MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 5156–5164 Vol. 17, No. 9
0270-7306/97/$04.00+0

Anchoring of CREB Binding Protein to the Human T-Cell Leukemia Virus Type 1 Promoter: a Molecular Mechanism of Tax Transactivation

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Received 11 February 1997/Returned for modification 4 March 1997/Accepted 21 June 1997

The human T-cell leukemia virus type 1 (HTLV-1)-encoded Tax protein activates viral transcription through interaction with the cellular transcription factor CREB (cyclic AMP response element [CRE] binding protein). Although Tax stabilizes the binding of CREB to the Tax-responsive viral CREs in the HTLV-1 promoter, the precise molecular mechanism by which Tax mediates strong transcriptional activation through CREB remains unclear. In this report, we show that Tax promotes high-affinity binding of the KIX domain of CREB binding protein (CBP) to CREB-viral CRE complexes, increasing the stability of KIX in these nucleoprotein complexes by up to 4.4 kcal/mol. Comparable KIX binding affinities were measured for both phosphorylated and unphosphorylated forms of CREB, and in all cases high-affinity binding was dependent upon both Tax and the viral CRE. Tax also promoted association of KIX to a truncated form of CREB containing only the 73-amino-acid basic leucine zipper (bZIP) domain, indicating that the entire amino-terminal CBP-interacting domain of CREB is nonessential in the presence of Tax. Functional studies upheld the binding studies, as expression of the bZIP domain of CREB was sufficient to support Tax transactivation of HTLV-1 transcription in vivo. Finally, we show that transfection of a KIX expression plasmid, which lacks activation properties, inhibited Tax transactivation in vivo. This suggests that KIX occupies the CBP binding site on Tax, and therefore CBP is likely a cofactor in mediating Tax stimulation of HTLV-1 transcription. Together, these data support a model in which Tax anchors CBP to the HTLV-1 promoter, with strong transcriptional activation resulting from the CBP-associated activities of nucleosome remodeling and recruitment of the general transcription machinery.

Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus responsible for an aggressive and fatal malignancy called adult T-cell leukemia (for a review, see reference 16). The viral genome encodes a unique oncoprotein, called Tax, which is a key regulatory protein that appears to facilitate the transition from viral latency to high levels of virion production in the infected T cell. Tax mediates the emergence from latency via strong transcriptional activation of the HTLV-1 genome. The precise molecular mechanism by which Tax activates viral transcription has been widely studied but is not fully understood. Tax does not bind DNA directly (3, 8, 19, 32) but interacts with host cell proteins to stimulate viral transcription through three 21-bp repeat sequences in the transcriptional control region of the virus (10, 17, 23, 31, 36, 38, 39). The three 21-bp repeats each contain an off-consensus core octanucleotide sequence with similarity to the cyclic AMP (cAMP) response element (CRE). A short run of GC-rich nucleotides immediately flanks the core CRE sequences within each of the 21-bp repeats. Together, the CRE and GC-rich flanks form a critical DNA element (called the viral CRE) that is obligatory for Tax transactivation in vivo (11, 18, 23, 29, 30). These viral CREs have been shown to serve as binding sites for several members of the basic leucine zipper (bZIP) family of cellular transcription factors. Specifically, the CRE binding protein (CREB) appears to have the most prominent role in mediating Tax transcriptional activation through the viral CREs in the HTLV-1 promoter (1, 2, 9, 11, 15, 27, 45–49). Recent studies have shown that in the absence of Tax, the interaction between CREB and the viral CRE is highly unstable, resulting in rapid dissociation of CREB from the viral promoter. In the presence of Tax, the dissociation rate of CREB from the viral CRE is decreased and the equilibrium binding affinity is increased (7, 11, 44, 45). Several lines of evidence indicate that Tax interacts primarily, although not exclusively, with the bZIP region of CREB to stabilize CREB binding to the weak viral CRE sequences (2, 7, 11, 15, 46). This binding stabilization by Tax appears to be accomplished through both an increase in CREB dimerization and stabilization of the helical structure of CREB's bZIP domain (7, 37, 42). Together, these studies support a model in which Tax transactivates HTLV-1 gene expression by increasing the number of CREB molecules bound to the viral promoter, leading to transcriptional activation of the virus and enhanced virion production. Unfortunately, since the transcriptional activity of CREB is largely dependent upon phosphorylation by cAMP-dependent protein kinase A (PKA), a model proposing that Tax simply serves to stabilize CREB binding to the viral promoter incompletely explains the strong HTLV-1 transcriptional activation observed in the presence of Tax.

Recently, several studies have shown that PKA phosphorylation of CREB at serine 133 promotes the recruitment of the coactivator, CREB binding protein (CBP), leading to transcriptional stimulation of cAMP-responsive genes (4, 12, 25). A 224-amino-acid (aa) subdomain of CBP (aa 455 to 679), called the KIX domain, is sufficient for recognition of the phosphorylated form of CREB, and the interaction between KIX and serine 133-phosphorylated CREB serves to tether CBP to the promoter (35). Once anchored through interaction with CREB, CBP appears to facilitate transcriptional activation through chromatin remodeling and contact with the general transcription machinery (6, 25, 33, 43). The identification

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of CBP, together with well-established evidence indicating that Tax strongly activates HTLV-1 transcription in the absence of CREB phosphorylation, provides the foundation for a revised model of Tax transactivation in which Tax both stabilizes CREB binding to the DNA and facilitates recruitment of CBP to the viral promoter. In support of this model, a recent study (24) has demonstrated that the KIX domain of CBP forms a protein-protein complex with Tax, promoting the association of KIX to CREB bound to the viral CRE.

To further characterize the mechanism of Tax transactivation through CREB and CBP, we have utilized equilibrium binding studies and functional assays to investigate the interactions between these molecules. In this report, we demonstrate that Tax can recruit the KIX domain of CBP to DNA-bound CREB complexes with high affinity. Additionally, we show that the GC-rich flanking sequences adjacent to the viral CREs are critical for Tax recruitment of KIX and contribute >2 kcal/mol to the overall stability of the complex. The recruitment of KIX did not stabilize the Tax-CREB-DNA complex, suggesting that the association of CBP with the viral promoter is responsible, at least in part, for transcriptional activation. Tax recruits the KIX domain to both the unphosphorylated and phosphorylated forms of CREB and to the related bZIP protein ATF-1. A truncated form of CREB that contains only the 73-aa bZIP domain (CREB BR, aa 254 to 327) supported KIX recruitment in the presence of Tax in vitro and, consistent with a previous study (26), supported Tax transactivation in vivo. Finally, cotransfection of an expression plasmid for just the KIX domain of CBP inhibited Tax activation of viral CRE-dependent transcription. This suggests that KIX can act as a competitive inhibitor of Tax transactivation by occupying the CBP binding site on Tax.

