, it is likely that Tax may either directly or indirectly alter the activity of a variety of different transcription factors to increase the level of gene expression from *tax*-responsive genes.

The mechanism by which Tax activates HTLV-I gene expression has been the subject of intense investigation. Tax does not bind directly to DNA elements in the HTLV-I LTR or other promoters (3, 25, 27, 48). Instead, through the use of in vitro binding assays, Tax has been demonstrated to directly interact with cellular transcription factors which bind to the HTLV-I LTR, including members of the ATF/CREB family (53, 67, 72, 76, 77), HEB1 (4), and TFIID (8). Gel retardation analysis suggests that Tax stimulates the binding of CREB to the B motif within the 21-bp repeats (17, 72, 76), though it is not clear whether this association is stable during electro-

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phoresis (2, 17, 72, 76, 77). It is also controversial whether Tax has specificity for one or more members of the CREB/ATF family (2, 17, 72, 76). In fact, one study suggests that Tax can stimulate the DNA binding properties of a variety of basic leucine zipper-binding (bZIP) proteins to their cognate binding sites (2, 17, 72). This latter result raises questions about how Tax could increase the binding of a variety of cellular transcription factors yet still have relatively specific targets for the activation of gene expression. The demonstration that Tax will not activate gene expression from a variety of different promoters containing CRE sites further points to marked specificity requirements for *tax* activation (24).

A number of members of the ATF/CREB family have been demonstrated to bind to the 21-bp repeat (75). These include TREB7, which is identical to CRE-BP1 and differs by two amino acids from ATF-2, and TREB 36, which is identical to ATF-1 and is highly homologous to CREB (29, 33, 37, 75). On the basis of the terminology of the Nomenclature Committee of the Human Genome Project, we will refer to CREB as CREB1 and CRE-BP1 as CREB2 (12, 71). In this study, we addressed the specificity of Tax interaction with members of the ATF/CREB family and other leucine zipper proteins by using both in vitro interaction studies and the mammalian two-hybrid system. Furthermore, we used mutagenesis to determine motifs in CREB1 and Tax necessary for their interaction both in vivo and in vitro. Finally, we explored the effects of Tax on CREB1 binding to both consensus and nonconsensus CRE sites. These studies suggest that specific interactions between Tax and CREB1 in the context of nonpalindromic CRE binding sites are critical for Tax activation of the HTLV-I LTR.

MATERIALS AND METHODS

Plasmid constructs. All clones constructed after PCR were analyzed and confirmed by DNA sequence analysis. The full-length CREB1 cDNA (37), the ΔN2 (4-274), and the ΔC (267-341) CREB1 deletion constructs were cloned downstream of the glutathione *S*-transferase (GST) protein in pGEX-2T. A *Bam*HI-*Eco*RI full-length CREB1 cDNA fragment was cloned into the pGEX-2T vector. The amino-terminal truncation was created by PCR with an oligonucleotide primer (5′-GTTGCCATGGCATCCTCCCCAGC-3′) which created an in-frame *Nco*I site and a 3′ oligonucleotide primer (5′-AATCCCC ATGGAATCTGATTTGTGGCAGTAAAGG-3′), with CREB1 cDNA as a template. This PCR product was then cut with *Nco*I-*Xho*I and cloned into the pGEX-2T vector. The 5′ PCR primer creating the carboxy-terminal truncation of CREB1 was made with the 5′ oligonucleotide primer 5′-CAGAAAGTGAAGA TTCACAG-3′ and the 3′ primer 5′-TTCCCATGGATTCTTCAGCAGGCTGT GTAGG-3′. The PCR product was digested with *Kpn*I-*Sma*I and cloned into the pGEX-2T vector.

The wild-type Tax and amino-terminal Tax truncation ΔN2 (2-136) were inserted in the *Nco*I restriction site of plasmid pQE60 (Qiagen). The amino-terminal truncation of Tax ΔN2 was constructed by PCR with a 5′ oligonucleotide primer, 5′-TCCAGCCATGGGACTCCGGCCCCAAAACCTG-3′, which created an in-frame *Nco*I site, and with a 3′ primer, 5′-GTCGGCAAATATCG CATGC-3′. Both the Tax cDNA and the TaxΔN2 construct were digested with *Nco*I and cloned into *Nco*I-digested pQE60 vector. The two resulting plasmids, pQE60-Tax-E6K and pQE60-E6K TaxΔN2, produced a fusion protein which included the Tax coding sequence, the influenza virus hemagglutinin epitope, a 6x histidine tag, and a substrate sequence (RRASV) for protein kinase A phosphorylation.

The 5x GAL-E1B-CAT reporter plasmid containing five GAL4 DNA binding sites and the E1B TATA box linked to the chloramphenicol acetyltransferase (CAT) gene have previously been described (57). The Tax-GAL eucaryotic expression vector was constructed by fusing an *Nco*I-cut Tax cDNA (58) fragment in frame with the GAL4 (1-147) coding sequences in the pDP18 expression vector, which contains a Rous sarcoma virus promoter and a simian virus 40 polyadenylation site. The Tax amino- and carboxy-terminal truncations TaxΔN1 (Δ3-93), TaxΔN2 (2-136), TaxΔC1 (Δ337-353), TaxΔC2 (Δ317-353), and TaxΔC3 (Δ297-353) were constructed by PCR. The primers for PCR created in-frame *Nco*I sites on both the 5′ and 3′ ends. The PCR primers used for these constructs included the TaxΔN1 (Δ3-93) 5′ primer, 5′-CTTACCATGGCAATCACTCATA CAACCCCCAAC-3′, and 3′ primer, 5′-GTCGGCAAATATCGCATGC-3′; the TaxΔC1 (Δ337-353) 5′ primer, 5′-CCCACAAATTGATAAGTACTTG, and 3′ primer, 5′-GCCCCCCATGGATATTTGGGGCTCATG-3′, the TaxΔC2 (Δ317-353) 5′ primer, 5′-TAATACGACTCACTATAGG-3′, and 3′ primer, 5′-CGCT CCATGGATCCGGGGATGTTGGTGTATTC-3′, and the TaxΔC3 (Δ297-353) 5′ primer, 5′-TAATACGACTCACTATAGG-3′, and 3′ primer, 5′-CGCTCC ATGGATCCGAGGCCGTGTGAGAGTAG-3′. The PCR products were digested with *Nco*I and cloned in frame upstream of the GAL4 (1-147) sequence in the pDP18 vector.

CREB1-VP16, CREB2-VP16, and Jun-VP16 fusions were constructed by insertion of these complete cDNA fragments in frame upstream of the VP16 (412-490) activation domain and a simian virus 40 nuclear localization signal in the pDP18 expression vector. CREB deletion constructs CREBΔN1 (Δ4-136), CREBΔN2 (Δ4-274), and CREBΔC (Δ267-341) were constructed by PCR. The PCR primers used to construct amino- and carboxy-terminal truncations of CREB with *Nco*I sites at each end are the CREBΔN1 (Δ4-136) 5′ primer, 5′-C AACTACCATGGAAAGTGAAGATTCACAGGAG-3′, and 3′ primer, 5′-AT TCCCCATGGAATCTGATTTGTGGCAGTAAAGG-3′; the CREBΔN2 (Δ4-274) 5′ primer, 5′-GTTGCCATGGCATCCTCCCCAGC-3′, and 3′ primer, 5′-AATCCCCATGGAATCTGATTTGTGGCAGTAAAGG-3′; and the CREBΔC (Δ267-341) 5′ primer, 5′-CAGAAAGTGAAGATTCACAG-3′, and 3′ primer, 5′-TTCCCATGGATTCTTCAGFAGGCTGTGTAGG-3′. The PCR products were digested with *Nco*I and then cloned in frame upstream of the VP16 (412-490) sequence in the pDP18 vector.

To construct point mutations of Tax and CREB1, single-stranded M13 DNAs were isolated and site-directed mutagenesis was performed with an oligonucleotide-directed in vitro mutagenesis system (Amersham). These mutant Tax and CREB cDNAs were subcloned either upstream of GAL4 (1-147) or upstream of VP16 (412-490), respectively, in the pDP18 vector and confirmed by sequencing. The oligonucleotide primers for the Tax mutants M1 (H-3→S), M22 (G-137L→AS), and M47 (L-319L→RS) were 5′-CACCATGGCCTCCTTCCCAGGGTT TGGAC-3′, designed to change His-3 to Ser; 5′-CTTTTCCAGACCCCGCTAG CCGGCCCCAAAACCTG-3′, designed to change Gly-137 and Leu-138 to Ala and Ser, respectively; and 5′-CCAACATCCCCATTTCTAGATCTTTTAACG AAAAAGAGG-3′, designed to change Leu-319 and Leu-320 to Arg and Ser, respectively (65). The resulting DNA fragments were digested with *Nco*I and cloned upstream of the GAL4 DNA binding domain in the pDP18 vector. The point mutations of CREB (37), including those of basic region I (284-R-KR→GTT), basic region II (301-R-RKKK→RGSRK and (301-R-RKKK→GTQQE), and the leucine zippers I and II (L-311, L-318→V,V) were constructed with the oligonucleotide primers 5′-GCTGAAGAAGCAGGATCTA GAGAGGTCCGTCTAATG-3′, 5′-GCAGCTCGAGAGTGTCGTGGA-3′, 5′-TCTAGAAAAGAATATGTGAAATGTTTAG-3′, and 5′-GGGAAGCAGCT CGAGAGTGTGGTACCCAGCAGGAAGAATATGTGAAATGTTTAG-3′, respectively, and the same 3′ primer used to construct the CREB1 5′ deletion constructs. The resulting DNA fragments were digested with *Kpn*I-*Bam*HI and cloned into a *Kpn*I-*Bam*HI-digested CREB-VP16 vector. All mutant clones constructed by site-directed mutagenesis were confirmed by DNA sequence analysis.

