The Human T-cell Leukemia Virus-1 Transcriptional Activator Tax Enhances cAMP-responsive Element-binding Protein (CREB) Binding Activity through Interactions with the DNA Minor Groove*

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Tax-1, the transcriptional activation protein of human T-cell leukemia virus-1, increases transcription from the human T-cell leukemia virus-1 long terminal repeat and specific cellular promoters through interactions with cellular DNA-binding proteins. The Tax response elements (TxREs) of the long terminal repeat resemble cAMP response elements (CREs), the target of cAMP-responsive element-binding protein (CREB). CREB binds the TxRE with reduced affinity; however, the interaction is specifically enhanced by Tax. Using a fluorescence quenching method, we determined that CREB dimerizes in the absence of DNA, and that Tax does not enhance dimerization. DNA footprinting of the TxRE with 1,10-phenanthroline-copper complex demonstrates that Tax contacts DNA and extends the footprint of CREB to GC-rich sequences flanking the core CRE-like element. The minor groove-binding drug chromomycin A₃, but not distamycin A, disrupted Tax-enhanced CREB binding to the TxRE. Substitution of the guanine-rich sequences flanking the core of the TxRE with inosine residues also blocked the Tax effect. Finally, the IC₅₀-substituted TxRE binds CREB with increased affinity, suggesting flanking DNA influences the binding of CREB to the core CRE-like element. These data indicate that Tax does not regulate DNA binding of CREB by altering dimerization, but rather enhances DNA binding by additionally interacting with the minor groove of flanking DNA sequences.

Viruses typically exploit host cellular processes to promote their own replication. Human T-cell leukemia virus (HTLV-1), a human retrovirus, is the causative agent of adult T-cell leukemia/lymphoma (reviewed in Ref. 1). Adult T-cell leukemia/lymphoma develops only in a small percentage of patients, typically many years following infection with HTLV-1. The virus remains in a state of latency in infected T-cells, producing low levels of transcripts from the long terminal repeat (LTR). Activation of viral RNA synthesis depends on cooperation between virally encoded proteins and cellular factors. The pX region of the HTLV-1 genome encodes Tax, a 40-kDa protein that is essential for viral pathogenesis. Tax is a positive transcriptional activator of both cellular and viral promoters, but Tax is thought to activate transcription through interactions with cellular proteins rather than by binding to DNA itself (2–4). Through a positive feedback mechanism, Tax enhances transcription from the viral LTR through a complex enhancer containing a series of three 21 base pair repeats, which act as Tax-responsive elements (or TxREs) (5, 6). The core sequence (5′-TGACG-3′) of the 21-base pair TxRE resembles the cAMP-responsive element (or CRE), the target of the cellular transcriptional regulatory protein CREB (7). The central CRE-like core of each 21-base pair repeat is flanked by GC-rich sequences that are required for Tax transactivation in vivo (2, 8, 9). The TxRE is a low affinity CREB-binding site in vitro; however, the binding activity of CREB can be dramatically enhanced by the addition of Tax (10–19).

We and others have shown that Tax augmentation of CREB DNA binding activity is sequence-specific; Tax enhances binding of CREB (and cAMP-responsive element modulator, or CREM) to the TxRE but not to the perfect palindromic CRE derived from the somatostatin promoter (12–14, 16, 17, 19–21). In addition Tax forms a stable complex with CREB and DNA, also in a sequence-specific manner, dependent on flanking GC-rich DNA motifs (12, 14, 16, 17, 19). Although Tax does not associate with CREB bound to the somatostatin CRE, it will bind to CREB in the absence of DNA (22–24). The stable complex of Tax, CREB, and the TxRE is termed the “ternary complex,” although the precise stoichiometry is unknown. This DNA-directed ternary complex recruits the CREB coactivator CREB-binding protein in a phosphorylation-independent manner, probably through a direct association with CREB-bound Tax (19, 21, 25). Thus, through the activity of Tax, the TxRE DNA directs the assembly of a topologically distinct activator-coactivator complex at the HTLV-1 LTR promoter, permitting transcriptional activation through CREB in a cAMP-independent manner.

