

# Expansion of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282–284 near the conserved DNA-binding domain of CREB

(human T-lymphotropic virus type I/cAMP response element/leucine zipper/protein–protein interaction)

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**ABSTRACT** The transactivator protein of human T-lymphotropic virus type I (HTLV-I), Tax, forms multiprotein complexes with the ubiquitous transcription factor CREB and the CREB/ATF-1 heterodimer. The interaction between Tax and CREB is highly specific and results in increased binding of the Tax/CREB complexes to the HTLV-I 21-bp repeats. Despite the extensive sequence similarities between CREB and ATF-1, Tax interacts with ATF-1 only marginally. Compared with CREB, Tax/CREB exhibits greatly increased DNA recognition specificity and preferentially assembles on a consensus binding site, GGGGG(T/A)TGACG(T/C)(A/C)TA(T/C)C-CCCC, homologous to the HTLV-I 21-bp repeats. Here we report that Tax affects CREB binding to the Tax-inducible DNA elements by interacting with the basic–leucine zipper (bZip) domain of CREB. We show by domain switching that the basic region in CREB bZip can confer on c-Jun and ATF-1 leucine zippers the ability to interact with Tax *in vitro*. Mutational analyses further demonstrate that the amino acid residues of CREB critical for Tax/CREB interaction are Ala-Ala-Arg at positions 282–284 (AAR<sup>284</sup>), immediately upstream of the highly conserved DNA-binding domain (R/K)XX(R/K)N(R/K)XAAXX(S/C)RX(R/K)(K/R) characteristic of all bZip proteins. Specific amino acid substitutions in AAR<sup>284</sup> of CREB weakened or abolished Tax/CREB interaction, whereas reciprocal changes in ATF-1 allowed it to interact with Tax. These results support a model in which the specific interaction between Tax and the AAR<sup>284</sup> residues near the DNA-binding domain of CREB results in a multiprotein complex with altered DNA recognition property. This protein complex assembles selectively on the viral Tax-responsive 21-bp repeats to augment transcription.

Tax is a 40-kDa nuclear protein encoded by the 3' region of the genome of human T-lymphotropic virus type I (HTLV-I). It stimulates HTLV-I transcription via three imperfect 21-bp repeats in the proviral long terminal repeat (1–3). cAMP response element (CRE)-like motifs in the repeats are crucial for Tax transactivation (3). We previously detected three cellular protein factors in Jurkat T cells and HeLa cells that bound specifically to the CRE in the HTLV-I 21-bp repeats. Two of these factors interacted with Tax directly (4, 5). These three cellular factors were identified to be CREB homodimer, CREB/ATF-1 heterodimer, and ATF-1 homodimer (5). Tax interacts directly with the CREB subunits in CREB homodimer and CREB/ATF-1 heterodimer and stabilizes their binding to the HTLV-I 21-bp repeats (5). Both CREB and ATF-1 are members of the basic–leucine zipper (bZip) family of transcription factors and are highly similar in primary amino acid sequences (6–8). The interaction between Tax

and CREB is highly specific. Despite the extensive amino acid similarities shared between ATF-1 and CREB, Tax interacts marginally with ATF-1 (5). By selecting for preferred Tax/CREB or CREB binding sites *in vitro*, the Tax/CREB complex was found to exhibit greatly altered DNA recognition specificity compared with CREB (9). The consensus Tax/CREB binding site, GGGGG(T/A)TGACG(T/C)(A/C)TA(T/C)C-CCCC, is homologous to the HTLV-I 21-bp repeats and highly inducible by Tax *in vivo*. The G and C sequences flanking the CRE motif are crucial for Tax/CREB assembly and Tax trans-activation but do not appear to contact directly the Tax/CREB complex (9). In this study, we show that the basic region in the CREB bZip motif is crucial for Tax/CREB interaction. Amino acid sequence comparison between CREB and ATF-1, coupled with domain switching and targeted mutagenesis, identify the Ala-Ala-Arg residues at 282–284 (AAR<sup>284</sup>) in CREB bZip to be crucial for Tax/CREB interaction. These three amino acid residues lie in the immediate vicinity of the highly conserved DNA-binding domain (RXXKNRXXAAXXCRXRK) of CREB. Specific amino acid substitutions in AAR<sup>284</sup> of CREB weakened or abolished Tax/CREB interaction, whereas reciprocal amino acid substitutions in ATF-1 conferred an ability to interact with Tax. These results help explain the specificity of Tax/CREB interaction and implicate a mechanism for regulating the DNA recognition specificity and transcriptional activity of the large family of CREB/ATF proteins.

## METHODS

**Plasmid Constructs.** The glutathione S-transferase (GST)–bZip fusion proteins and various CREB and ATF-1 mutants were constructed by PCR. Their DNA sequences were confirmed by the dideoxy chain-termination method. Details of the constructions can be obtained upon request.