Expression and purification of bacterially produced CREB1 and Tax proteins. The bacterial expression vectors pGEX2T and pQE60 with either Tax or CREB cDNA inserts were transformed into *Escherichia coli* M15. Cultures (400 ml) of *E. coli* were grown to an optical density at 600 nm of 0.6 to 0.8 and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Cells were pelleted, resuspended in buffer A (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride), and mildly sonicated, and the debris was pelleted. For GST fusion proteins (64), the supernatant was incubated with 2 ml of glutathione-Sepharose beads for 60 min at 4°C. The beads were then washed five times with 10 ml of buffer A containing 1% Triton X-100 (63). The proteins were eluted by incubation with elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM glutathione) at 4°C. Proteins were dialyzed against protein storage buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10% glycerol, 1 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride). For 6x His-tagged proteins, bacterial cells were cultured and fractionated under these same conditions except that the cell lysate was incubated with 1 ml of nickel-nitrilotriacetic acid-agarose for 60 min at 4°C. The proteins were eluted with elution buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM DTT, 1 mM EDTA) containing from 20 to 200 mM imidazole. The proteins were dialyzed against protein storage buffer and kept at −80°C.

Gel retardation analysis. For gel retardation assays, 50 to 100 ng of either bacterially produced GST-CREB or bacterially produced CREB-6x His in either the presence or the absence of bacterially produced Tax-6x His or ΔN1Tax-6x His protein was incubated with 1 μg of poly(dI-dC) (Pharmacia) and labeled probe in binding buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM DTT, 5% glycerol, 50 mM NaCl). The reaction time was for 20 min at room temperature in a 40-μl final volume. Synthetic double-stranded oligonucleotides containing the somatostatin CRE (5′-GGTTCCTCCTTGGCTGACGTCAGAG AGAGA-3′) or HTLV-I LTR 21-bp repeat I (5′-TCGACGTCCTCAGGCGT TGACGACAACCCCTCAC-3′), 21-bp II (5′-CTCGGGCTAGGCCCTGAC GTGTCCCCCTGAA-3′), or 21-bp III (5′-CCAGACTAAGGCTCTGACGTC TCCCCCCGGACCT-3′) were end labeled with [γ-^{32}P]ATP, by using T4 polynucleotide kinase. Approximately 0.1 ng of ^{32}P-labeled DNA (50,000 cpm) was incubated with the CREB and Tax proteins. When competition was performed, unlabeled oligonucleotides in a 50-fold molar excess were added to the reaction mixture. DNA-protein complexes were resolved on a 5% polyacrylamide gel in

TABLE 1. Comparison of HTLV-1 21-bp repeats and consensus CRE sequences

Site	Sequence[a]
I	A A G G C T C T G A C G T C T C C C C C C
II	T A G G C C C T G A C G T G T C C C C C T
III	C A G G C G T T G A C G A C A A C C C C T
CRE	C C T A G G C T G A C G T C A G A G A G A

[a] The nucleotide sequences of the three HTLV-I 21-bp repeats are shown as is the sequence of the somatostatin CRE. The position of domain B in the 21-bp repeats and of the CREB binding site in the somatostatin CRE is indicated.

0.25× TBE (50 mM Tris-borate [pH 8.3], 1 mM EDTA). The gels were dried and autoradiographed with an intensifying screen at −80°C.

In vitro protein-protein binding. The bacterially produced Tax-E6K protein was labeled with [γ-^{32}P]ATP at the introduced protein kinase A substrate site, by the addition of the protein kinase A catalytic subunit for 15 min at 30°C. A total of 20 to 50 μg of GST-CREB, GST-CREB ΔN2, or GST-CREB ΔC protein was incubated with glutathione-agarose beads for 1 h at 4°C. Approximately 1 μg or 300,000 cpm of either Tax-E6K or TaxΔN1-E6K labeled proteins was then added with GST-CREB, GST-CREB ΔN, or GST-CREB ΔC beads in TIB buffer (10% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 50 mM KCl, 50 nM $ZnCl_2$, 2.5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride) for 1 h at 4°C. The protein-protein complexes were washed five times at 4°C with 10 volumes of TIB buffer. The Tax proteins which remained attached to the beads were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), the gels were dried, and autoradiography was performed.

Analysis of CREB-VP16 and GAL-Tax protein. To analyze the expression of the GAL-Tax constructs, each of the constructs was cloned into the pTM1 expression construct downstream of the T7 promoter (14). HeLa cell plates (diameter, 60 mm) were transfected by the calcium phosphate transfection procedure with 10 μg of each pTM1 expression construct. Protein expression was induced by infection with a T7 RNA polymerase encoding helper virus at a multiplicity of infection of 5 as previously described (59). Cells were lysed and harvested 24 h later in protein gel loading dye, and cleared whole-cell lysates from 2×10^5 cells were used in Western immunoblotting with Tax monoclonal antibody obtained from the AIDS Reagent Repository.

To analyze the expression of the Rous sarcoma virus CREB1-VP16 expression constructs, each of the constructs was transfected into the adenovirus E1A- and E1B-transformed human embryonic kidney cell line 293 (31). At 48 h posttransfection, the cells were lysed and harvested in protein gel loading dye, and the cleared whole-cell lysates from 2×10^5 cells were used in Western immunoblotting with CREB polyclonal antibody (42).

Transfection and CAT assays. Jurkat cells were maintained in RPMI and 10% fetal bovine serum with penicillin and streptomycin. Cells were diluted 1:2 in fresh medium on the day prior to transfection, and 5×10^6 cells were transfected with 0.2 mg of DEAE-dextran. Cells were harvested after 48 h and one quarter of the cell extract was used for CAT assays (30).

RESULTS

Tax stimulates CREB1 binding to nonpalindromic compared with palindromic CRE sites. The HTLV-I LTR contains three 21-bp repeats, each of which contain sequences known as CRE motifs which have homology to sequences found in cyclic AMP-responsive promoters (23, 54, 61). However, none of the 21-bp repeats contains palindromic CRE sites such as found in the cyclic AMP-regulated somatostatin promoter (51). We compared the binding of bacterially synthesized CREB1 protein to each of the three HTLV-I 21-bp repeats and the somatostatin CRE. CREB1 was the first member of the ATF/CREB family to be identified (50, 51). This 43-kDa protein has been demonstrated to bind to both consensus and nonconsensus CRE elements, and its transcriptional activating properties are regulated by phosphorylation in response to cyclic AMP-dependent protein kinase (28, 37, 50, 51, 74). As shown in Table 1, the nonpalindromic sequences of the CRE site in each of the 21-bp repeats differ from the palindromic sequence found in the somatostatin promoter.

Gel retardation analysis indicated that bacterially produced CREB1 bound weakly to oligonucleotides corresponding to each of the 21-bp repeats (Fig. 1A, lanes 1, 4, and 7). However, it was noted that CREB1 consistently bound better to 21-bp repeat III (Fig. 1A, lane 7). In contrast, with the same concentration of CREB1 protein, a much greater degree of CREB1 binding to the somatostatin CRE was seen (Fig. 1A, lane 10). CREB1 binding was also assayed in the presence of bacterially produced Tax. Tax was able to markedly stimulate CREB1 binding to each of the HTLV-I 21-bp repeats (Fig. 1A, lanes 2, 5, and 8). There was no stimulation of CREB1 binding with the addition of other unrelated proteins such as GST (data not