The proposed molecular mechanisms of Tax-enhanced binding of CREB to DNA and ternary complex formation are controversial. Wagner and Green (26) have proposed that Tax augments the dimerization of CREB as well as other B/ZIP factors. Although Tax interacts specifically with the basic region of B/ZIP proteins (18, 24, 27), the contribution of Tax to dimerization, mediated by the leucine zipper motif, is unclear. Tax increases the DNA binding of chimeric peptides consisting of the GCN4 basic region and unrelated dimerization motifs...
(27) and, in the context of CREB, specific residues within the leucine zipper may be essential for the Tax effect on DNA binding (17, 20). Nevertheless, by using nonspecific cross-linking, Wagner and Green (26), and Perini et al. (27), suggest that Tax increases dimeric association of the GCN4 B/ZIP through interactions with the basic segment. The DNA sequence specificity of the Tax effect, however, argues against the idea that enhanced dimerization accounts for the increased DNA binding activity. Indeed, the ability of Tax to stimulate the DNA binding activity of cross-linked basic region peptides independently of dimerization (15) suggests that Tax might associate with specific DNA sequences, despite the lack of evidence to date for direct Tax-DNA interactions (12, 26).

In this report, we directly measure CREB dimerization by using a solution-binding assay based on fluorescence quenching and demonstrate that Tax does not affect this aspect of CREB function. By DNA footprinting with 1,10-phenanthroline-copper complex, a minor groove-selective cleavage reagent, we demonstrate that Tax interacts with the minor groove of the DNA flanking the core CRE-like motif of the TaxRE, in the context of the CREB-Tax-DNA ternary complex. The GC-selective minor groove-binding drug chromomycin A₃ selectively disrupts the Tax effect on CREB recruitment. Furthermore, alteration of minor groove structure by substitution of GC with IC base pairs in the flanking region disrupts Tax-enhanced DNA binding by CREB. These findings support a model of Tax acting through minor groove DNA contacts, specifically increasing the association of CREB with the HTLV-1 LTR TaxRE.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Expression Vectors**—The bacterial expression vector for HTLV-1 Tax has been described previously (28). A cDNA encoding a single cysteine form of CREB₃₂₇ was prepared by site directed mutagenesis of cysteines at codons 286 and 323 to serines using the Altered Sites system (Promega) (29). Wild type CREB₃₂₇ and the single cysteine mutant of CREB₃₂₇ (cysteine → serine 286, cysteine 296, cysteine → serine 323) were each subcloned into the prokaryotic expression vector pET11d as a NcoI-BamHI fragment (Novagen) and expressed as full-length proteins without polyhistidine tags in Escherichia coli (DE3) by identical procedures. Sequences were confirmed by the dideoxy chain termination method (Sequenase, U. S. Biochemical Corp.).

**Protein Purification—**E. coli BL21(DE3) cells harboring the CREB expression vectors were grown as described previously (29). Cells were harvested after 3 h at 37 °C, washed in ice-cold phosphate-buffered saline, resuspended in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT (TED buffer) and broken with two passages through a French pressure cell at 14,000 p.s.i. Ammonium. Lysates were cleared of insoluble cellular debris by centrifugation at 30,000 × g for 30 min. The lysate was then heated to 70 °C for 15 min, and centrifuged at 12,000 × g for 10 min. Nuclear acid was precipitated from the supernatant by addition of 0.2% polyethyleneimine following adjustment of the supernatant to 0.3 M NaCl. CREB was then precipitated from the supernatant with 20% (v/v) ammonium sulfate. Pellets were solubilized in TED and dialyzed, and the soluble protein was applied to a Q-Sepharose column (Amersham Pharmacia Biotech). Proteins were then eluted with a continuous concentration gradient from 0 to 1 M NaCl in TED. Fractions containing CREB were then adjusted to 2 M NaCl and applied to a phenyl-Sepharose column, washed, and eluted with low salt. Fractions containing CREB were pooled and fractionated by heparin-agarose chromatography. CREB was dialyzed into TED + 100 mM NaCl and 5% glycerol, aliquoted, and stored at −70 °C. CREB protein concentrations were determined as described previously (31).

**RESULTS**

**Measurement of CREB Dimerization by Fluorescence Quenching**—We utilized an approach based on fluorescence resonance energy transfer to detect self-association of CREB in the absence of DNA binding and to determine whether Tax enhances the DNA binding activity of CREB through enhancement of dimerization. Fluorescence resonance energy transfer measurements have been used previously to study dimerization of Fos and Jun, the components of the AP-1 complex (35). The specificity of this approach relies on the strict distance dependence of nonradiative energy transfer (36).