Together, these data provide the framework for a model in which Tax functions to form a ternary complex with CREB bound to the CREs in the viral promoter. Concomitantly, Tax serves as a high-affinity bridging molecule that directly recruits CBP to the viral promoter. The stable association of CBP with the viral promoter occurs in the absence of CREB phosphorylation and results in HTLV-1 transcriptional activation achieved through the CBP-associated activities of nucleosome remodeling and recruitment of the general transcription machinery.

MATERIALS AND METHODS

GST-KIX cloning and purification. The GST-KIX fusion construct was created by inserting the *Eco*RI fragment (nucleotides 1351 to 2159, containing KIX domain aa 451 to 720) derived from the CBP expression plasmid pRC/RSV-CBP (kindly provided by Richard Goodman, Vollum Institute, Oregon Health Sciences University [28]) into the *Eco*RI site of the glutathione *S*-transferase (GST)-containing plasmid pGEX-2T (Pharmacia). pGST-KIX was transformed into *Escherichia coli* XL1Blue, and individual colonies were expanded in broth containing ampicillin at 50 μg/ml and tetracycline at 20 μg/ml and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and sonicated in phosphate-buffered saline with 1 mM phenylmethylsulfonyl fluoride, aprotinin at 5 μg/ml, leupeptin at 5 μg/ml, and 1 mM dithiothreitol (DTT). Following sonication, Tween 20 was added to 1% (vol/vol) and the lysate was centrifuged at 9,500 rpm for 10 min in a Beckman JA20 rotor. The supernatant was added to glutathione-agarose beads (Sigma) and incubated at 4°C for 40 min with gentle agitation. The column was extensively washed, and bound protein was eluted with 50 mM Tris (pH 8.0)–5 mM reduced glutathione–1 mM DTT. The eluted protein was dialyzed overnight at 4°C against Superdex buffer (25 mM HEPES [pH 7.9], 12.5 mM $MgCl_2$, 10 μM $ZnSO_4$, 150 mM KCl, 20% [vol/vol] glycerol, 0.1% [vol/vol] Tween-20, 4 mM β-mercaptoethanol) for use in electrophoretic mobility shift assays (EMSA). The protein was aliquoted and stored at −70°C. The concentration of the purified protein was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). GST-KIX was purified to >90% homogeneity by this procedure.

Expression and purification of DNA binding proteins and Tax. The bacterial expression plasmids for CREB (15), the CREB binding region (bZIP aa 254 to 327), ATF-1, and the ATF-2 binding region (22) were transformed into *E. coli* BL21(pLysS) (40), and the proteins were expressed and purified as previously described (22). The proteins were additionally subjected to heparin-agarose chromatography and dialyzed against TM–0.1 M KCl (50 mM Tris [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA [pH 8.0], 20% [vol/vol] glycerol, 0.025% [vol/vol] Tween 20, 1 mM DTT) (14) for use in transcription assays or Superdex for use in EMSA. The concentration and purity of each protein were estimated by SDS-PAGE. Each protein was purified to >90% homogeneity by this procedure, and the specificity of DNA binding was shown by competition assays (data not shown; see references 5 and 15). CREB was phosphorylated by using the catalytic subunit of PKA by incubating 1.6 μM CREB in a reaction mixture containing 3.3 μM ATP (or UTP for mock reactions), 5 mM $MgCl_2$, and 55 U of PKA (Sigma) in 25 mM potassium phosphate buffer, pH 6.6. Successful phosphorylation was monitored by EMSA, as the phosphorylated protein migrates with reduced mobility in the native gel. The HTLV-1 Tax protein was expressed in *E. coli* XL1Blue from the $pTaxH_6$ expression plasmid (48) and purified by 1 M ammonium sulfate precipitation, followed by nickel chelate chromatography using a 0 to 400 mM imidazole gradient. Eluted protein was dialyzed against Superdex for use in EMSA. The concentration of Tax was estimated by SDS-PAGE. Tax was purified to >90% homogeneity by this procedure.

EMSA. EMSA were performed by incubation of the indicated amount of purified DNA binding protein, Tax, or GST-KIX in 0.5× Superdex buffer with 0.2 nM ^{32}P-end-labeled double-stranded DNA probes and poly(dA) · poly(dT) at 250 ng/ml in a 20-μl sample. Binding reaction mixtures were incubated on ice for 30 min and analyzed on 5% nondenaturing polyacrylamide gels (49:1 [wt/wt] acrylamide–*N*,*N*′-methylenebisacrylamide) in buffer containing 0.04 M Tris · HCl, 0.306 M glycine (pH 8.5), and 0.1% (vol/vol) Nonidet P-40. Gels were dried and visualized by PhosphorImager analysis, processed by Adobe Photoshop, and annotated and printed from Claris Draw.

Identification of the polypeptides in the quaternary complex was performed by sequential EMSA and immunoblot analysis. One picomole of unlabeled viral CRE, cellular CRE, or nonspecific DNA (paired homeodomain binding site) fragments 80 bp in length was incubated with 300 ng of CREB, 90 ng of Tax, 80 ng of GST-KIX, and 500 ng of poly(dA) · poly(dT) under conditions described above for EMSA binding reactions. To identify the location of the quaternary complex, binding reaction mixtures containing 2.5 ng of CREB, 150 ng of Tax, 240 ng of GST-KIX (as indicated), 10 ng of poly(dA) · poly(dT), and 4 fmol of the ^{32}P-end labeled viral CRE fragment were run in lanes adjacent to the test reaction mixtures containing the unlabeled DNAs. The protein-DNA complexes were resolved by electrophoresis on a 5% native polyacrylamide gel and autoradiographed for 1 h to identify the location of the quaternary complex. Gel slices corresponding to the quaternary complex (see Fig. 1B) were excised and incubated in SDS sample buffer at 65°C for 15 min, electrophoresed on an SDS–12% polyacrylamide gel, and transferred to nitrocellulose for analysis in the second dimension by immunoblotting. The nitrocellulose was incubated with an antiserum cocktail containing appropriate dilutions of anti-CREB, anti-Tax, and anti-KIX antibodies, followed by reaction with ^{125}I-labeled protein A. The membrane was washed, and antibody binding was detected by PhosphorImager analysis. The antibodies used in this assay are specific and do not cross-react with other proteins (data not shown).