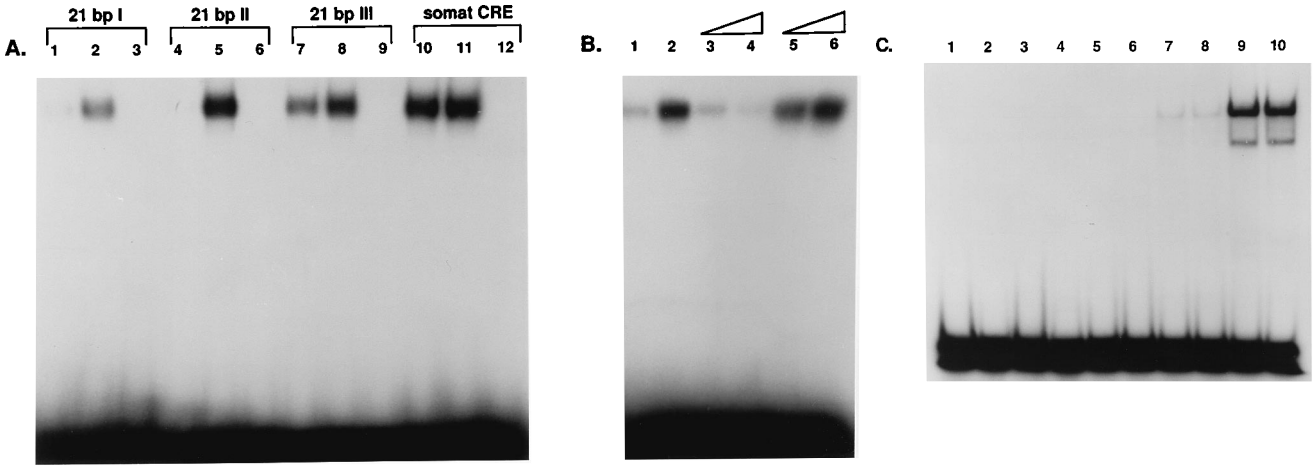

FIG. 1. Tax stimulates the binding of CREB1 to HTLV-I 21-bp repeats. (A) Oligonucleotides corresponding to HTLV-I 21-bp repeat I (lanes 1 to 3), 21-bp repeat II (lanes 4 to 6), and 21-bp repeat III (lanes 7 to 9) and the somatostatin (somat) CRE (lanes 10 to 12) were end labeled with [γ-^{32}P]ATP and used in gel retardation assays. Lanes 1, 4, 7, and 10, 100 ng of bacterial CREB1-6x His alone; lanes 2, 5, 8, and 11, 100 ng of bacterial CREB1-6x His and 100 ng of bacterially expressed Tax-6x His; lanes 3, 6, 9, and 12, 100 ng of Tax-6x His alone. (B) Gel retardation analysis with 21-bp repeat I and either 100 ng of CREB1-6x His alone (lane 1) or 100 ng of TAX-6x His (lane 2). Specific competition of CREB1 binding to 21-bp repeat I was performed by preincubation of either a 50 (lane 3)- or 150 (lane 4)-fold molar excess of unlabeled 21-bp repeat oligonucleotide or similar quantities of unlabeled and B motif-mutated 21-bp repeat oligonucleotides (lanes 5 and 6). (C) Gel retardation analysis with oligonucleotides corresponding to the somatostatin CRE (lanes 1 to 10) were performed in both the absence (lanes 1, 3, 5, 7, and 9) and the presence of 100 ng of Tax (lanes 2, 4, 6, 8, and 10). Either no CREB1 (lanes 1 and 2) or increasing amounts of CREB1 (0.2 ng [lanes 3 and 4], 2 ng [lanes 5 and 6], 20 ng [lanes 7 and 8], and 200 ng [lanes 9 and 10]) were added to the gel retardation assay.

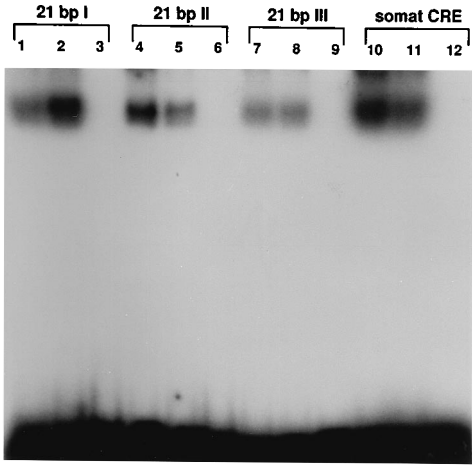

FIG. 2. Tax does not markedly stimulate the binding of CREB2. Oligonucleotides corresponding to HTLV-I 21-bp repeat I (lanes 1 to 3), 21-bp repeat II (lanes 4 to 6), and 21-bp repeat III (lanes 7 to 9) and the somatostatin (somat) CRE (lanes 10 to 12) were end labeled and used in gel retardation analysis. Lanes 1, 3, 7, and 10, 100 ng of bacterially expressed GST-CREB2 alone; lanes 2, 4, 8, and 11, 100 ng of GST-CREB2 and 100 ng of Tax-6x His; lanes 3, 6, 9, and 12, 100 ng of Tax-6x His alone.

shown) or nonfunctional Tax mutants (see below). Tax itself did not bind to any of the HTLV-I 21-bp repeats as has previously been reported (Fig. 1A, lanes 3, 6, 9, and 12). Competition analysis was also performed to demonstrate the specificity of this binding. Wild-type (Fig. 1B, lanes 3 and 4) but not mutated (Fig. 1B, lanes 5 and 6) 21-bp repeat oligonucleotides were able to compete for CREB1 binding to the HTLV-I 21-bp repeats.

It was also interesting that Tax resulted in only minimal stimulation of CREB1 binding to oligonucleotides corresponding to the somatostatin CRE (Fig. 1A, lanes 10 and 11). To further investigate the lack of Tax stimulation of CREB1 binding to the somatostatin CRE, a titration of CREB1 protein ranging from 0.2 to 200 ng was performed (Fig. 1C, lanes 3 to 10). Tax was unable to significantly stimulate the binding of CREB1 to the somatostatin CRE over a wide concentration range of CREB1, and this was confirmed by PhosphorImager scanning (Fig. 1C). Thus, Tax markedly stimulated the binding of CREB1 to the 21-bp repeats but not to a consensus CRE.

Tax does not stimulate the binding of CREB2 to the HTLV-I 21-bp repeats. Recently it was reported that Tax was able to stimulate the DNA binding properties of a variety of different DNA-binding proteins, including those containing bZIP binding domains (2, 17, 72). We wished to determine whether Tax was able to stimulate the binding of another bZIP member of the ATF/CREB family of DNA-binding proteins, designated CRE-BP1 or CREB2 (33, 46, 47, 75). CREB2 was produced in bacteria as a GST fusion protein and was purified with glutathione-agarose beads, and its binding properties were tested with oligonucleotides corresponding to both the HTLV-I 21-bp repeats and the somatostatin CRE. At the same protein concentrations as used for CREB1 (Fig. 1), CREB2 bound to each of the HTLV-I 21-bp repeats (Fig. 2, lanes 1, 4, and 7) and to the somatostatin CRE (Fig. 2, lane 10). However, Tax did not markedly stimulate the binding of CREB2 to any of the 21-bp repeats (Fig. 2, lanes 2, 5, and 8) or to the somatostatin CRE (Fig. 2, lane 11). Again, Tax did not bind to any of these DNA probes (Fig. 2, lanes 3, 6, 9, and 12). Thus, Tax stimulated the binding of CREB1 but not CREB2 to the HTLV-I 21-bp repeats. Tax also did not stimulate the binding of other cellular factors such as AP-2 and PRDII-BF1 that have been demonstrated to bind to the HTLV-I 21-bp repeats (data not shown).

To further investigate the effects of Tax on CREB1 and CREB2 binding to the HTLV-I 21-bp repeats, a variety of additional experiments was performed. To determine the role of differences in the protein concentrations of CREB1 and CREB2 on Tax stimulation of binding to the 21-bp repeats, we performed titrations of both CREB1 and CREB2 on the HTLV-I 21-bp repeat III, with a constant amount of Tax. The titrations of CREB1 and CREB2 were conducted over a range of protein concentrations, from 40 to 200 ng (Fig. 3). The amount of CREB binding was determined by PhosphorImager quantitation of the amount of 21-bp repeat probe bound in both the presence and the absence of Tax. The maximal amount of Tax stimulation of CREB1 binding in this experiment was approximately sevenfold at a CREB1 concentration of 100 ng (Fig. 3A, lanes 5 and 6). At higher CREB1 concentrations, the amount of Tax stimulation was only about twofold (Fig. 3A, lanes 7 and 8). In contrast to the results with CREB1, CREB2 binding to the 21-bp repeats was not affected and in fact it was slightly inhibited by the addition of Tax over a similar protein concentration range (Fig. 3B). Graphs of the amount of CREB1 and CREB2 which were bound to the 21-bp repeats in both the presence and the absence of Tax are shown in Fig. 3C and D.

Next, we wished to further investigate why Tax was unable to stimulate the binding of CREB2 to the 21-bp repeats. The failure of Tax to stimulate the binding of CREB2 was not because CREB2 was expressed as a fusion with GST (Fig. 4A). Though Tax was able to stimulate the binding of CREB1 to 21-bp repeat III (Fig. 4A, lanes 3 and 4), neither the binding of GST-CREB2 (Fig. 4A, lanes 5 and 6) nor increasing amounts of authentic CREB2 generated by cleavage with thrombin (Fig. 4A, lanes 7 to 10) were stimulated by Tax. Coomassie-stained polyacrylamide gels confirmed that thrombin cleavage of GST-CREB2 liberated native CREB2 (data not shown). Finally, we mixed both CREB1 and CREB2 in the same gel retardation reaction mixture with the 21-bp repeat III probe in both the presence and the absence of Tax (Fig. 4B). Tax stimulated the binding of CREB1 to the 21-bp repeat approximately three- to fourfold (Fig. 4B, lanes 3 to 10). However, again there was no stimulation by Tax of the binding of the upper CREB2 doublet to 21-bp repeat III (Fig. 4B, lanes 3 to 10). These experiments further established that Tax would specifically stimulate the binding of CREB1 but not CREB2 to the HTLV-I 21-bp repeats. Studies using on and off rates indicated that Tax stimulated the on rate of CREB binding, as previously demonstrated (data not shown) (72).