For this assay, we used a single cysteine form of CREB₃₂₇ in which cysteines at positions 286 and 323 were changed to serine residues by site-directed mutagenesis, preserving a single cysteine residue at amino acid 296 (at the first ε position within the coiled-coil heptad repeat, Fig. 1A). Previous studies have demonstrated that these cysteines are dispensable for high affinity DNA binding. For example, the BZIP of GCN4 lacks these cysteine residues, yet binds CRE sequences with high affinity (37, 38). So forgo-Rivera et al. (39) demonstrated that tetrahydroamidomethyl carbamylmethylation of CREB B/ZIP peptides at these cysteines did not alter DNA binding activity, but rather diminished aggregation and enhanced solubility of these peptides in solution. Mutation of a cysteine to a serine in the basic regions of Fos and Jun had a similar stabilizing effect (40). Finally, Richards et al. (29) demonstrated that DNA bind-
ing of CREB341 lacking these three cysteine residues was not altered. The structure of the CREB B/ZIP is not known, but may be modeled on the structure of the GCN4 B/ZIP bound to DNA (41, 42). Based on GCN4, the cysteine at position 296 is predicted to lie on the outer face of the dimeric coiled-coil leucine zipper motif, away from the dimerization interface. The sulfur-sulfur distance between subunits of a homodimer of single cysteine CREB is predicted to be approximately 18 Å.

We labeled single cysteine CREB327 (Cys-296) with thiol-reactive fluorescein-maleimide (F-CREB) or tetramethylrhodamin-5-maleimide (Rh-CREB). The Förster distance, or the distance of 50% energy transfer efficiency, for the fluorescein-rhodamine pair is about 46 Å (36); thus, we expected heterodimerization of F-CREB with Rh-CREB would result in significant energy transfer between fluorochromes. In the binding assay a trace amount of fluorescein-conjugated CREB is mixed with an increasing concentration of tetramethylrhodamine-labeled CREB, pairing F-CREB with Rh-CREB under conditions of Rh-CREB excess. Energy transfer from donor fluorescein to acceptor rhodamine, detected by a quenching of the fluorescein fluorescence intensity, depends on the precise steric positioning of dimerization. Because of the sixth power dependence of efficiency of energy transfer, no quenching will occur in the absence of dimerization.

Fig. 1A shows an emission spectrum of fluoresceinated-CREB in the absence and presence of tetramethylrhodamine-CREB. A, coiled-coil model of the single cysteine CREB327 leucine zipper (cysteine → serine 286, cysteine 296, cysteine → serine 323). Cysteine 296 used for labeling with either fluorescein-maleimide or tetramethylrhodamine-5-maleimide is indicated by arrows in the idealized dimer. B, fluorescence emission spectra of F-CREB and F-CREB with Rh-CREB. The fluorescence emission spectra of 5 nM F-CREB alone (closed symbols) and 5 nM F-CREB with 50 nM Rh-CREB (open symbols) were recorded between 500 and 600 nm following excitation at 485 nm.

Fig. 1B shows an emission spectrum of fluoresceinated CREB in the absence or presence of a saturating concentration of rhodamine-CREB, with an excitation wavelength of 485 nm, the optimum for fluorescein. In the absence of Rh-CREB, F-CREB has an emission maximum of 515 nm. In the presence of Rh-CREB, we observed reduced fluorescence intensity at 515 nm, and increased fluorescence intensity at the tetramethylrhodamine emission peak of 575 nm, indicating energy trans-
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Fig. 2. Kinetics of CREB dimer exchange in the absence of DNA. A, association of CREB dimers under native conditions. A 5 nM solution (1 ml) of F-CREB in binding buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 5% glycerol, 50 μg/ml BSA) was rapidly mixed with either 10 μl of a 10 μM solution of Rh-CREB in a 1-ml cuvette (solid line), or 10 μl of storage buffer (dashed line). Fluorescence emission at 515 nm was monitored at 1-s intervals for 900 s following excitation at 485 nm. B, F-CREB and Rh-CREB were mixed as described in A, except F-CREB with Rh-CREB were in 5 M guanidine hydrochloride, 25 mM Tris-HCl, 1 mM EDTA, 5% glycerol. Arrows indicate injection time. The fluorescence intensity was normalized to the initial value for F-CREB alone.

fer. The kinetics of fluorescence quenching were determined by a rapid mixing experiment, in which 5 nM F-CREB was mixed in a cuvette with 100 nM Rh-CREB in binding buffer, and then fluorescence emission intensity was measured at 515 nm at 1-s intervals for 900 s (Fig. 2A). The kinetics of dimer exchange (the dissociation of F-CREB and Rh-CREB dimers by dilution, followed by association of F-CREB and Rh-CREB into heterodimers) are slow. The decay fits a single exponential, with a half-life of 104 ± 1 s. These results suggest a high affinity for dimerization. Injection of F-CREB alone into sample buffer had no effect on fluorescence intensity with time (Fig. 2A).