Equilibrium binding studies were performed to estimate the apparent dissociation constant of GST-KIX for the various protein-DNA complexes. Binding reaction mixtures were incubated for 1 h on ice until equilibrium was reached (empirically determined; data not shown) and then subjected to nondenaturing PAGE and PhosphorImager analysis as described above. To calculate the apparent binding affinity, we used the following equilibrium binding equation: K_D = [KIX] [TF-Tax-DNA complex]/[KIX-TF-Tax-DNA complex], where TF is any of the transcription factors that were tested. Tax is included in the binding equation, although its presence is not always discernible. When 50% of the GST-KIX complex (quaternary complex) is formed, the equation is simplified, as under this condition the concentrations of the ternary and quaternary complexes are equal and therefore the apparent K_D is equal to the concentration of free GST-KIX in the binding reaction mixture. The concentration of the free GST-KIX domain is approximately equal to the total concentration of GST-KIX, as the amount of bound GST-KIX is small (less than 2% of the total). The total concentration of GST-KIX at which 50% of the quaternary complex formed was quantitatively determined by plotting the fraction of GST-KIX-containing complexes formed versus the total concentration of GST-KIX protein in each reaction mixture. The free-energy values were calculated from the apparent affinities by using the equation $\Delta G = -RT\ln(1/K_D)$, where R = 0.00198 kcal · mol^{-1} · K^{-1} and T = 277 K.

For the dissociation kinetics studies, two binding reaction mixtures containing the viral CRE probe were incubated with 75 ng of CREB and 900 ng of Tax, with or without 1.7 μg of GST-KIX. The conditions were optimized to promote approximately 20% of the probe bound in complex. The binding reactions were allowed to reach equilibrium and then challenged with a 1,000-fold molar excess of unlabeled viral CRE. Aliquots were loaded onto a running gel at the appropriate time points following challenge and resolved by EMSA, and the percent bound was determined by PhosphorImager analysis. Computer analysis of the data was performed with the KaleidaGraph software program.

DNA probes. Complementary double-stranded DNA oligonucleotides representing the viral CRE and the cellular CRE were cloned into the *Bam*HI site of pUC19, and the *Eco*RI-*Hin*dIII fragments were isolated, purified, and 5′ end

labeled with ^{32}P for use in EMSA reactions as previously described (11). The nucleotide sequences of the top strands of the oligonucleotide inserts are as follows: cellular CRE (from the human chorionic gonadotropin gene), 5′-GATC TCCA**TGACGTCA**ATTGA-3′; viral CRE (from the promoter-proximal 21-bp repeat), 5′-GATCTCAGGCGT**TGACGACA**ACCCCTCACCTCA-3′. The octanucleotide CRE cores are in boldface.

Recombinant plasmids, cell culture, and transfections. The luciferase reporter plasmid pminLUC-viral CRE contains exactly the same promoter sequences as previously reported (constructed from pminCAT-21 bp repeat and pminCAT-CRE; see reference 11); however, the promoter fragment has been cloned into the pGL2Basic (Promega) luciferase reporter plasmid. The Tax expression plasmid pHTLV-I Tax has been previously described (10). The expression plasmid RSV-CREB was a generous gift from Marc Montminy (20). The CREB BR eucaryotic expression plasmid was constructed by amplification of the bZIP region by using the PCR primers 5′-GGGATCCCAGTCCATTTTCCACCAC ATAG-3′ and 5′-AAGCTTATGGCGTCCTCCCCAGCACTTCCT-3′. The amplification products were digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI-*Hin*dIII site of the mammalian expression plasmid RSV-CREB, replacing the full-length CREB with the bZIP CREB. Plasmid RSV-KIX was constructed by inserting a PCR fragment corresponding to aa 459 to 679 of mouse CBP into a modified pRc/RSV plasmid.

The HTLV-1-negative human T-lymphocyte cell lines CEM and Jurkat were maintained in Iscove's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Transient cotransfection assays were performed with Jurkat T cells grown to a density of 10^6/ml. The cells were transfected with Lipofectamine (Life Technologies) and a constant amount of DNA and assayed for luciferase activity in accordance with the manufacturer's directions. F9 embryonic teratocarcinoma cells were maintained in high-glucose Dulbecco modified Eagle medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, and antibiotics. At 50% confluency, a 1:15 dilution was made, and after 6 h, cells were transfected by calcium phosphate precipitation with a constant amount of DNA. The cultures were grown in 3% CO_2 for 16 to 20 h before harvesting and assayed for luciferase activity in accordance with the manufacturer's directions. A Turner Designs TD 20-e Luminometer was used to measure luminescence.

RESULTS

EMSA reveal a specific interaction between the KIX domain of CBP and the Tax-CREB-DNA complex. We and others have previously shown that Tax enhances the equilibrium DNA binding affinity of CREB and that in the presence of the viral CRE (from the third 21-bp repeat of HTLV-1), a highly specific ternary complex is formed (11, 21, 34, 41, 44, 49). However, in the absence of CREB phosphorylation (and the subsequent recruitment of the coactivator CBP), one would predict that Tax stimulation of CREB binding to the viral promoter would not significantly activate viral transcription. Because of this discordance, we were interested in determining whether Tax, in addition to stimulating CREB DNA binding activity, may also directly recruit CBP to the viral promoter.

To test this hypothesis, we cloned a 269-aa region of CBP (aa 451 to 720) which contains the phospho-CREB-interacting KIX domain and expressed and purified this protein as a C-terminal fusion to GST. To determine whether Tax might promote an interaction between KIX and CREB, we measured protein-DNA complex formation in an EMSA with both mock-phosphorylated and PKA-phosphorylated forms of CREB on both the labeled viral and cellular CREs. These CREs differ significantly in the sequences adjacent to the CRE octanucleotide. Specifically, the viral CRE contains GC-rich nucleotides flanking the CRE whereas the cellular CRE flanking sequences are AT rich (see Materials and Methods). As shown in Fig. 1A, and as expected, Tax promoted ternary complex formation with both the unphosphorylated and phosphorylated forms of CREB only on the viral CRE (lanes 3 and 7). When both Tax and the KIX domain were present in the DNA binding reaction mixtures, both forms of CREB entered into much slower-migrating complexes (lanes 4 and 8), suggesting that KIX was stably incorporated into a quaternary complex. Although high concentrations of KIX (>1 μM) interact specifically with phosphorylated CREB (data not shown; 35), the lower concentrations of KIX (~0.2 μM) used in the experiment presented in Fig. 1A did not produce a detectable interaction with either form of CREB in the absence of Tax (lanes 2 and 6). The use of truncated forms of KIX in the binding reaction mixtures produced complexes with increased mobilities, confirming that KIX is physically incorporated into the complexes (data not shown). Furthermore, replacement of the GST moiety with a six-histidine tag produced a KIX fusion