CREB1 but not other leucine zipper proteins directly interacts with Tax. The results of gel retardation analysis indicated that Tax could stimulate the binding of CREB1 to the HTLV-1 21-bp repeats. However, no gel-retarded complex indicative of a stable complex comprising TAX and CREB1 was detectable. Thus, it was not clear whether Tax and CREB1 might be able to directly interact and then dissociate during gel electrophoresis or whether some other mechanism was responsible for Tax stimulation of CREB1 binding. Two approaches to further investigate interactions between Tax and CREB1 were attempted. First, we used in vivo analysis with the mammalian two-hybrid system (45), and second, we used direct in vitro binding studies to determine whether there was a stable interaction between CREB1 and Tax. For two-hybrid analysis (16), constructs in which Tax was fused at its carboxy terminus to the DNA binding domain of the yeast transcription factor GAL4 were made. Several previous studies have used Tax fusions with GAL4 to demonstrate that Tax contains a weak transcrip-

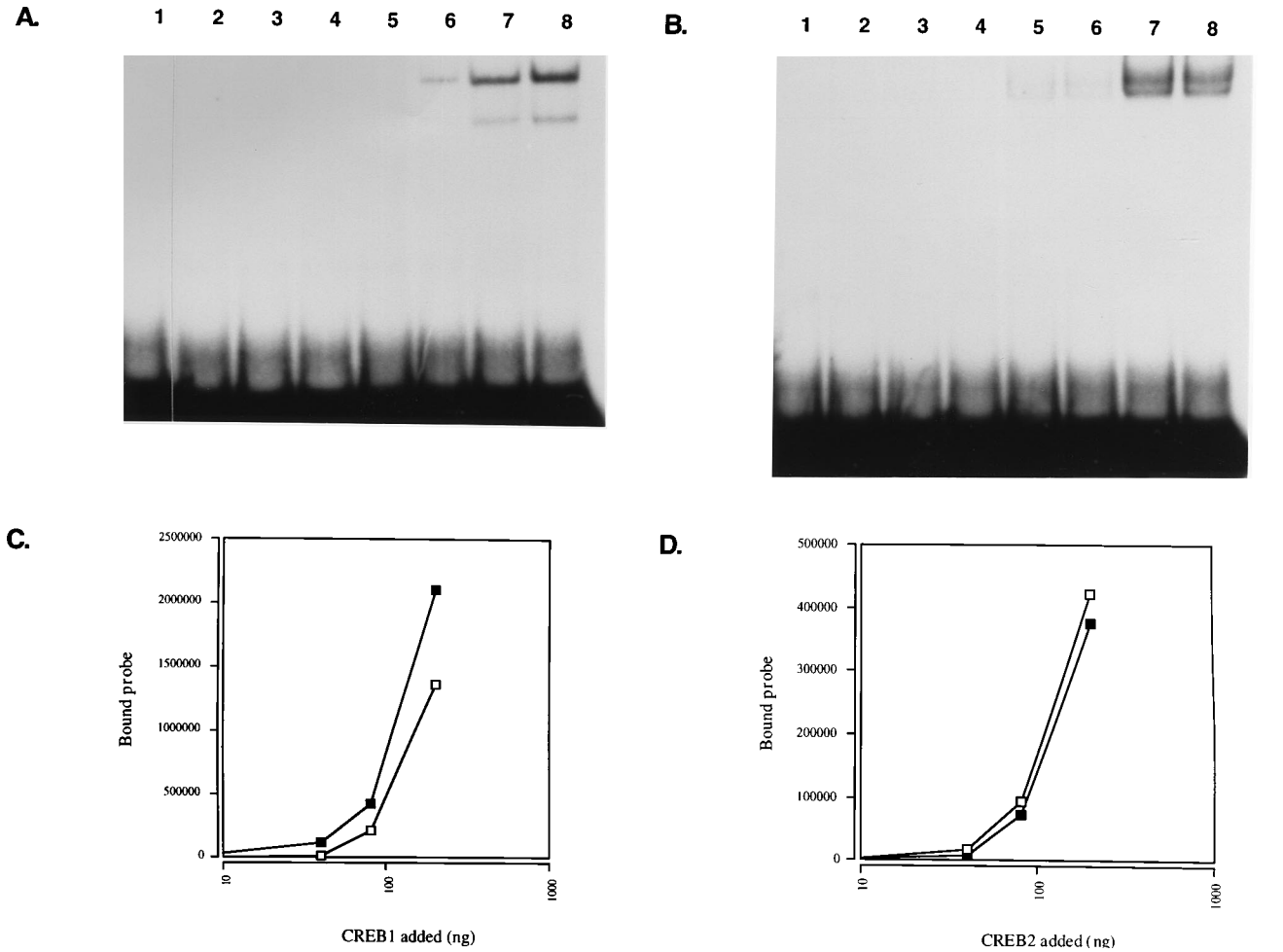

FIG. 3. Titration of CREB1 and CREB2 binding to the HTLV-I 21-bp repeat. Oligonucleotides corresponding to HTLV-I 21-bp repeat III were end labeled and used in gel retardation analysis with either CREB1 (A and C) or CREB2 (B and D) in either the absence (lanes 1, 3, 5, and 7) or the presence of 100 ng of Tax-6x His (lanes 2, 4, 6, and 8). Increasing amounts of CREB1 (A) or CREB2 (B) (0 ng [lanes 1 and 2], 40 ng [lanes 3 and 4], 80 ng [lanes 5 and 6], or 200 ng [lanes 7 and 8]) were added to each reaction mixture, and the relative amount of each retarded species in both the presence (■) and the absence (□) of Tax was quantitated by PhosphorImager analysis (C and D).

tional activation domain (9, 21, 25). The CREB1 protein was fused at its carboxy terminus to the transcriptional activation domain of the herpesvirus transactivator VP16. A 5x GAL-EIB TATA CAT reporter plasmid (57) was used to assay potential interactions following transfection of these constructs into the T-lymphocyte cell line Jurkat, as illustrated in Fig. 5A (57). Interactions between Tax and CREB in the two-hybrid assay would increase the level of CAT activity because of the effects of positioning the strong acidic activator VP16 near the transcription initiation complex on the E1B promoter (45). Transfections were normalized by the addition of a β-galactosidase control plasmid, and each transfection was repeated at least three times.

The Tax-GAL construct alone gave little increase in gene expression from the reporter construct in the presence of VP16 which was not bound to CREB1 (Fig. 5B, lane 3). This was in agreement with the results of a previous study which showed that a GAL-Tax construct which bound to GAL4 DNA binding sites which lacked the flanking 21-bp repeat sequences was a weak transactivator (9). Likewise, a GAL construct lacking Tax was not activated by CREB1-VP16 (Fig. 5B, lane 2). However, in the presence of both Tax-GAL and CREB1-VP16 constructs, gene expression was stimulated approximately 10-fold, indicating a direct and stable interaction between the CREB1 and Tax proteins (Fig. 5B, lane 4). Similar results were seen by switching the GAL4 and VP16 moieties and using CREB1-GAL and Tax-VP16 constructs, but the magnitude of stimulation of gene expression was less than seen with Tax-GAL and CREB1-VP16 constructs (data not shown). For this reason, only Tax-GAL and CREB1-VP16 constructs were further tested. We also assayed whether other proteins containing bZIP domains such as CREB2 or Jun when fused to VP16 were capable of interacting with Tax-GAL. Cotransfection of these bZIP constructs with Tax-GAL and the 5x GAL reporter construct did not increase CAT activity (Fig. 5B, lanes 6 and 8).

It was important to determine whether the failure of the CREB2-VP16 and Jun-VP16 to interact with the Tax-GAL construct could be due to the failure to express these proteins. Also, we wished to assess whether CREB2-VP16 and Jun-VP16 were able to potentially interact with other leucine zipper proteins. As shown in Fig. 5C, we found that CREB2-VP16 was able to interact with the leucine zipper protein Fos by using a Fos-GAL construct (Fig. 5C, lane 2), while it was not able to interact with Tax-GAL (Fig. 5C, lane 3). Jun-VP16 was

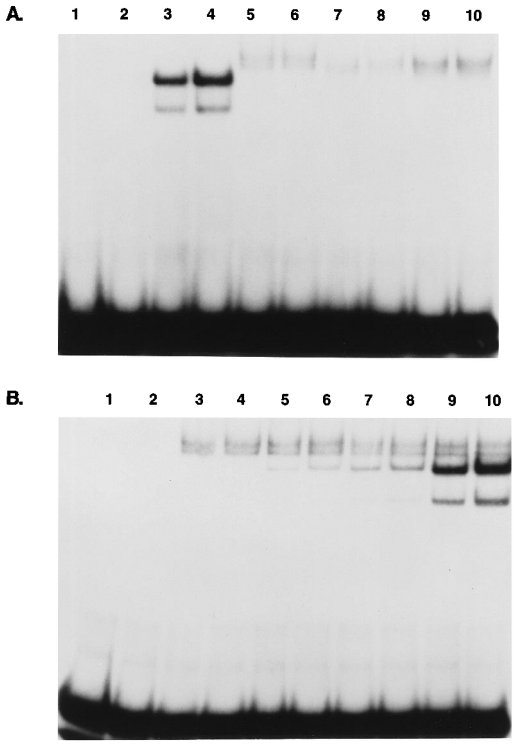

FIG. 4. Tax preferentially increases the binding of CREB1 but not CREB2 to the HTLV-I 21-bp repeats. Oligonucleotides corresponding to HTLV-I 21-bp repeat III were end labeled and used in gel retardation analysis with either CREB1 or CREB2 in separate reactions (A) or combined (B) in both the absence (lanes 1, 3, 5, 7, and 9) and the presence (lanes 2, 4, 6, 8, and 10) of 100 ng of Tax (A and B). (A) Gel retardation assay with HTLV-I 21-bp repeat III with no added CREB1 (lanes 1 and 2), 200 ng of CREB1 (lanes 3 and 4), 100 ng of GST-CREB2 (lanes 5 and 6), and either 100 ng (lanes 7 and 8) or 200 ng (lanes 9 and 10) of thrombin-cleaved GST-CREB2. (B) Gel retardation assay with 100 ng of HTLV-I 21-bp III with no added CREB (lanes 1 and 2), 200 ng of CREB2 alone (lanes 3 and 4), 200 ng of CREB2 and 40 ng of CREB1 (lanes 5 and 6), 200 ng of CREB2 and 80 ng of CREB2 (lanes 7 and 8), and 200 ng of both CREB2 and CREB1 (lanes 9 and 10).