We demonstrated the specificity of the assay by performing the same experiment under conditions where the dimerization of CREB could not occur. We first performed the same kinetic experiment in the presence of guanidine hydrochloride. The same concentrations of F-CREB and Rh-CREB were rapidly mixed in 5 M guanidine hydrochloride, 25 mM Tris-HCl, 1 mM EDTA, 5% glycerol. Under these conditions, we detected no fluorescence quenching (Fig. 2B). We performed a similar experiment by using a single cysteine CREB destabilized by amino acid substitutions in the e and g positions of the leucine zipper. This mutant, which fails to form homodimers and bind DNA (32) also fails to interact in this assay (data not shown).

We then measured the equilibrium dissociation constant ($K_d$) for CREB dimerization and asked whether HTLV-1 Tax enhanced CREB dimerization. For these experiments, 500 pM F-CREB was titrated with increasing concentrations of Rh-CREB in the absence of specific DNA and allowed to come to equilibrium. Fig. 3 shows the extent of quenching as a function of CREB concentration in the absence or presence of bacterially expressed Tax. First, the equilibrium dissociation constant for dimerization of CREB as measured by this method is low, with a $K_d$ of 0.6 nM. Tax had no significant effect on the affinity of dimerization, as shown by the half-maximal quenching concentration ($K_d = 0.8$ nM); however, Tax did reduce the maximum efficiency of energy transfer, suggesting that the association of Tax with the CREB dimer increases the intersubunit distance. The fluor-conjugated proteins bound the somatostatin CRE with an affinity identical to wild type CREB as determined by electrophoretic mobility shift assay (data not shown). The modified proteins were also recruited to the TxRE by Tax in a manner identical to the wild type proteins (data not shown), demonstrating that the conjugation does not alter the interaction of CREB with DNA, or the effect of Tax.

Tax Extends the Footprint of the CREB-DNA Complex—Previous studies from several laboratories have demonstrated that Tax forms a stable complex with CREB both in the absence (22, 24) and presence of DNA (16, 17, 19). We and others demonstrated previously that the association of Tax with CREB depends on the DNA context (16, 17, 19). Tax and CREB form a ternary complex with the proximal TxRE, but not with the palindromic somatostatin CRE, correlating with the ability of Tax to enhance the affinity of CREB only for the former element. The DNA sequence dependence of the Tax effect suggests that Tax itself participates in DNA recognition in the context of a complex with CREB and DNA, even though Tax alone does not bind DNA. Previous studies of the Tax-DNA interaction by DNase I footprinting and dimethyl sulfate methylation interference failed to detect differences between the footprint formed by CREB alone bound to the TxRE and the footprint formed by the complex of CREB-Tax (12). We also failed to demonstrate differences between CREB alone and CREB-Tax.
in methylation protection or methylation interference assays (data not shown). These results are surprising given the dependence of the Tax effect on flanking CG-rich sequences (8, 12); however, the approaches utilized address only a limited portion of the DNA available for interaction with DNA-binding proteins. For example, the primary site of modification of DNA by dimethyl sulfate under neutral conditions is N-7 of guanine, in the major groove (30). Because of the possibility that Tax might interact with the minor groove of DNA, we employed the 2:1 complex of 1,10-phenanthroline-copper ((OP)_{2}Cu^{+}), a chemical nuclease that cleaves selectively in the minor groove (34, 43). (OP)_{2}Cu^{+} cleaves the phosphodiester bond in the floor of the minor groove through oxidative attack of the C-1 hydrogen of deoxyribose, a cleavage reaction sensitive to protein binding in the minor groove, or major groove binding that disrupts the structure of the minor groove (43). We consequently asked whether Tax extended or altered the footprint of the CREB-binding site on the TxRE, using (OP)_{2}Cu^{+}, reasoning that Tax might make minor groove contacts with DNA in the GC-rich sequences flanking the central CRE-like element of the 21 base pair repeats. Fig. 4 shows the results of the footprinting of CREB alone or CREB-Tax on the HTLV-1 LTR, demonstrating a protection of cleavage around the promoter-proximal TxRE (−113 to −77). The protection of CREB-bound DNA from (OP)_{2}Cu^{+} cleavage is modest in the absence of Tax. Nevertheless, the cleavage pattern is diminished within the immediate 5’ and 3’ GC-rich regions flanking the CRE-like core (−97 to −90) of the TxRE in the presence of CREB+Tax relative to CREB alone. Tax M47 (L319R, L320S), a mutant that fails to transcriptionally activate the HTLV-1 LTR promoter (59), also fails to augment the footprint (data not shown).