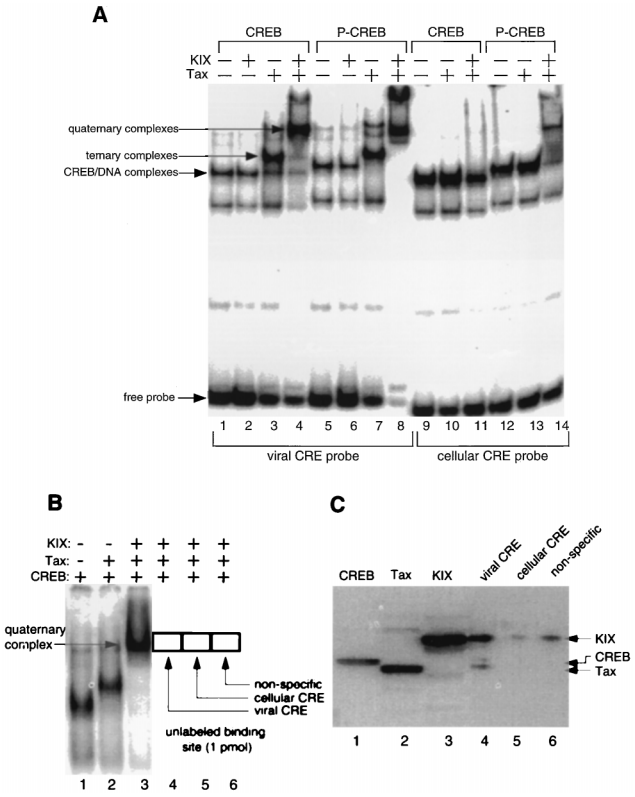

FIG. 1. (A) Tax recruits the CBP KIX domain to both phosphorylated CREB (P-CREB) and mock-phosphorylated CREB on a viral CRE. Binding reaction mixtures contained 4 fmol of either the viral CRE (lanes 1 to 8) or the cellular CRE (lanes 9 to 14) probe, mock-phosphorylated CREB (2.5 ng, lanes 1 to 4 and 9 to 11), or phosphorylated CREB (1.25 ng, lanes 5 to 8 and 12 to 14). Reaction mixtures also contained 150 ng of Tax and/or 240 ng of GST-KIX, as indicated. Binding reactions were analyzed by EMSA. PKA-phosphorylated CREB migrated with slower mobility in the native gel. The positions of the free probe and relevant protein-DNA complexes are shown. The minor bands that migrated below the major CREB homodimer are truncated forms of CREB present in the purified preparation. The bands migrating near the wells in lanes 4 and 8 are likely the result of aggregation from the high GST-KIX concentration. Similarly, the slower-migrating complexes especially obvious in the CREB and Tax lanes resulted from high concentrations of CREB with binding enhancement by Tax. These bands were probably due to CREB aggregation. They displayed slightly different mobility than the complex observed in the presence of the GST-KIX domain (data not shown). (B and C) Identification of the polypeptides present in the quaternary complex by EMSA-Western blotting. Binding reaction mixtures with 4 fmol of the labeled viral CRE probe (B, lanes 1 to 3) each contained 2.5 ng of CREB, 150 ng of Tax (B, lanes 2 and 3), and 240 ng of GST-KIX (B, lane 3), as indicated. Binding reaction mixtures with 1 pmol of the unlabeled viral CRE fragment (B, lane 4), the cellular CRE fragment (B, lane 5), or a nonspecific DNA fragment (B, lane 6) also contained 300 ng of CREB, 90 ng of Tax, 80 ng of KIX, and 500 ng of poly(dA) · poly(dT). Protein-DNA complexes were resolved by electrophoresis on a 5% native polyacrylamide gel and then autoradiographed for 1 h to locate the position of the quaternary complex. Portions of the EMSA (boxed) corresponding to the position of the quaternary complex were excised, soaked in SDS sample buffer, and electrophoresed on an SDS–12% polyacrylamide gel. (C, lanes 4 to 6). Immunoblot analysis of the polypeptides excised from the EMSA by using an antiserum cocktail containing anti-CREB, anti-Tax, and anti-KIX antibodies. Purified CREB (70 ng, lane 1), Tax (70 ng, lane 2), and KIX (80 ng, lane 3) were electrophoresed in adjacent lanes.

protein competent for quaternary complex formation, indicating that the KIX domain is specifically required for the interaction (data not shown). Like the ternary complex that forms in the presence of Tax and CREB, the quaternary complex was found to be strongly dependent upon the presence of the viral CRE; complexes formed with the cellular CRE appear significantly less stable under these electrophoretic conditions (Fig. 1A, compare lanes 4 and 8 with lanes 11 and 14).

To confirm that CREB, Tax, and KIX were each present in the quaternary complex, we performed SDS-PAGE–immunoblot analysis of the protein-DNA complexes isolated by EMSA. In this experiment, 1 pmol of unlabeled DNA representing the viral CRE, the cellular CRE, or an unrelated site was incubated with CREB, Tax, and KIX and the samples were resolved by EMSA (Fig. 1B, lanes 4 to 6). To identify the position of the quaternary complex on the EMSA, parallel binding reaction mixtures were prepared that contained 4 fmol of the labeled viral CRE and the appropriate proteins (Fig. 1B, lanes 1 to 3). Following brief autoradiography, gel slices corresponding to the position of the quaternary complex were excised (Fig. 1B, boxes) and analyzed by immunoblot analysis using a cocktail of antibodies against CREB, Tax, and KIX (Fig. 1C). In the reaction mixture that contained the viral CRE, polypeptides corresponding to CREB, Tax, and KIX were observed (Fig. 1C, lane 4). In control reaction mixtures containing the cellular CRE or nonspecific DNA, neither CREB nor Tax was detected (Fig. 1C, lanes 5 and 6). We did observe a small amount of KIX in these control reaction mixtures which likely resulted from comigration of the quaternary complex with unliganded KIX protein (observed by silver staining of the EMSA; data not shown). These data indicate that CREB, Tax, and KIX enter into a stable nucleoprotein complex specifically on the viral CRE. Stoichiometric analysis of the polypeptides indicated that equimolar amounts of Tax, KIX, and dimeric CREB were present in the quaternary complex.