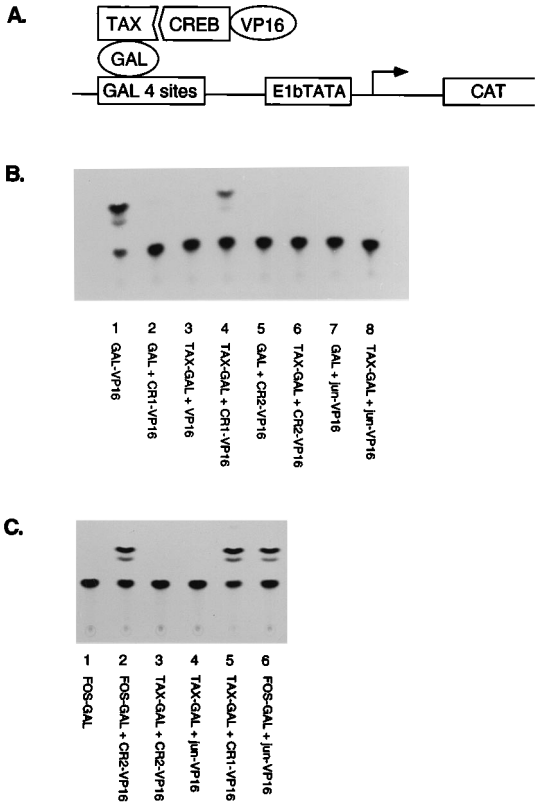

FIG. 5. Tax interacts with CREB1 in the mammalian two-hybrid system. (A) Schematic diagram of the mammalian two-hybrid system, including the 5x GAL-E1B TATA CAT reporter construct and the GAL-Tax and CREB-VP16 fusion proteins. (B) Percent CAT conversions for the 5x GAL reporter construct transfected into Jurkat cells with GAL-VP16 (74%) (lane 1), GAL and CREB1-VP16 (1.8%) (lane 2), Tax-GAL and VP16 (1.7%) (lane 3), Tax-GAL and CREB1-VP16 (18%) (lane 4), GAL and CREB2-VP16 (1.6%) (lane 5), Tax-GAL and CREB2-VP16 (1.4%) (lane 6), GAL and *jun*-VP16 (1.6%) (lane 7), or Tax-GAL and *jun*-VP16 (1.4%) (lane 8). (C) Percent CAT conversions for the 5x GAL reporter construct transfected into Jurkat cells for Fos-GAL (7.2%) (lane 1), Fos-GAL and CREB2-VP16 (45%) (lane 2), Tax-GAL and CREB2-VP16 (6.3%) (lane 3), Tax-GAL and *jun*-VP16 (6.5%) (lane 4), Tax-GAL and CREB1-VP16 (61%) (lane 5), and Fos-GAL and *jun*-VP16 (58%) (lane 6).

also unable to interact with Tax-GAL (Fig. 5C, lane 4), while it was able to strongly interact with Fos-GAL (Fig. 5C, lane 6). In this same experiment CREB1-VP16 strongly interacted with Tax-GAL (Fig. 5C, lane 5). Western blot analysis indicated the stable expression of these proteins following transfection (data not shown). Thus, the mammalian two-hybrid system demonstrated interactions between Tax and CREB1 but not between Tax and other bZIP proteins, including CREB2 and Jun.

The amino terminus of Tax is critical for interactions with CREB1. Extensive mutagenesis of the Tax protein has indicated that the multiple regions of this protein are required for transcriptional activation (60, 64). Two studies which have investigated the transactivation properties of GAL-Tax fusion proteins when bound to GAL4 binding sites have demonstrated that Tax mutants which are defective for transactivation in this assay were also defective for activation of the HTLV-I LTR, indicating the relevance of this system for studying Tax function (21, 25). To determine which regions of Tax were involved in interactions with CREB1, both deletion and oligonucleotide-directed mutagenesis of the Tax protein were performed. A schematic of the Tax mutants used in this study is shown in Fig. 6A. Three carboxy-terminal truncations of Tax, each of which was fused to the DNA binding domain of GAL4, were tested for their abilities to interact with CREB1-VP16 and increase gene expression from the 5x GAL reporter construct. Two of these carboxy-terminal truncations, TaxΔC1-GAL and TaxΔC2-GAL, which deleted either 16 or 36 amino acids, respectively, were each able to interact with CREB1-VP16 (Fig. 6B, lanes 5 and 7). However, a further carboxy-terminal deletion of Tax, TaxΔC3-GAL, which removed a total of 56 amino acids, was not able to interact with CREB1-VP16 (Fig. 6B, lane 9). Truncations of the amino terminus of Tax were also tested. Two amino-terminal truncations of Tax, TaxΔN1-GAL and TaxΔN2-GAL, which deleted either 93 or 136 amino acids, respectively, were both unable to interact with CREB1-VP16 and increase gene expression from the reporter construct (Fig. 6B, lanes 11 and 13).

In addition to amino- and carboxy-terminal truncations of Tax, we also tested the activity of three previously described site-directed mutants in Tax designated M1, M22, and M47, which were reported to have differential abilities to activate either HTLV-I or HIV-1 CAT constructs (64). The M1 mutant, which changed the histidine at amino acid 3 of Tax to serine, and the M47 mutant, which changed two leucine residues at 319 and 320 of Tax to arginine and serine, were reported to be very defective for HTLV-I activation but yet were active for HIV-1 activation (64). In contrast the M22 mutation,