Minor Groove-binding Drugs Prevent Tax Enhancement of CREB DNA Binding Activity and Ternary Complex Formation—Distamycin A and chromomycin A₃ are reversible minor groove-binding drugs with sequence preference for AT- or GC-rich regions, respectively. This relative specificity in DNA interaction has proven useful for selective disruption of the DNA binding activity of minor groove-binding proteins (44, 45). The Tax-dependent interaction of CREB with the TxRE depends on flanking GC-rich sequences (8, 16, 17); thus, we expected that minor groove-binding drugs with GC preference might specifically disrupt Tax activity. Because CREB, like other B/ZIP factors, interacts with the major groove of DNA, we reasoned that these drugs would not disturb the interaction of CREB to DNA, but rather specifically disrupt the Tax enhancement of DNA binding.

Gel mobility shift assay was used to measure the association of CREB with either the consensus somatostatin CRE or the TxRE derived from the promoter proximal 21 base pair repeat of the HTLV-1 LTR (21) in the absence or presence of 250 nM bacterially expressed Tax. As we demonstrated previously by using fluorescence polarization, CREB binds with high affinity to the somatostatin CRE (Fig. 5B, upper panel). In contrast, CREB binds poorly to the promoter proximal TxRE, although the core CRE-like sequence differs from the somatostatin CRE by only one base pair (-TGACGTA- versus -TGACGACA-) (Fig. 5A, upper panel). Tax enhances the DNA binding activity of CREB to the TxRE by greater than 10-fold. In addition, we reproducibly observe a modest supershift of the complex consistent with the findings of other laboratories (indicated by * complex, Fig. 5A, upper panel) (14, 25, 46), reflecting either the increased size of the CREB-Tax-DNA complex, or some alteration in the shape of the complex, such as DNA bending (47).

We then asked whether minor groove-binding drugs could prevent the Tax-enhanced DNA binding of CREB. For these experiments, gel shift reactions were pre-incubated with the minor groove-binding drug and all components except protein for 30 min. After the addition of protein, the reactions were equilibrated by incubation at 20 °C for 1 h. Distamycin A at concentrations up to 2 μM had no effect on CREB binding to the CRE or on Tax enhanced binding of CREB to the TxRE (Fig. 6), a concentration that completely blocks the interaction of TBP with the TATA element (45). Binding of CREB to the somatostatin CRE was likewise unaffected by distamycin A (data not shown). In contrast, chromomycin A₃ at 0.1 μM nearly completely blocked Tax-enhanced binding of CREB to the TxRE, but had no effect on the low affinity basal interaction of CREB with the TxRE in the absence of Tax (Fig. 5A) or the high affinity interaction of CREB with the somatostatin CRE (Fig. 5B). At higher concentrations of drug, the interaction of CREB with both DNAs is partially disrupted, perhaps due to distortion of the major groove from minor groove saturation (48).

Chromomycin A₃, but not distamycin A, also prevented assembly of Tax into the CREB-TxRE complex. We previously demonstrated, using a DNA-directed protein complex assembly assay (19), that Tax recruitment was DNA sequence-dependent, and was not seen in the context of the consensus somatostatin CRE. The CREB-TxRE complex is not supershifted by Tax in the presence of chromomycin A₃ by gel shift assay (see Fig. 5A, middle and lower panels), suggesting that either Tax is not present in the complex or that Tax is present, but unable to alter the conformation of the ternary complex to shift electrophoretic mobility. In contrast, distamycin A fails to disrupt the
supershift observed for the Tax-CREB-DNA complex (Fig. 6).

These results suggest that Tax mediates effects on CREB DNA binding through the minor groove of DNA, and support both the biochemical and functional data implicating the GC-rich flanking sequences of the TxRE in mediating assembly of the ternary complex.