The CBP-interacting domain of CREB is dispensable for KIX association in the presence of Tax in vitro and Tax transactivation in vivo. Previous studies have shown that the KIX domain of CBP and the serine 133-phosphorylated kinase-inducible domain (aa 88 to 160) of CREB are required for the interaction between these two proteins (35). Since Fig. 1A shows that phosphorylation of CREB is dispensable for association with CBP in the presence of Tax, we were interested in determining whether the entire kinase-inducible domain of CREB might also be dispensable for this interaction. To test this hypothesis, we compared full-length CREB with a truncated form of CREB containing only the 73-aa DNA binding and dimerization domain (bZIP domain) (CREB BR; aa 254 to 327; reference 22) in the presence of the KIX domain and Tax. Neither full-length CREB nor CREB BR formed detectable complexes with the KIX domain alone (Fig. 2A, lanes 1, 7, and 13). However, in the presence of Tax, KIX associated with both full-length CREB and CREB BR on the viral DNA (Fig. 2A, compare lanes 4 to 6 and 10 to 12). Unlike full-length CREB, CREB BR did not form a detectable ternary complex with Tax and the viral CRE (Fig. 2A, compare lanes 3 and 9). These data indicate that Tax can recruit KIX into the CREB-viral DNA complex in the absence of the amino-terminal CBP-interacting region of CREB.

Tax stabilization of the KIX-CREB BR-viral DNA complex led to the intriguing question of whether CREB BR might support Tax transactivation of HTLV-1 in a functional assay in vivo. Figure 2B shows the results of a transient transfection assay performed with undifferentiated F9 cells (a mouse embryonal teratocarcinoma cell line that is deficient in CREB and

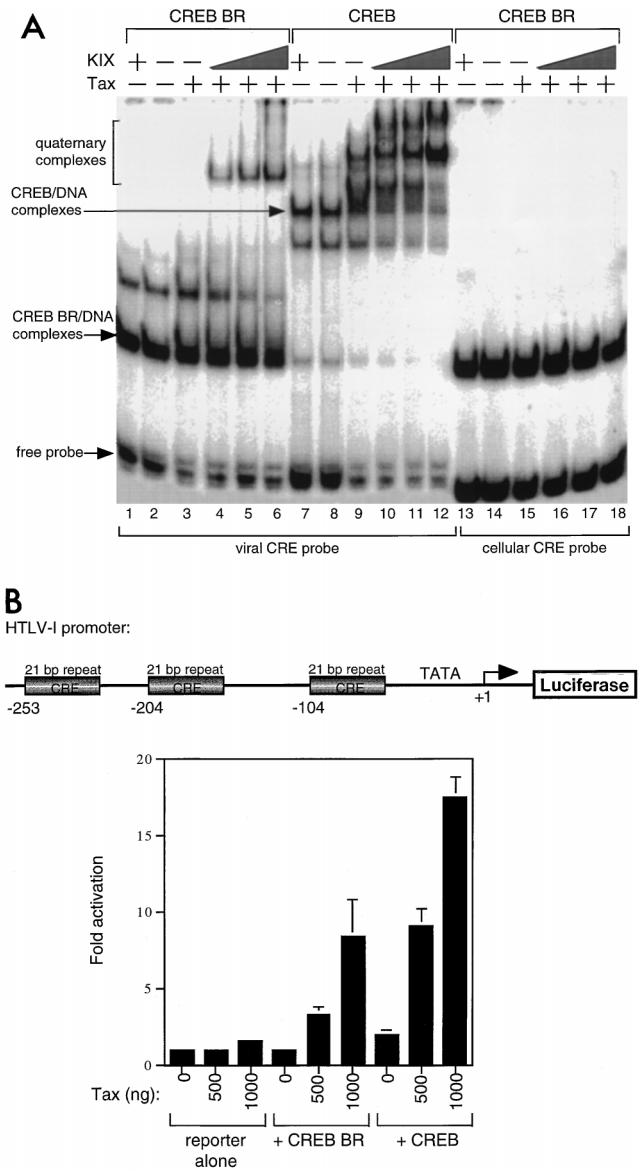

FIG. 2. The bZIP domain of CREB is sufficient for KIX complex formation and Tax transactivation. (A) Binding reaction mixtures contained 4 fmol of either the viral CRE (lanes 1 to 12) or the cellular CRE (lanes 13 to 18) probe, full-length CREB (2.5 ng, lanes 7 to 12), or the 73-aa bZIP domain of CREB (CREB BR) (0.12 ng in lanes 1 to 6 and 0.02 ng in lanes 13 to 18). Reaction mixtures contained 200 ng of Tax and increasing amounts of GST-KIX (24, 48, and 120 ng; lanes 1, 7, and 13 contained 120 ng of GST-KIX). Binding reactions were analyzed by EMSA. The positions of the free probe and relevant protein-DNA complexes are shown. As in Fig. 1A, the slower-migrating complexes in the CREB or CREB BR (with or without Tax) lanes are CREB aggregates typically observed with a high CREB concentration (note that most of the probe is shifted into a complex with CREB). (B) Transient cotransfection assay comparing the effects of CREB and CREB BR on Tax transactivation in vivo. Calcium phosphate precipitations were performed with F9 cells and 2 μg of the full-length CREB or CREB BR expression plasmid, 2 μg of the HTLV-1 luciferase reporter plasmid (shown schematically), and the indicated amounts of the Tax expression plasmid. Luminescence was quantitated with a luminometer, and fold activation was calculated relative to the reporter alone in the absence of Tax. Values shown are the mean fold activation ± the standard error from four independent experiments.

unresponsive to PKA-stimulated transcription; see reference 13). This cell line allows us to compare the abilities of full-length CREB and CREB BR to support Tax transactivation on a reporter construct carrying the HTLV-1 transcriptional con-

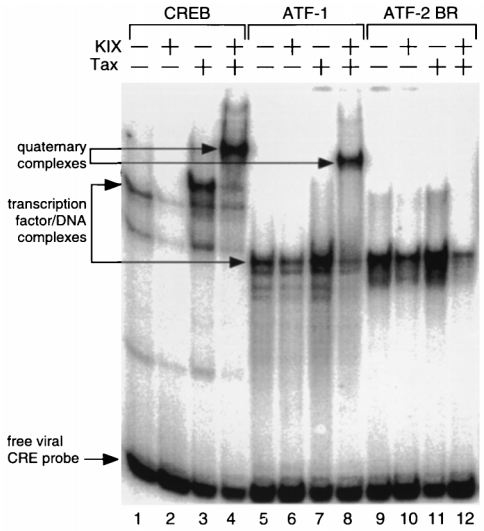

FIG. 3. Tax recruits KIX to the related protein ATF-1. Binding reaction mixtures contained 4 fmol of the viral CRE probe, 1.25 ng of full-length CREB (lanes 1 to 4), 0.21 ng of full-length ATF-1 (lanes 5 to 8), or 0.44 ng of the 155-aa bZIP domain of ATF-2 (ATF-2 BR, aa 350 to 505) (22). Reaction mixtures contained 150 ng of Tax and/or 240 ng of GST-KIX, as indicated. Binding reactions were analyzed by EMSA. The positions of the free probe and relevant protein-DNA complexes are shown.