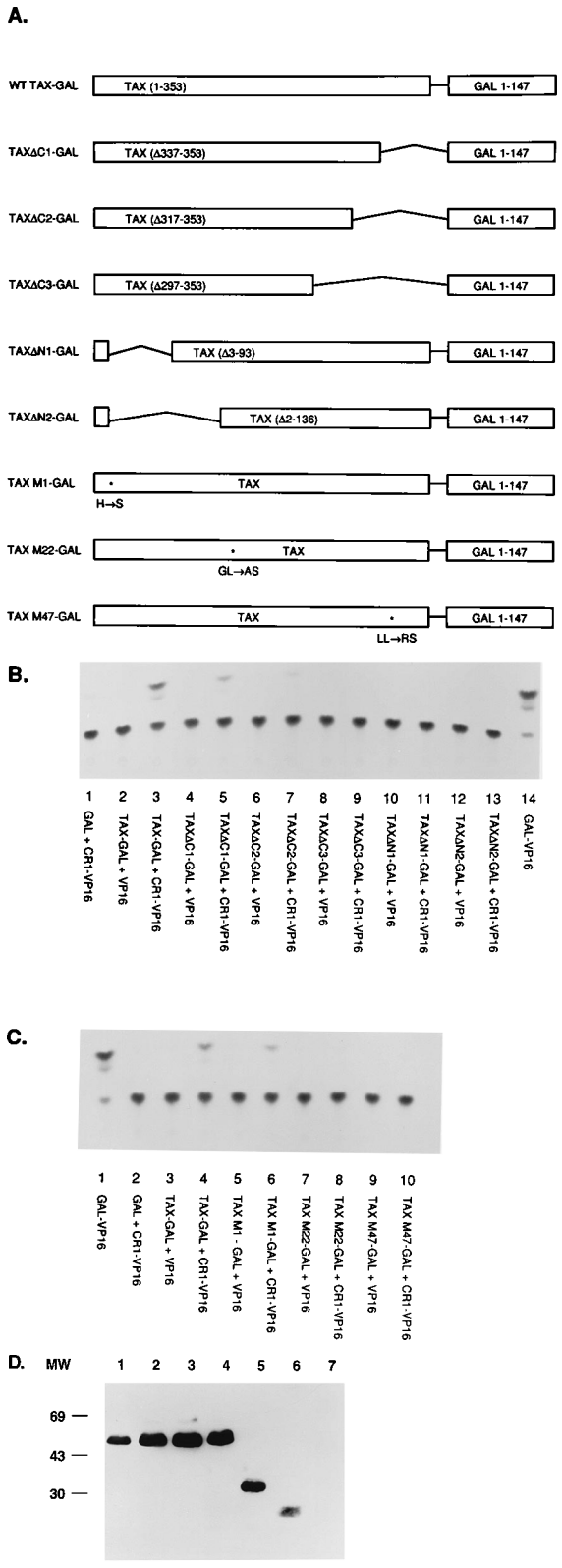

FIG. 6. Mutagenesis of Tax defines domains which interact with CREB1. (A) Schematic of Tax proteins fused to the DNA binding domain of GAL4, including three carboxy-terminal truncations (ΔC1, ΔC2, and ΔC3), two amino-terminal truncations (ΔN1 and ΔN2), and three site-directed mutants (M1, M22, and M47), are indicated. (B) Either wild-type or truncations of Tax fused to the DNA binding domain of GAL4 were transfected with CREB1 (CR1) proteins fused to VP16 and a 5x GAL4 reporter construct. The percent CAT conversion is shown for the 5x GAL reporter construct with GAL and CR1-VP16 alone (2.6%) (lane 1), Tax-GAL and VP16 (1.4%) (lane 2), Tax-GAL and CR1-VP16 (38%) (lane 3), Tax-GALΔC1 and VP16 (1.5%) (lane 4), Tax-GALΔC1 and CR1-VP16 (11%) (lane 5), TaxΔC2-GAL and VP16 (1.2%) (lane 6), TaxΔC2-GAL and CR1-VP16 (4.0%) (lane 7), TaxΔC3-GAL and VP16 (1.3%) (lane 8), TaxΔC3-GAL and CR1-VP16 (1.3%) (lane 9), TaxΔN1-GAL and VP16 (0.9%) (lane 10), TaxΔN1-GAL and CR1-VP16 (0.8%) (lane 11), TaxΔN2-GAL and VP16 (0.8%) (lane 12), TaxΔN2-GAL and CR1-VP16 (1.4%) (lane 13), and GAL-VP16 (89%) (lane 14). (C) Point mutants in Tax were assayed, and the percent CAT conversion is shown for the 5x GAL reporter with GAL-VP16 (83%) (lane 1), GAL and CR1-VP16 (1.5%) (lane 2), Tax-GAL and VP-16 (1.3%) (lane 3), Tax-GAL and CR1-VP16 (20%) (lane 4), Tax M1-GAL and VP16 (1.3%) (lane 5), Tax M1-GAL and CR1-VP16 (11%) (lane 6), Tax M22-GAL and VP16 (1.3%) (lane 7), Tax M22-GAL and CR1-VP16 (1.8%) (lane 8), Tax M47-GAL and VP16 (1.7%) (lane 9), and Tax M47-GAL and CR1-VP16 (1.7%) (lane 10). (D) A variety of the Tax-GAL constructs tested in two-hybrid analysis for panels B and C were cloned downstream of the T7 promoter in the expression vector pMT1 and transfected into HeLa cells and then infected with a recombinant virus which expresses T7 polymerase. Western blot analysis was performed on the cellular extracts with a monoclonal antibody directed against the C terminus of Tax for Tax-GAL (lane 1), Tax-M1GAL (lane 2), Tax-M22GAL (lane 3), Tax-M47 GAL (lane 4), Tax-ΔN1 GAL (lane 5), and Tax-ΔN2 GAL (lane 6). Lane 7, mock infection.

which changed glycine and leucine residues at amino acids 137 and 138 of Tax to alanine and serine, was reported to partially activate the HTLV-I LTR but not the HIV-1 LTR. Thus, it was suggested that these mutants might have altered specificities for activation of CREB and NF-κB binding sites (64). The Tax M1-GAL construct was able to interact, though at reduced levels, with CREB1-VP16 (Fig. 6C, lane 6), while the M22 and M47 mutants were found to be defective for interactions with CREB1-VP16 (Fig. 6C, lanes 8 and 10). All three of these mutants were found to be defective for activation of HTLV-I LTR CAT gene expression following transfection into Jurkat cells (data not shown). The reason for the differences in the phenotype of these Tax mutants on HTLV-I gene expression compared with that of a previous study (64) could not be determined, but altered phenotypes of several similar types of Tax mutants have been noted (60). However, the results of our mutagenesis of the Tax protein, in agreement with the results of other studies (60, 64), indicated that the carboxy terminus of Tax in contrast to the remainder of the protein was dispensable for activation of HTLV-I gene expression and interactions with CREB1.

With these different Tax constructs, it was critical to determine whether they were able to express equivalent levels of Tax protein. The Tax-GAL constructs were cloned downstream of the T7 promoter in the expression vector pTM1 (14). Following transfection of these constructs into HeLa cells, the cells were infected with a recombinant vaccinia virus which expressed T7 polymerase (14). Vaccinia virus expression constructs have previously been used to study the stability and function of mammalian transcription factors (39). Cellular extracts were prepared, and Western blot analysis was performed with a monoclonal antibody directed against the C-terminal portion of Tax (between amino acids 330 and 353). As shown in Fig. 6D, roughly equivalent amounts of wild-type Tax and mutants M1, M22, M47, and ΔN1 were produced (Fig. 6D, lanes 1 to 5). There was a somewhat reduced expression of the ΔN2 Tax mutant (Fig. 6D, lane 6). Since the Tax monoclonal antibody would not detect C-terminal truncations of Tax, we also placed the wild-type GAL-Tax and the ΔC3 mutant constructs into pTM1 and attached the 12-amino-acid influenza virus hemagglutinin epitope, which reacts with the commercially available monoclonal antibody 12CA5. The ΔC3 Tax protein was also expressed at levels equivalent to that of wild-type Tax (data not shown). These results indicated that the two

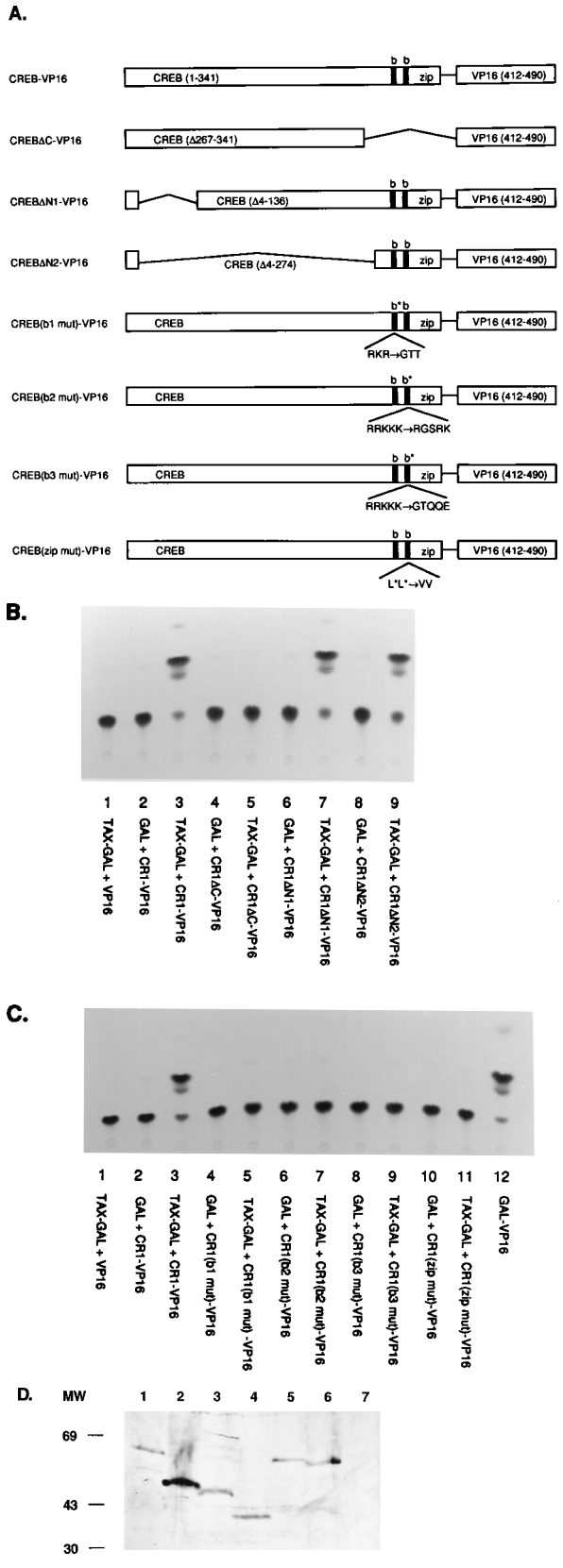

FIG. 7. Mutagenesis of CREB1 defines domains which interact with Tax. (A) Schematic of fusion proteins of CREB1 with the activation domain of VP16 including deletions of the CREB1 carboxy terminus, (CREBΔC), deletions of the amino terminus, (CREBΔN1 and CREBΔN2), and oligonucleotide-directed mutants of either the basic region including CREB (b1 mut), CREB (b2 mut), and CREB (b3 mut) or the leucine zipper, CREB (zip mut). (B) Truncations of CREB1 were assayed and the percent CAT conversion of the 5x GAL reporter construct is shown for transfections with Tax-GAL and VP16 (3.1%) (lane 1), GAL and CR1-VP16 (2.2%) (lane 2), Tax-GAL and CR1-VP16 (88%) (lane 3), GAL and CR1ΔC-VP16 (1.7%) (lane 4), Tax-GAL and CR1ΔC-VP16 (1.3%) (lanes 5), GAL and CR1ΔN1-VP16 (2.4%) (lane 6), Tax-GAL and CR1ΔN1-VP16 (84%) (lane 7), GAL and CR1ΔN2-VP16 (2.7%) (lane 8), and Tax-GAL and CR1ΔN2-VP16 (93%) (lane 9). (C) Site-directed mutants of the bZIP domain of CREB were assayed and the percent CAT conversion is shown for Tax-GAL and VP16 (1.5%) (lane 1), GAL and CR1-VP16 (2.2%) (lane 2), Tax-GAL and CR1-VP16 (83%) (lanes 3), GAL and CR1 (b1 mut)-VP16 (1.3%) (lane 4), Tax-GAL and CR1 (b1 mut)-VP16 (1.5%) (lane 5), GAL and CR1 (b2 mut)-VP16 (1.1%) (lane 6), Tax-GAL and CR1 (b2 mut)-VP16 (1.5%) (lane 7), GAL and CR1 (b3 mut)-VP16 (6%) (lane 8), Tax-GAL and CR1 (b3 mut)-VP16 (1.1%) (lane 9), GAL and CR1 (zip mut)-VP16 (1.2%) (lane 10), Tax-GAL and CR1 (zip mut)-VP16 (2.0%) (lane 11), and GAL-VP16 (92%) (lane 12). (D) A variety of CREB1-VP16 constructs tested in two-hybrid analysis for panels B and C were transfected into 293 cells, and the cellular extract obtained was used in Western blot analysis with rabbit polyclonal antibody directed against CREB1 for CR1-VP16 (lane 1), CR1ΔC-VP16 (lane 2), CR1ΔN1-VP16 (lane 3), CR1ΔN2-VP16 (lane 4), CR1 (zip mut)-VP16 (lane 5), and CR(b2 mut)-VP16 (lane 6). Lane 7, mock infection.

critical Tax deletion mutants used in this study, ΔN1 and ΔC3, in addition to the Tax point mutants were expressed at levels equivalent to that of wild-type Tax.