Disruption of Minor Groove Interactions with Inosine-Cytosine-substituted Oligonucleotides—The disruption of Tax-enhanced DNA binding by the GC-selective minor groove-binding drug chromomycin A₃ suggested that the effect of Tax on DNA binding of CREB was mediated through the minor groove of the GC-rich DNA flanking the core CRE-like sequence. We therefore asked whether components of the minor groove of the GC-rich flanking sequences of the promoter proximal TxRE were required for Tax action. We synthesized a synthetic duplex oligonucleotide corresponding to the DNA sequence of the promoter proximal TxRE, except that guanine:cytosine base pairs in the 5' and 3' flanking domains were replaced with inosine:cytosine base pairs (IC-TxRE). Inosine differs from guanine only in the absence of an exocyclic amino group at the 2-position of the purine ring. Thus, the IC base pair has the appearance of a AT base pair in the minor groove, while preserving the stereochemistry of a GC base pair in the major groove (49).

FIG. 5. The minor groove-binding drug, chromomycin A₃, blocks Tax-enhanced DNA binding. Electrophoretic mobility shift assay of CREB binding to either the TxRE or the somatostatin CRE. A, increasing concentrations of CREB, were incubated with a duplex ³²P-end labeled oligonucleotide corresponding to the TxRE in the absence or presence of Tax, with either 0 µM (upper panel), 0.1 µM (middle panel), or 1 µM (lower panel) chromomycin A₃ (CA₃). B, increasing concentrations of CREB, were incubated with a duplex ³²P-end labeled oligonucleotide corresponding to the somatostatin CRE, with or without chromomycin A₃. The CREB concentration for each titration point is either 0 nM (lanes 1, 6, and 11), 0.5 nM (lanes 2, 7, and 12), 1 nM (lanes 3, 8, and 13), 10 nM (lanes 4, 9, and 14), or 100 nM (lanes 5, 10, and 15). Lanes 1–5 and 11–15 are without Tax; lanes 6–10 with 250 nM Tax. The supershifted complex is indicated by “.*

DISCUSSION

We proposed previously that Tax activates transcription of viral and cellular CRE-containing promoters through distinct mechanisms depending on the DNA context and the cellular CRE-binding protein that Tax targets (19, 21). In this model, the DNA sequence of the CRE plays a critical role in determining the topology of transcription factors in the activator complex. The finding that Tax enhances DNA binding of CREB via interactions through the minor groove of DNA flanking the core CRE-like sequences of the TxRE is consistent with this model. We suspect that Tax does not merely enhance CREB dimerization, but rather contributes to the recognition of specific DNA sequences and directs the assembly of a specific multiprotein complex that includes CRE-binding proteins and the transcriptional coactivator CREB-binding protein (19, 21, 25).
concentrations for each lane are as indicated in Fig. 6.

For B/ZIP factors, dimerization is a prerequisite for DNA binding. Dimerization through the leucine zipper is complex, depending on hydrophobic interactions within the core coiled-coil interface (a and d positions) as well as interhelical electrostatic interactions between e and g positions between peptide strands (50, 51). Although peptides corresponding to the basic regions of B/ZIP proteins may specifically interact with DNA with low affinity (52, 53), mutations in the leucine zipper motif have deleterious effects on DNA binding affinity (32). Despite the importance of dimerization on DNA binding activity, and the potential of this step for regulation of DNA binding, few studies quantitatively address this interaction. In fact, most arguments that favor an effect of Tax on dimerization use the measurement of DNA binding as a surrogate for dimerization potential (15). Studies of the yeast B/ZIP factor GCN4 suggest that Tax enhances DNA binding through enhancement of dimerization (26, 27); however, the GCN4 B/ZIP may not be an appropriate model for all B/ZIP factors. Weiss et al. (38) demonstrated that the affinity of GCN4 B/ZIP for the CRE is at least 10–20-fold lower than the affinity of CREB for the same site (29). Also, unlike CREB, GCN4 binds both CRE and AP-1 as well as interhelical electrostatic interactions between e and g positions between peptide strands (50, 51). Although peptides corresponding to the basic regions of B/ZIP proteins may specifically interact with DNA with low affinity (52, 53), mutations in the leucine zipper motif have deleterious effects on DNA binding affinity (32). Despite the importance of dimerization on DNA binding activity, and the potential of this step for regulation of DNA binding, few studies quantitatively address this interaction. In fact, most arguments that favor an effect of Tax on dimerization use the measurement of DNA binding as a surrogate for dimerization potential (15). Studies of the yeast B/ZIP factor GCN4 suggest that Tax enhances DNA binding through enhancement of dimerization (26, 27); however, the GCN4 B/ZIP may not be an appropriate model for all B/ZIP factors. Weiss et al. (38) demonstrated that the affinity of GCN4 B/ZIP for the CRE is at least 10–20-fold lower than the affinity of CREB for the same site (29). Also, unlike CREB, GCN4 binds both CRE and AP-1 elements (37, 38).