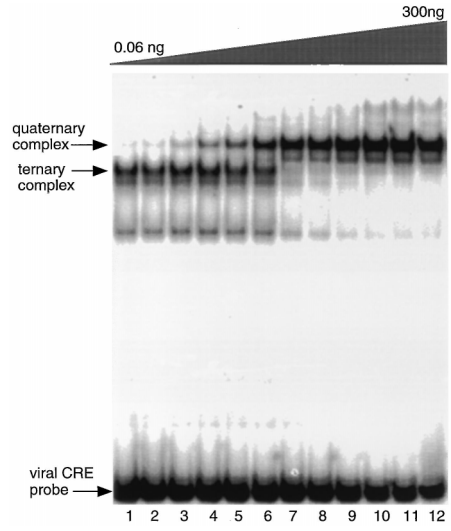

FIG. 4. Equilibrium binding of KIX to the phosphorylated CREB-Tax-viral CRE complex. Equilibrium binding reaction mixtures contained 4 fmol of the viral CRE probe, 1.25 ng of phosphorylated CREB, and 200 ng of Tax. GST-KIX was added in increasing amounts from 0.06 to 300 ng, as indicated. The amounts of GXT-KIX added were 0.06, 0.12, 0.3, 0.6, 1.2, 3.0, 6.0, 12, 30, 60, 120, 300 ng. Binding reactions were analyzed by EMSA. The positions of the free probe and protein-DNA complexes are shown.

trol region linked to the luciferase gene (Fig. 2B). As shown in Fig. 2B, cotransfection of either full-length CREB or CREB BR expression plasmids had little effect on luciferase activity in the absence of Tax. However, in the presence of increasing amounts of the Tax expression plasmid, both CREB and CREB BR supported increased levels of HTLV-1 transcription.

Tax promotes association of KIX with the related bZIP protein ATF-1. Since the bZIP region of CREB shares homology with several members of the ATF/CREB family of proteins, we were interested in determining whether Tax can stabilize the KIX domain in protein-DNA complexes with other members of the bZIP family which bind the CRE. Figure 3 shows that on the viral CRE, Tax promoted KIX complex formation only with the closely related protein ATF-1, which shares 85% similarity with CREB within the bZIP region (lanes 4 and 8). ATF-1 did not form a detectable ternary complex with Tax and the viral CRE (lane 7). A truncated form of the more distantly related bZIP protein ATF-2 (ATF-2 BR; aa 350 to 505, which shares 44% similarity with CREB within the bZIP region) (lanes 9 through 12) did not interact with KIX in the presence of Tax. In addition, full-length ATF-2, c-Jun, and the zinc finger protein Sp1 also failed to interact with KIX in the absence or presence of Tax (data not shown). Finally, mutations in the core viral CRE abolished quaternary complex formation with ATF-1, Tax, and KIX. Together, these data suggest that Tax specifically stabilizes KIX in complexes with the highly homologous bZIP proteins CREB and ATF-1 and a viral CRE.

Determination of apparent KIX binding affinities for transcription factor-DNA complexes in the presence of Tax. The data presented above suggest that Tax may function by recruiting CBP to the HTLV-1 promoter. Because the rate of initiation of transcription may depend on the strength of the interaction between CBP and the Tax-CREB-DNA complexes, we performed equilibrium binding assays to examine the relative KIX domain binding affinities for various protein-DNA complexes. In these experiments, the amounts of labeled DNA, transcription factor, and Tax were kept constant and that of the KIX protein was varied over a large concentration range (>1,000-fold). The binding reactions were allowed to reach equilibrium, and the protein-DNA complexes were analyzed by EMSA. Figure 4 shows a representative experiment examining the binding of the KIX domain to phosphorylated CREB in the presence of Tax on a labeled viral CRE. To calculate the apparent binding affinity, the fraction of quaternary complex formed was plotted versus the total KIX concentration and the concentration of KIX required for half-maximal quaternary complex formation was determined (see Materials and Methods). The results of these studies are presented in Table 1, together with the deduced free energies of the binding reactions and whether a ternary complex was observed in the EMSA.

For comparison, we measured KIX domain-transcription factor binding affinities on the viral CRE and, where possible, on the cellular CRE. The values reported in Table 1 reflect apparent affinities determined in the presence of Tax. Under

TABLE 1. Apparent affinities[a] of GST-KIX for various transcription factors on the viral and cellular CREs in the presence of Tax

Transcription factor	Binding site	Apparent K_D	Free energy (kcal/mol)	Ternary complex
CREB	Viral CRE	25 nM	−9.6	Yes
Phospho-CREB	Viral CRE	1.7 nM	−11	Yes
CREB BR[b]	Viral CRE	200 nM	−8.5	No
ATF-1[b]	Viral CRE	125 nM	−8.7	No
CREB	Cellular CRE	3.8 μM	−7.0	No
Phospho-CREB	Cellular CRE	90 nM	−8.9	No

[a] All reported values are averages of three independent trials.
[b] KIX complex formation on the cellular CRE was undetectable in the presence or absence of Tax.