The bZIP region of CREB1 is critical for interaction with Tax. The activity of the 43-kDa CREB protein is mediated by protein kinase A phosphorylation of a serine residue at amino acid 133 (28, 29, 50, 74). CREB1 also contains a leucine zipper domain near its carboxy terminus, with four leucine residues spaced seven residues apart preceded by a region of basic amino acids (28, 37). The leucine zipper functions in CREB1 dimerization, which has been demonstrated to be critical for its DNA binding, while the basic residues are required for DNA binding but not dimerization (13). We wished to map the domains in CREB1 which were required for interactions with Tax by testing a variety of amino- and carboxy-terminal truncations in addition to several site-directed mutants (Fig. 7A). Each of these CREB1 mutants was fused to the activation domain of VP16 and then tested for interactions with Tax by the mammalian two-hybrid system. Two amino-terminal truncations of CREB1, CREBΔN1-VP16 and CREBΔN2-VP16, which deleted either 136 or 274 amino acids, respectively, were each able to directly interact with Tax-GAL in the two-hybrid system (Fig. 7B, lanes 7 and 9). Thus, the amino terminus of CREB1, including the site for phosphorylation by protein kinase A, was not critical for interactions with Tax. A carboxy-terminal truncation of CREB1, CREBΔC-VP16, which deleted between amino acids 267 and 341, did not interact with Tax (Fig. 7B, lane 5). Since this mutant removed the basic and leucine zipper regions of CREB1, it suggests that the basic and leucine zipper regions of CREB1 were likely targets for interaction with Tax.

To better define the interactions of CREB1 with Tax, site-directed mutations were constructed in either of two basic amino acid regions in CREB1 or the adjacent leucine zipper region (28, 33, 37). Each of these CREB1 mutants was then fused to the VP16 activation domain and tested for its ability to interact with Tax-GAL. A CREB1 basic region mutant, CREB (b1 mut), which changed amino acids 284 to 286, failed to interact with Tax-GAL (Fig. 7C, lane 5). Likewise, two different sets of mutations in another portion of the basic domain of CREB1 between amino acids 301 and 305, CREB (b2 mut) and CREB(b3 mut), were also defective in their abilities to interact with Tax (Fig. 7C, lanes 7 and 9). Finally, we tested whether mutation of two of the four leucine residues in the

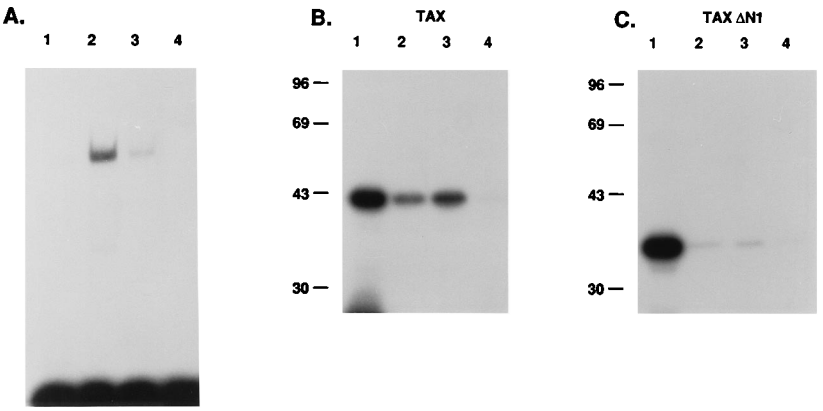

FIG. 8. N-terminal truncations of Tax do not interact with CREB1. (A) Oligonucleotides corresponding to 21-bp repeat I (lane 1) were end labeled and used in gel retardation with 50 ng of GST-CREB and 100 ng of Tax-6x His (lane 2), 50 ng of GST-CREB and 100 ng of TaxΔN1-6x His (lane 3), or 100 ng of Tax-6x His alone (lane 4). Either wild-type Tax-6x His (B) or TaxΔN1-6x His (C) labeled with ^{32}P was subjected to PAGE either alone (lane 1) or following incubation with agarose beads containing GST-CREB (lane 2), GST-CREBΔN1 (Δ4-136) (lane 3), or GST-CREBΔC (lane 4). The positions of the molecular weight markers (in thousands) are shown.

leucine zipper of CREB1, CREB (zip mut), resulted in a protein with altered interactions for Tax. This mutant did not interact with Tax-GAL (Fig. 7C, lane 11). Thus, mutation of either the basic domain or the leucine zipper of CREB1 prevented its ability to interact with Tax.

With these different CREB VP-16 expression constructs, it was critical to determine whether any of the CREB1 mutations altered the stability of the proteins following transfection. A variety of the CREB1-VP16 constructs used for Fig. 7B and C were transfected into 293 cells, which have a very high transfection efficiency, and Western blot analysis was performed with CREB1 antibody. Two-hybrid analysis with Tax-GAL was performed on a portion of each of these samples, and the results corresponded with those obtained for Fig. 6 and 7 with Jurkat cells (data not shown). Roughly equivalent levels of protein were detected with the wild-type CREB1-VP16 construct (Fig. 7D, lane 1) and a variety of N-terminal deletion mutants (Fig. 7D, lanes 3 and 4) as well as CREB-VP16 constructs with substitutions of leucine zippers 1 and 2 (zip mut) (Fig. 7D, lane 5) and a portion of the CREB1 basic domain (b2mut) (Fig. 7D, lane 6). The only mutant that expressed to levels different from those of wild-type CREB1-VP16 constructs was the CREBΔC-VP16 construct (Fig. 7D, lane 2), whose expression level was higher than that of the wild-type construct. This mutant failed to interact with Tax-GAL (Fig. 7B). These experiments indicated that the CREB-VP16 constructs expressed similar levels of protein when transfected into cells.

Wild-type but not mutant Tax proteins directly interact with CREB1 in vitro. Since the results of the two-hybrid analysis with Tax and CREB1 expression constructs suggested that these proteins were capable of direct interactions, we attempted to correlate these results by in vitro interaction studies. First, we tested whether an amino-terminal deletion mutant of Tax, TaxΔN1, that was unable to interact with CREB1 in two-hybrid analysis could stimulate the binding of CREB1 to the HTLV-I 21-bp repeat. By gel retardation analysis, the wild-type Tax protein stimulated the binding of CREB1 to the 21-bp repeat (Fig. 8A, lane 2), while the TaxΔN1 mutant did not stimulate CREB1 binding (Fig. 8A, lane 3). Thus, a Tax mutant which did not interact with CREB1 in two-hybrid analysis also did not stimulate CREB1 binding in vitro.

Next, we wished to address whether either wild-type or mutant Tax proteins could directly interact with CREB1, even though a gel-retarded species consistent with a complex composed of both Tax and CREB1 was not detected. ^{32}P-labeled Tax protein was incubated with either GST-CREB1 or GST fusions with CREB1 mutants which deleted either its amino terminus (CREBΔN1) or its carboxy terminus (CREBΔC). Tax bound strongly to GST-CREB1 (Fig. 8B, lane 2) and equally well to the GST-CREBΔN1 protein which contained a deletion between amino acids 4 and 136 (Fig. 8B, lane 3). However, Tax was unable to bind to the GST-CREBΔC mutant protein which deleted between amino acids 267 and 341, including both the basic and the leucine zipper domains (Fig. 8B, lane 4). In contrast to the results with the wild-type Tax protein, the amino-terminal truncated and ^{32}P-labeled TaxΔN1 protein was unable to bind to GST fusions with either wild-type CREB1 or amino- and carboxy-terminal truncations of CREB1 (Fig. 8C, lanes 2 to 4). These results indicate that Tax and CREB1 specifically interacted, using both in vitro and in vivo assays. Furthermore, the bZIP protein of CREB1 and the majority of Tax excluding its carboxy terminus were critical for this interaction.

DISCUSSION

The mechanism by which the HTLV-I transactivator Tax is able to activate viral and cellular gene expression is not fully known. Tax is unable to bind directly to DNA (3, 25, 27); thus, its mode of action likely involves either direct or indirect interactions with cellular transcription factors (48). Tax is able to activate the HTLV-I LTR through three regulatory elements known as 21-bp repeats each of which contains nonpalindromic CREs (6, 23, 54, 61). In addition, Tax has been demonstrated to activate gene expression from both the HIV-I LTR (5, 60, 64, 66) and the IL-2 receptor α (5, 10, 43, 60) through NF-κB binding sites and the c-*fos* promoter (19–21) through the serum response element binding factor p67^{srf}. These results indicate that Tax is able to modulate the binding or transcriptional activating properties of multiple cellular transcription factors to facilitate increases in gene expression.

A number of studies have indicated that Tax can directly interact with specific cellular transcription factors that bind to promoter elements that are activated by Tax. Cellular extracts containing Tax have been demonstrated to contain increased NF-κB binding activity in gel retardation analysis (43, 44). In fact, immunoprecipitation experiments indicate that Tax and

both the p105 and the p100 forms of NF-κB form a stable intracellular complex (5, 35, 36). Mutagenesis of one of the cytoplasmic inhibitors of NF-κB nuclear localization, IκB γ, which contains the C-terminal portion of p105, indicated that the ankyrin repeats of this protein were the site of interaction with Tax (36). Thus, one mode of Tax activation of promoters containing NF-κB binding sites may be direct interaction with IκB γ or other members of the IκB family, resulting in the translocation of the p65 form of NF-κB from the cytoplasm to the nucleus, with resultant activation of gene expression.