The dimerization affinity that we measured for CREB in the absence of DNA is higher than expected based on comparison to the GCN4 B/ZIP, and represents the first determination of the dimerization affinity of an intact B/ZIP transcription factor. Weiss et al. (38) indirectly measured GCN4 B/ZIP association by following changes in a-helical content by circular dichroism at varying peptide concentrations. In the absence of DNA, the GCN4 B/ZIP underwent a helical transition at micromolar concentrations of peptide, consistent with folding of the leucine zipper into a coiled-coil conformation. The peptide corresponding to the isolated GCN4 leucine zipper may have a lower relative affinity of association (54), suggesting that protein sequences outside of the leucine zipper per se may contribute to the folding of the dimerization domain. Santiago-Rivera et al. (39) likewise examined the behavior of the CREB B/ZIP module by circular dichroism, and suggested that CREB dimerizes with micromolar affinity. The B/ZIP peptide used for that study bound DNA with 200-fold lower affinity than either native, full-length CREB (29, 55) or bacterially expressed CREB B/ZIP,2 however. Estimations of the dimerization affinities of Fos and Jun are also widely divergent, with measured values over nearly a 100-fold range (35, 56, 57). This variation perhaps also reflects differences in the size of the peptides used, or in the methods of determination.

The observation that Tax does not enhance the dimerization of CREB is not surprising, given the DNA sequence specificity of the Tax effect. Factors that strengthen dimerization of B/ZIP proteins would be expected to enhance the binding to all target DNA sequences because of the coupled equilibrium for dimerization and the subsequent dimer-DNA interaction in the DNA-binding pathway. For Tax and CREB at least, this seems not to be the case, although several investigators have demonstrated that Tax enhances the general affinity of the GCN4 B/ZIP peptide for DNA (15, 26, 27). The discrepancy between the results with CREB and the studies of GCN4 may relate to a lower affinity of dimerization for the yeast DNA-binding protein. In addition, Tax itself may dimerize (27, 46), and appears to interact with specific residues within the basic region of B/ZIP factors (24, 27). The enhancement of the DNA binding activity of CREB occurs at a half-maximal effective concentration of 10^-7 M Tax (data not shown), although dimerization of Tax is proposed to occur at low nanomolar concentrations (46). Consequently, dimerized Tax may contribute to dimerization of B/ZIP factors that have low affinity leucine zippers, such as GCN4, through symmetric intersubunit contacts between the basic regions.

Our experiments suggest that Tax enhances the DNA binding activity of CREB for the TxRE through minor groove interactions with the GC-rich flanking sequences. However, the observation that Tax extends the CREB footprint within the context of the TxRE contrasts with previous studies. For example, Pacca-Uccaralertkun et al. (12) did not observe an extension of the CREB footprint on the Tax-responsive element by using either DNase I footprinting or methylation interference assays. However, these methods lack the resolution to detect subtle differences in protein-DNA interactions. For example, dimethyl sulfate modification of guanine occurs primarily at the N-7 of the purine ring in the major groove and would not interfere with minor groove interactions.

The GC-rich sequences flanking the core CRE-like element are essential for Tax enhanced binding of CREB to the TxRE (8, 12, 16). Although the direct interaction of Tax with DNA cannot be demonstrated with the techniques described, our conclusion that Tax interacts with the GC-rich portion of flanking DNA is based on three lines of experimental evidence. First, Tax extends the footprint of CREB produced by the chemical nuclease, 1,10-phenanthroline-copper complex ([OP]2Cu+)2, a minor groove-selective cleavage agent. [OP]2Cu+ cleavage is, however, partially sequence-dependent and cleavage could potentially be altered by distortion of the minor groove by a major groove protein binding (43). The inability to detect major groove interactions by methylation interference makes this

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2 J. Richards, unpublished observation.
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possibility unlikely. Second, chromomycin A₉, a reversible minor groove-binding drug with GC selectivity, specifically blocks the Tax-enhanced DNA binding activity of CREB to the TxRE and prevents formation of the Tax-CREB-TxRE ternary complex. Finally, the specificity of contact in the minor groove is confirmed by substitution of deoxyinosinose for deoxyguanosinose in the sequences immediately flanking the core CRE-like portion of the TxRE. The dI-dC base pair has the appearance of a single base pair, yet has more than a 20-fold lower affinity for CREB (19). Surprisingly, CREB binds the dI-dC-substituted TxRE with increased affinity relative to the native TxRE, and Tax fails to enhance binding further or associate with the CREB-DNA complex. The hierarchies of affinities of CREB for various CRE sequences have not yet been systematically determined, although functional studies suggest that flanking sequences contribute to activity (58). In addition, the affinity of CREB for the even more divergent tyrosine aminotransferase (TAT) promoter-derived CRE (5’-TGACCGCAG-3’) differs by less than 5-fold from that of the somatostatin CRE, as measured by fluorescence polarization (29). The most plausible explanation for our observations is that the efficacy of the CRE-like sequence within the viral LTR is determined in part by the context of flanking DNA.