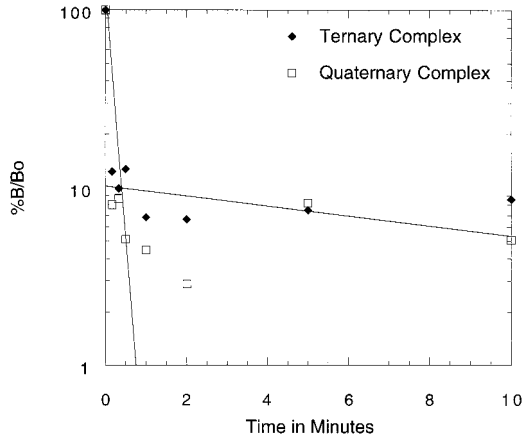

FIG. 5. Kinetics of CREB-Tax dissociation in the presence and absence of KIX. CREB was incubated with Tax and the viral CRE in the absence or presence of GST-KIX. Equilibrium binding reaction mixtures were challenged with a 1,000-fold molar excess of the unlabeled binding site, and the kinetics of dissociation were analyzed by EMSA. CREB-Tax dissociation was quantitated, and the concentration of CREB-Tax remaining bound, relative to the concentration bound at time zero (no added competitor) (%B/Bo), was plotted as a function of time following the challenge. CREB-Tax dissociation from the viral CRE in the absence (diamonds) and presence (squares) of GST-KIX is shown. The fast and slow phases of the dissociation plot are indicated. The graph represents average values of two independent experiments.

our binding conditions, the KIX domain had the highest apparent affinity for the complex containing Tax, phosphorylated CREB, and the viral CRE, with an apparent K_D of 1.7 nM. Substitution of the cellular CRE for the viral CRE in the Tax-containing binding reaction mixture resulted in a dramatic reduction of apparent stability of the KIX domain in the complex (>2 kcal/mol). In all cases, Tax dramatically stabilized the KIX domain-containing protein-DNA complexes and, where measurable, conferred a 4.4-kcal/mol increase in the free energy of the complex. Together, the equilibrium binding studies reported here indicate the importance of both Tax and the viral CRE sequence for the stabilization of KIX in the transcription factor-DNA complexes.

Dissociation kinetics reveal no differences in the half-life of CREB-Tax-viral CRE complexes in the presence or absence of KIX. The association of CBP with the Tax-CREB-viral DNA complex likely results in transcriptional activation through properties associated with CBP. However, it is also possible that CBP simply stabilizes Tax on the viral promoter and transcriptional activation results from an activation domain intrinsic to Tax. To determine whether CBP might stabilize the Tax-CREB-viral DNA complex, we analyzed the dissociation kinetics of the CREB-Tax complex from the viral CRE in both the presence and absence of KIX. The viral CRE probe was incubated with CREB and Tax in the presence or absence of KIX. Binding reaction mixtures were then challenged with a large excess of unlabeled viral CRE, and the kinetics of complex dissociation were determined by quantitative analysis of the EMSA. A plot of these data is shown in Fig. 5. In both the absence and the presence of KIX, the initial dissociation (to <10% bound) of the protein complexes from the viral CRE was very rapid, with a half-life of less than 25 s. The dissociation kinetics of the two reactions were nearly identical, suggesting that the KIX domain does not stabilize the CREB-Tax complex on the viral CRE. While it is possible that full-length CBP behaves differently, these data support a model in which Tax activation occurs, at least in part, through properties associated with CBP.

The KIX domain of CBP inhibits Tax transactivation in vivo. The observation that Tax efficiently recruits the KIX domain of CBP in vitro led to the hypothesis that the addition of a KIX expression plasmid might compete with active CBP in the cell, thus inhibiting Tax transactivation in vivo. To test this hypothesis, transient transfection assays were performed with HTLV-1-negative human Jurkat T cells. The viral CRE chimeric promoter construct was fused to the luciferase gene (Fig. 6) and cotransfected with expression plasmids for Tax and KIX. Figure 6 shows that addition of the KIX expression plasmid to the chimeric promoter carrying the viral CREs had a negligible effect on luciferase activity, indicating that the plasmid was not toxic to the cells. When the Tax expression plasmid was transfected into the cells, the predicted increase in luciferase activity was observed. However, cotransfection of the KIX expression plasmid decreased Tax transactivation in a dose-dependent fashion. The observation that cotransfection of the KIX expression plasmid inhibited Tax stimulation on the viral CRE suggests that KIX may compete with endogenous CB for association with Tax on the promoter. Together, these data provide evidence that CBP plays a fundamental role in mediating Tax transactivation of HTLV-1 transcription in vivo.

DISCUSSION

The role of CREB in mediating Tax transactivation of HTLV-1 gene transcription has been the subject of considerable research in recent years; however, the precise molecular mechanism by which Tax stimulates viral transcription mediated through CREB and the 21-bp repeats remains incompletely understood. Many previous studies strongly support a role for Tax in the stabilization of CREB binding to the relatively weak CREs in the viral promoter. This stabilization, however, cannot fully account for the strong transcriptional activation observed in the presence of Tax, as the transcriptional activity of DNA-bound CREB is dependent upon recruitment of CREB's coactivator, CBP, which requires CREB phosphorylation by cAMP-dependent PKA. In this report, we

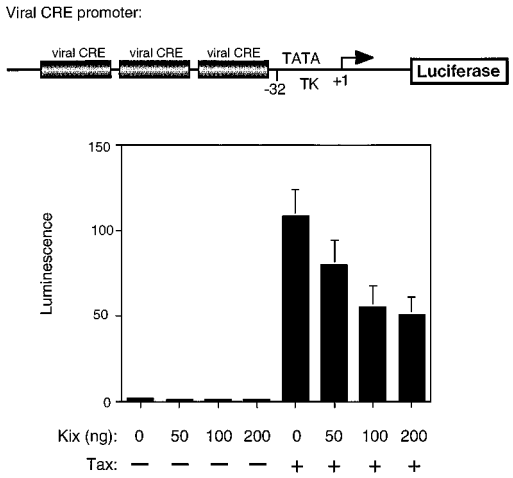

FIG. 6. KIX represses Tax transactivation of the viral CRE in vivo. Transient cotransfection assays were performed with HTLV-1-negative human Jurkat T cells. Cotransfections were performed with 400 ng of the viral CRE promoter luciferase reporter plasmid (shown schematically), 50 ng of the Tax expression plasmid, and/or the indicated amount of the KIX expression plasmid. Luminescence was quantitated with a luminometer. The values shown are mean luminescence ± the standard error from two independent experiments performed in triplicate.

account for the strong Tax transcriptional activation by showing that Tax is brought to the HTLV-1 promoter through interaction primarily with the bZIP region of CREB bound to the viral CREs. Tax serves as an attachment site for CBP, tethering the coactivator to the viral promoter. This tethering most likely results in the subsequent activation of transcription seen in vivo. The bridging function of Tax increases the stability of CBP on the viral promoter by 4.4 kcal/mol (compared with the phosphorylated CREB-CBP interaction in the absence of Tax [~6 μM; data not shown]). This defines the primary function of Tax as a bridging molecule for CBP, bypassing CREB phosphorylation in the recruitment of the coactivator.