In addition to effects on the cellular localization of NF-κB, Tax has been shown to activate gene expression from the c-*fos* promoter through the serum response regulatory element (18, 19). The serum response regulatory element contains a region of dyad symmetry with a CArG box which binds a factor designated $p67^{srf}$ (20). Normally, $p67^{srf}$ can form a complex with a mitogen-inducible factor, $p62^{tcf}$, which enhances $p67^{srf}$ binding. Tax has been demonstrated to directly bind to $p67^{srf}$ in vitro and directly interact with $p67^{srf}$ in the mammalian two-hybrid system (20). Mutagenesis experiments with $p67^{srf}$ indicate that the carboxy-terminal half of $p67^{srf}$, which lacks domains for DNA binding, dimerization, and ternary complex formation with $p62^{tcf}$, was sufficient for interaction with Tax.

Tax has also been demonstrated to stimulate the binding of a variety of DNA-binding proteins containing leucine zippers, including members of the ATF/CREB family to the HTLV-I 21-bp repeats (2, 3, 68, 72, 75–77). However, since the activation properties of Tax for both viral and cellular promoters is restricted, the functional significance of these findings remains to be determined. In this study, we wished to address questions concerning the mechanism by which Tax activates gene expression of the HTLV-I LTR. Though a nonpalindromic CRE is present within each 21-bp repeat, questions have remained about the role of CREB in *tax* activation of the HTLV-I LTR. It is not clear how the elements flanking the CRE in the 21-bp contribute to Tax induction, though a recent study suggests that these elements specify an extended binding site for a protein complex comprising both CREB and Tax (53). However, both our in vivo and our in vitro analyses suggest that the extended binding site is not essential for Tax and CREB interaction. Also, if CREB is important for activation of HTLV-I, why is Tax unable to activate other promoters containing CRE sites? Does Tax stably interact with one or a variety of members of the ATF/CREB family? What domains in the Tax and CREB proteins are required for their interaction, and is this interaction critical for Tax activation of HTLV-I gene expression?

We found that Tax stimulated the DNA binding of CREB1 but not CREB2 to the HTLV-I 21-bp repeats, indicating specificity for Tax function. This is in contrast to several other studies which indicated that Tax could stimulate the binding of a variety of other leucine zipper DNA-binding proteins (2, 72). Furthermore, though Tax stimulated the binding of CREB1 to the 21-bp repeat, it did not stimulate the binding of CREB1 to the somatostatin CRE at similar protein concentrations. This may be due to the fact that the HTLV-I 21-bp repeats contain a nonconsensus CRE in contrast to the consensus CRE found in the somatostatin CRE or to the effects of the GC-rich sequences found in the A and C domains of the 21-bp repeats which may be critical for Tax function. CREB1 bound with a higher affinity to the consensus compared with a nonconsensus CRE site. Thus, the restriction of *Tax* activation of promoters containing consensus CRE sites may in part be determined by the endogenous cellular concentration of CREB1 protein. CREB1 may be present in concentrations which allow stimulation of its binding to the 21-bp repeat by Tax but yet be present in high enough quantities that do not allow Tax stimulation of CREB1 binding to other promoters with CRE sites (24).

We demonstrated, with in vitro binding assays, that Tax was able to associate directly with CREB1. Several mutants that have previously been demonstrated to inactivate the transcriptional activating properties of either Tax or CREB1 in transfection assays were unable to interact in vitro, suggesting a functionally significant interaction (60, 64). The relevance of these in vitro interactions between CREB1 and Tax were extended with the mammalian two-hybrid system. The specificity of these interactions was tested by demonstrating that Tax could directly interact with CREB1 but not CREB2 or a variety of other bZIP proteins such as AP-4, Jun, and Fos. Consistent with these findings, it was demonstrated that the adenovirus E1A protein, but not Tax, activated gene expression from a reporter construct which bound a CRE-BP1 or CREB2 fusion protein (46). Thus, our studies correlated both in vitro and in vivo studies and demonstrated that Tax specifically interacted with CREB1.

Deletion and oligonucleotide-directed mutants of Tax and CREB1 were used to define domains involved in interaction between these proteins, by two-hybrid analysis. Tax proteins containing deletions of the carboxy terminus were able to interact with CREB1, while Tax proteins containing amino-terminal deletions were unable to interact with CREB1. Three previously characterized point mutations in Tax, designated M1, M22, and M47, which were grouped according to their differential activation phenotype for either CREB binding sites (M1 and M47) and NF-κB binding sites (M22), were also tested for their abilities to interact with CREB1 (64). We found that the M1 Tax mutant was able to interact with CREB1, though to a lesser degree than wild-type Tax. Both the M22 and M47 Tax mutants were unable to interact with CREB. Transfection of M1, M22, or M47 Tax mutants along with an HTLV-I CAT reporter construct indicated that each of these mutants was very defective for activation of HTLV-I gene expression. The failure of the M1 mutant to transactivate the HTLV-I LTR, yet partially interact with CREB1 in two-hybrid analysis when fused to GAL4, may be explained by potential effects of this mutation on interrupting Tax nuclear localization (64, 65), which may be compensated by the GAL4 nuclear localization signal, or perhaps disrupting a domain in Tax required for either transcriptional activation or interaction with other transcription factors. A previously characterized mutant, d320, was also found to directly interact with CREB in gel retardation analysis, yet it failed to activate HTLV-I LTR gene expression in transfection experiments (68). Both point mutants and deletion mutants of Tax indicated that the majority of the Tax protein excluding its carboxy terminus was critical for both transactivation of HTLV-I LTR and interaction with CREB1. Similar results have been obtained using both wild-type and mutated GAL-Tax (21, 25) and Tax (60, 64) constructs.

Mutagenesis was also performed to determine the region of CREB1 that was responsible for interaction with Tax in two-hybrid analysis. The effects seen with different Tax and CREB1 proteins in this assay were not due to differences in protein stability as determined by Western blot analysis. Amino-terminal truncations of CREB1 that deleted domains that have been demonstrated to be important for transcriptional activation were still able to interact with Tax. However, carboxy-terminal deletions of the CREB1 protein were unable to interact with Tax. This suggested that the bZIP region of CREB1 was involved in interactions with Tax. Oligonucleotide-directed mutation of two portions of the basic region or the leucine zipper

region of CREB1 eliminated interactions with Tax. A recent study indicated that three residues (282 to 284) in the basic domain of CREB1 were sufficient to confer recognition specificity by Tax (1). A similar mutant, CREB (b1 mut), in this study was defective for interactions with Tax in the two-hybrid analysis. However, other basic and leucine zipper mutants of CREB1 were also defective for interactions with Tax. The failure to detect these other regions was likely due to the fact that fusions of CREB and ATF1 were used in the previous study and these proteins have a very high degree of homology in other portions of their bZIP domains (1).

The ability to perform in vivo analysis of CREB1 and Tax interactions rather than gel retardation assays exclusively allows one to more clearly elucidate potential interactions. For instance, mutations of the CREB1 leucine zipper will inhibit its dimerization and subsequent DNA binding properties (13). Likewise, mutations of the CREB1 basic domain will inhibit its DNA binding properties (13). Thus, it becomes difficult to determine the domains required for CREB1 interaction with Tax by gel retardation analysis if in fact these same domains are required for CREB1 binding to DNA. The two-hybrid assay used in this study offers many advantages to study interactions between CREB1 and Tax. In summary, our in vivo and in vitro results indicate that in the absence of CREB1 binding to its cognate DNA site, both its basic and its leucine zipper regions were critical for interactions with Tax.

The direct interaction between the CREB1 and Tax proteins demonstrated by both in vivo and in vitro studies could potentially result in Tax-mediated increases in CREB1 dimerization and subsequent increases in CREB1 binding to the 21-bp repeats. The failure in our study to identify complexes composed of both Tax and CREB1 by gel retardation analysis (1, 68, 76, 77) has been noted in several other studies (2, 17, 72). This may be due to the unstable nature of the CREB1 and Tax complex with dissociation during PAGE. Tax could potentially interact with two CREB proteins via their basic domains or leucine zipper. In this latter model, Tax may be in direct contact with the CRE binding site in conjunction with a CREB dimer. An alternative but less likely model would be that a heterodimer composed of a CREB1 monomer and Tax could also potentially bind to the CRE. Finally, a transient interaction between CREB1 and Tax which either induces CREB1 dimerization and/or directly increases the rate of CREB DNA binding is possible. Both structural and mutagenesis studies of the CREB1 and Tax proteins are under way in an attempt to address these possibilities.

The ability of Tax to modulate CREB1 binding is likely a key feature in the activation of HTLV-I gene expression. However, it is likely that Tax interactions with either TFIID or general transcriptional factors will also be important for activation of HTLV-I gene expression (8). Thus, Tax may have several cellular targets which are involved in the activation of HTLV-I gene expression and serve as an adapter molecule which may serve as a bridge between the CREB1 and the TFIID complex. Tax, like the adenovirus E1A protein which binds to ATF-2, can interact with a member of the ATF/CREB family (45). Other HTLV-I binding proteins such as Ets and cellular factors binding to the A and C motifs flanking the CRE in the 21-bp repeats may also be important for Tax activation (58). However, an understanding of the mechanism by which Tax mediates the interactions and binding properties of CREB to the HTLV-I 21-bp repeats will provide important insights into the properties of this interesting viral transactivator protein. Our study demonstrates that CREB1 and Tax interact in vivo and provide a convenient means to carefully elucidate the protein domains involved in the interaction between an important viral transactivator and a critical cellular transcription factor.

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