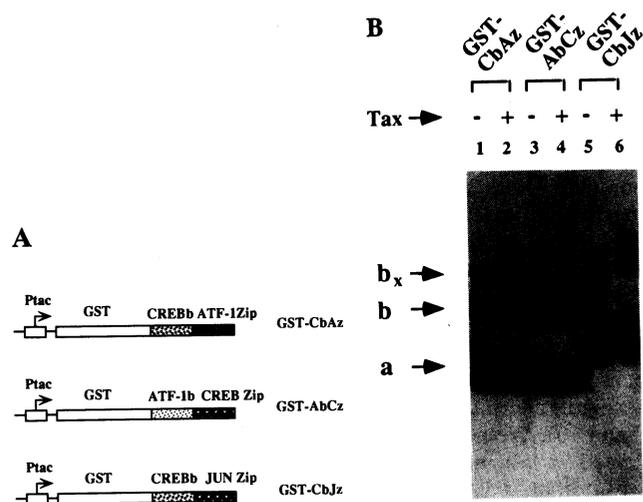
**Coimmunoprecipitation of Tax and CREB.** The TNT T7 coupled reticulocyte lysate system from Promega was used to translate the various CREB/ATF-1 mutant proteins *in vitro*. Protein A-Sepharose was prepared in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.5% dry milk. Tax-C antibody (5  $\mu$ l) was then added to the protein A-Sepharose (50  $\mu$ l) for 1 hr at 4°C. The Sepharose was then washed twice with the same buffer containing 0.1% Nonidet P-40. Translated proteins were precleared by incubation with protein A-Sepharose/Tax-C antibody complex for 30 min at 4°C. Equivalent amounts of precleared wild-type or mutant CREB and ATF-1 proteins (8  $\mu$ l each) were incubated with purified Tax (400 ng) in 1 $\times$  gel-shift binding buffer (5) for 30

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Abbreviations: BLV, bovine leukemia virus; bZip, basic–leucine zipper; CRE, cAMP response element; GST, glutathione S-transferase; HTLV-I, human T-lymphotropic virus type I.

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**Fig. 2.** The basic domain of CREB confers ability to interact with Tax. (A) The GST fusion proteins contained the basic domain of CREB and the leucine zipper of ATF-1 (GST-CbAz), the basic domain of ATF-1 and the leucine zipper of CREB (GST-AbCz), or the basic domain of CREB and the leucine zipper of c-Jun, GST-CbJz. (B) Tax interacts with the basic domain of CREB. Gel shift assay was as in Fig. 1C. Purified GST-CbAz was used in lanes 1 and 2, GST-AbCz in lanes 3 and 4, and GST-CbJz in lanes 5 and 6. Lanes 2, 4, and 6 contained 100 ng of Tax.

GST-CbAz contains the basic domain of CREB and the leucine zipper of ATF-1, and GST-AbCz contains the basic domain of ATF-1 and the leucine zipper of CREB. Both fusion proteins bound to the DNA probe to produce complexes a and b (Fig. 2B, lanes 1 and 3). As indicated, Tax specifically altered the mobility of complex b formed by GST-CbAz (lane 2), but not that of a similar complex formed by GST-AbCz (lane 4). These results indicate that the basic domain, instead of the leucine zipper, of CREB confers the ability to interact with Tax. Consistent with these results, a fusion protein, GST-CbJz (Fig. 2A), containing the basic domain of CREB and the leucine zipper of c-Jun also interacted with Tax (compare lanes 5 and 6). The complex b formed by GST-CbJz appeared as a doublet, most likely due to protein degradation at the relatively long carboxyl terminus of the c-Jun zipper. The GST-JunbZip protein did not form a detectable level of nucleoprotein complex with the DNA probe used (unpublished data).

**Specific Amino Acid Substitutions Immediately Adjacent to the Conserved DNA-Binding Domain of CREB or ATF-1 Abrogate or Confer Ability to Interact with Tax *in Vitro*.** CREB and ATF-1 share extensive amino acid sequence homology in their bZip domains. The specific interaction between Tax and the basic domain of CREB implies that those amino acid residues unique in this region of CREB must play a critical role in interacting with Tax. Amino acid sequences of the basic domains of CREB and ATF-1 (Fig. 1B) differ in six residues, EEAAR<sup>284</sup> and V<sup>288</sup> in CREB vs. DDPQL<sup>214</sup> and I<sup>218</sup> in ATF-1, immediately upstream of the DNA-binding domain, RXXKNRXXAAXXCXRK, of CREB. To determine which of these amino acids were important for Tax interaction, reciprocal amino acid substitutions in this region of CREB and ATF-1 were constructed (Fig. 3A). Since AAR<sup>284</sup> (CREB) and PQL<sup>214</sup> (ATF-1) represent the most significant amino acid sequence divergence between the two proteins, our mutational analyses were targeted towards these residues. Three CREB mutants, CREB AAR<sup>284</sup> → PQL, CREB A<sup>282</sup> → P, and CREB R<sup>284</sup> → L were constructed (Fig. 3A). A corresponding ATF-1 mutant, ATF-1/C, was similarly created by replacing aa 201–214 (including PQL<sup>214</sup>) of ATF-1 with aa 271–284 (including