We utilized equilibrium binding assays to measure the apparent affinity of the CBP KIX domain for various nucleoprotein complexes in the presence of Tax. We demonstrate that Tax can recruit the KIX domain to protein-DNA complexes containing either the unphosphorylated or the phosphorylated form of CREB. However, the complex containing phosphorylated CREB associates with the KIX domain with an approximately 15-fold higher affinity than does that containing unphosphorylated CREB. This phosphorylation difference contributes approximately 1.4 kcal/mol to the free energy of the KIX association. These results differ from those reported by Kwok et al. (24) and may be related to the different technical approaches used to evaluate equilibrium binding affinities. The significantly higher affinity of KIX for Tax and phosphorylated CREB, versus unphosphorylated CREB, provides strong support for the presence of distinct regions within the KIX domain of CBP that bind independently and simultaneously to both phosphorylated CREB and Tax. It is surprising, given the large size of CBP (2,441 aa), that both Tax and phosphorylated CREB interact with neighboring regions in this domain of CBP. It is possible that the juxtaposition of these interaction domains enables CBP to adopt a functionally similar promoter-bound conformation whether it is interacting with phosphorylated CREB or with Tax. While we can measure a significant increase in the KIX binding affinity for the phosphorylated CREB complex in the presence of Tax, it is well established that Tax strongly transactivates HTLV-1 transcription in the absence of cAMP stimulation and that phosphorylation of CREB by PKA only modestly increases viral transcription in the presence of Tax. It is possible that the interaction between Tax and unphosphorylated CREB is sufficiently strong to recruit saturating amounts of CBP to the viral promoter, and therefore PKA phosphorylation of CREB provides an insignificant contribution to the overall stability of the complex. This mechanism is consistent with a strategy for virion production in the infected T cell that occurs largely independently of cAMP stimulation.

The data presented above, and those of Kwok et al. (24), suggest that either unphosphorylated or phosphorylated CREB can function as a binding site for Tax on the viral promoter, enabling Tax to recruit CBP. The observation that Tax promotes the association of the KIX domain to protein-DNA complexes containing just the bZIP region of CREB (CREB BR) implies that DNA-bound CREB serves only as a scaffold for Tax in the recruitment of CBP to the DNA. Therefore, on the viral promoter, CREB is not likely involved in direct CBP interaction, except when phosphorylated by PKA. This model is further supported by functional studies (this study and reference 26) showing that CREB BR is capable of supporting transcriptional activation of the viral promoter, but only when Tax is present. The measured affinity of KIX for the CREB BR-Tax-DNA complex was approximately eightfold lower than that observed with full-length CREB, indicating that the N-terminal amino acids in CREB contribute approximately 10% of the free energy of the complex in the presence of Tax. This may account for the reduced Tax transactivation on the viral promoter observed in vivo (Fig. 2B). Finally, we show that Tax can recruit the KIX domain into a protein-viral DNA complex with the closely related bZIP protein ATF-1. While there is no evidence indicating that the KIX domain recognizes ATF-1 directly, our data are consistent with a previous report suggesting that Tax weakly interacts with this protein (49) and therefore can exploit this interaction to recruit KIX (we have not observed ATF-1-supported Tax transactivation in vivo).

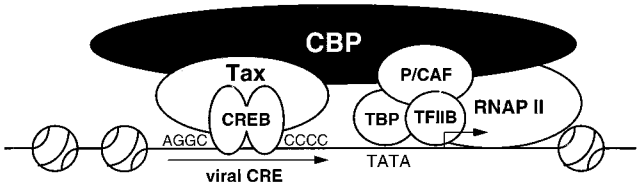

FIG. 7. Model showing Tax recruitment of the coactivator CBP to the HTLV-1 promoter. For simplicity, only one of the three 21-bp repeats is shown. Tax in association with CREB and the viral CRE creates a high-affinity binding site that anchors CBP to the viral promoter. Once bound, CBP activates HTLV-1 transcription through chromatin remodeling and recruitment of the general transcription machinery. TFIIB, transcription factor IIB; P/CAF, p300/CBP-associated factor; RNAP II, RNA polymerase II.

It is well established that the viral CREs are critical elements in mediating ternary complex formation, binding stabilization, and Tax transactivation in vivo. In this report, we provide strong molecular evidence showing that the association of the KIX domain with nucleoprotein complexes is also dependent upon the viral CREs. These important sequences carry GC-rich nucleotides immediately adjacent to the CREB binding site core. In the presence of Tax, the viral CRE, compared to the cellular CRE, conferred an up to 150-fold increase in the apparent affinity of KIX for the nucleoprotein complexes ($\Delta\Delta G$, $\leq$2.6 kcal/mol). It is likely that the increased affinity of KIX is indirectly a consequence of Tax binding stabilized by the viral sequence, as the viral CREs appear to have a direct (albeit ill-defined) role in the stable incorporation of Tax into DNA-bound complexes.

The data presented here form the basis of a model in which Tax serves as a bridging molecule to recruit CBP to the viral promoter (Fig. 7). Tax is initially stabilized at the viral promoter through contacts principally with the bZIP domain of CREB and contributions from the GC-rich nucleotides that flank the viral CRE. Once associated, Tax serves as a high-affinity binding site to anchor the coactivator CBP to the viral promoter. The precise sequence of molecule recruitment, however, has not been established, and it is also possible that Tax is brought to the viral promoter in a preformed complex with CBP which then stably associates with CREB on the viral CRE. In either case, once CBP is associated, it strongly potentiates Tax transactivation, establishing CBP as a critical cofactor in Tax function. The molecular events potentiated by CBP may involve the recruitment of the general transcription machinery through recruitment of transcription factor IIB (25) and nucleosome remodeling and/or displacement through the extrinsic and intrinsic histone acetylase activities associated with CBP (6, 33, 43). The combination of these CBP-associated activities likely accounts for the strong transcriptional activation of HTLV-1 transcription observed in the presence of Tax.

ACKNOWLEDGMENTS

We thank Paul Laybourn and Brian Lenzmeier for generous advice and critical reading of the manuscript. We also thank Richard Goodman for the CBP expression plasmid and Marc Montminy for the RSV-CREB expression plasmid.

This work was supported by American Cancer Society Junior Faculty Research Award 506 (to J.K.N.) and Public Health Service grant CA-55035 from the National Cancer Institute (to J.K.N).

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