The TxREs of the HTLV-1 LTR vary considerably (see Ref. 1), not only in the extent of divergence of the core sequence from the canonical CRE, but also in the specific sequence of the flanking GC-rich DNA, although core elements are conserved. Oligonucleotide binding site selection experiments using the CREB-Tax complex demonstrate the imprecise nature of DNA sequence recognition by Tax and suggest that Tax targets elements with similar sequence composition but non-identical sequences (12). Features other than primary DNA sequence per se, such as DNA deformability, or conformational microheterogeneity, may define which CREs are responsive to Tax. For example, optimal binding of CREB to the TxRE may require alteration of the DNA structure (47). Structural characterization of different GCN4-BZIP-DNA complexes demonstrates how this class of proteins might interact with dissimilar sequences. The GCN4 BZIP binds both the AP-1 site (-TGACCTA-) and the CRE site, which contains an additional central GC base pair. Comparison of the structures of these GCN4 BZIP-DNA complexes demonstrates that this is accomplished primarily by local distortion of the DNA helix (41, 42). Thus, Tax may interact with the minor groove to cause a local DNA unwinding or melting and therefore allow CREB to overcome an unfavorable binding energy by altering the DNA structure.

The conversion of the TxRE from a low affinity to a relatively high affinity CREB-binding site by substitution of inosine for guanine bases in flanking sequences favors an architectural role for Tax in DNA binding. DNA with inosine substitution will have altered thermal stability due to the loss of the third hydrogen bond of a dG-dC pair, and altered base pair stacking. In this model, Tax would act through DNA bending, melting or otherwise altering the structure of an unfavorable CREB-binding site to reduce the free energy of CREB binding. CREB provides the specificity, targeting the Tax-CREB complex to the core CRE-like motif, augmented by Tax interactions with GC-rich flanking DNA sequences. The Tax interaction may require the exocyclic 2-amino group of guanine as a hydrogen bond donor. Alternatively, the narrow minor groove in IC-substituted DNA might preclude other minor groove-Tax interactions with, for example, backbone sugars or bases. A model suggesting that Tax facilitates “prefolding” of the basic segment of the BZIP domain (15) cannot account for the dependence of the Tax effect on GC-flanking sequences.

Fig. 8 shows a model for the interaction of CREB with DNA, indicating the positions of postulated flanking sequence minor groove interactions. In this model, Tax interacts with the outer face of the CREB basic region segment as it lies in the major groove of the core CRE-like DNA sequence, and additionally interacts with flanking GC-rich DNA on the same face of the DNA helix through minor groove contacts. The model suggests two molecules of Tax per CREB dimer bound to DNA; however, the stoichiometry of the components within this complex is unknown.

Are these processes unique to the Tax-responsive sequences of the viral promoter? The hepatitis B virus X protein may employ a similar mechanism for enhancement of the DNA binding activity of CREB and other BZIP factors to variant CREs within the hepatitis B virus enhancer (60–62). In addition, subsets of cellular genes responsive to Tax may also have elements that are specifically recognized by the Tax-CREB complex. Low et al. (63) showed that the enkephalin CRE might be a target of Tax, through recruitment of the CRE-binding protein, ATF-3.

This model also raises the possibility that cellular CREs divergent from the canonical somatostatin CRE may also utilize accessory cellular factors to specifically recruit CREB in vivo. Other examples of complexes in which the DNA binding specificity of individual components is expanded by protein-protein interactions include the p65-C/EBP protein complex (64), and AP-1/glucocorticoid receptor interactions (65). The
model suggests a greatly increased DNA binding specificity for CREB, and provides a basis for the selectivity of the transcriptional response to this ubiquitous factor.

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Note Added in Proof—While this manuscript was under review, Lenzmeier et al. (66) reported that recruitment of CREB to the Tax-responsive element depends on an interaction of Tax with the DNA minor groove.

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