AAR<sup>284</sup>) of CREB. DNA sequences of these mutants were then confirmed by the dideoxy method. The mutant proteins were expressed by using the pET11 vector (Novagen), purified by DNA cellulose affinity chromatography, and used in gel shift assays with <sup>32</sup>P-labeled 21(1) DNA. All CREB mutants bound 21(1) (Fig. 3B, lanes 3, 5, and 7) to yield protein–DNA complexes that comigrated with the wild-type CREB/21(1) complex (lane 1, indicated by arrow at left). Tax interacted and formed slower-migrating ternary complex with wild-type CREB/21(1) (lane 2) and CREB A<sup>282</sup> → P/21(1) (lane 6) but failed to affect the CREB AAR<sup>284</sup> → PQL/21(1) (lane 4) or CREB R<sup>284</sup> → L/21(1) (lane 8) complex. This result was further supported by the Tax-C antibody-induced supershifts of Tax/CREB/21(1) and Tax/CREB A<sup>282</sup> → P/21(1) ternary complexes (lanes 9 and 11). As anticipated, Tax and Tax-C antibody had little effect on the CREB AAR<sup>284</sup> → PQL/21(1) (lane 10) or CREB R<sup>284</sup> → L/21(1) (lane 12) complex. These data indicated that the AAR<sup>284</sup> sequence of CREB plays a critical role in interaction with Tax. This conclusion is further strengthened by the reciprocal experiment carried out with the mutant ATF-1/C protein engineered to contain the amino acid sequence required for Tax interaction. As previously reported (5) and as shown in Fig. 3B, the ATF-1/21(1) complex could not interact with Tax (compare lanes 13 and 14). In contrast, Tax formed a slower-migrating ternary complex with 21(1) and mutant ATF-1/C containing AAR in place of PQL<sup>214</sup> (compare lanes 15 and 16). Further, Tax and Tax-C antibody specifically caused a supershift of the ternary complex (lane 18) but had no effect on the ATF-1/21(1) complex (lane 17).

**Coimmunoprecipitation of Various Wild-Type and Mutant CREB and ATF-1 Proteins with Tax *in Vitro* Substantiates the Critical Role of the CREB AAR<sup>284</sup> Residues in Tax Interaction.** To examine further the role of the CREB AAR<sup>284</sup> residues in interaction with Tax, we tested the ability of CREB, ATF-1, and various mutants to coimmunoprecipitate with Tax. Approximately 1 μg of CREB and ATF-1 expression plasmids (pET11-CREB, pET11-ATF-1, and derivatives) were added to the *in vitro* transcription/translation system for protein expression. Under these conditions, similar amounts of [<sup>35</sup>S]methionine-labeled proteins were synthesized (Fig. 4D). This allows the degree of interaction of various proteins with Tax to be quantified. Equivalent amounts of labeled CREB, ATF-1, and their mutants were incubated with (Fig. 4A, lanes 2, 4, 6, 8, and 10) or without (Fig. 4A, lanes 1, 3, 5, 7, 9, and 11) purified Tax and precipitated with Tax-C antibody in the absence of DNA. Tax interacts strongly with CREB (lanes 2, compare with control in lane 1) but only marginally with ATF-1 (lanes 3 and 4). Approximately 5% of the input <sup>35</sup>S-labeled CREB was precipitated, indicating that the amount of CREB in the reaction mixture was not limiting. Consistent with results from the gel mobility-shift assays, substitutions of PQL for AAR<sup>284</sup> drastically reduced mutant CREB interaction with Tax to 4% that of the wild-type protein (Fig. 4A, lanes 7 and 8, and Fig. 4B). Individual amino acid changes, R<sup>284</sup> → L (lanes 11 and 12) and A<sup>282</sup> → P (lanes 9 and 10) weakened mutant CREB interaction with Tax to 24% and 57% (Fig. 4B) that of the wild-type, respectively. As expected, in sharp contrast to ATF-1, ATF-1/C interacted strongly with Tax (Fig. 4A, compare lanes 4 and 6). In the absence of Tax, nonspecific binding of the labeled proteins to Tax-C antibody was negligible (Fig. 4A, lanes 1, 3, 5, 7, 9, and 11). Further, the immunoprecipitation with Tax-C antibody was effectively blocked by the Tax-C peptide (NEKEAD-NDHEPQISPGGLEPPSEKHFRETEV, residues 322–353) against which the Tax-C antibody was raised (Fig. 4C, lanes 3 and 4) but not by a nonspecific peptide (WSELVAS-RKIRLDSPLKLQLENEWLSRLF) (Fig. 4C, lane 5). These results are in agreement with those obtained with the gel mobility-shift assays and indicate the importance of

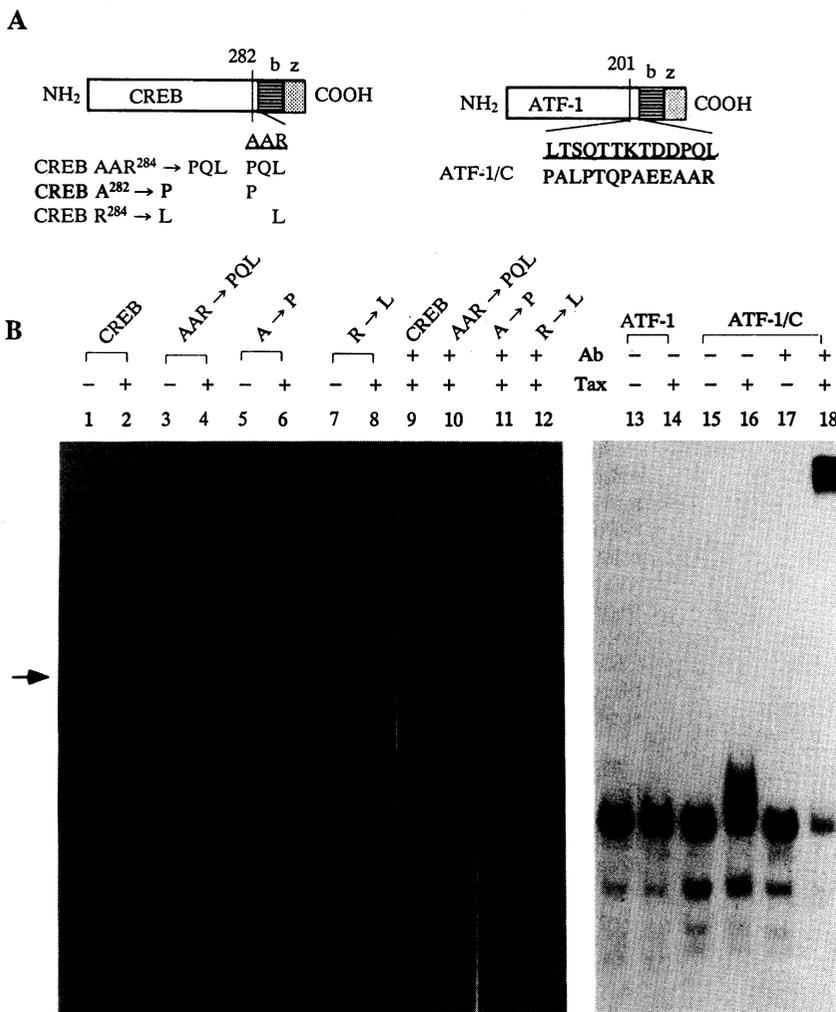


FIG. 3. Specific amino acid substitutions immediately adjacent to the conserved DNA-binding domain of CREB or ATF-1 abrogate or confer ability to interact with Tax. (A) Amino acid residues AAR<sup>284</sup> in CREB were changed to PQL (CREB AAR<sup>284</sup> → PQL), A<sup>282</sup> to P (CREB A<sup>282</sup> → P), and R<sup>284</sup> to L (CREB R<sup>284</sup> → L). Residues 202–214 of ATF-1 were replaced with residues 272–284 of CREB to generate ATF-1/C. Amino acid residues present in the wild-type proteins are underlined. Letters b and z indicate the basic domain and leucine zipper, respectively. (B) Gel shift was as in Fig. 1 with a 46-bp *Bgl* II-*Nco* I DNA fragment containing only the promoter-proximal 21(1) repeat. Arrows mark the major CREB or ATF-1 protein/21(1) complexes. The various CREB or ATF-1 proteins (10–20 ng) used are indicated on the top of each lane. Lanes 2, 4, 6, 8–12, 14, 16, and 18 contained ≈100 ng of Tax, and lanes 9–12, 17, and 18 contained 0.5 μl of the Tax-C antibody.

residues AAR<sup>284</sup> upstream of the conserved DNA-binding domain in CREB in interaction with Tax.

DISCUSSION

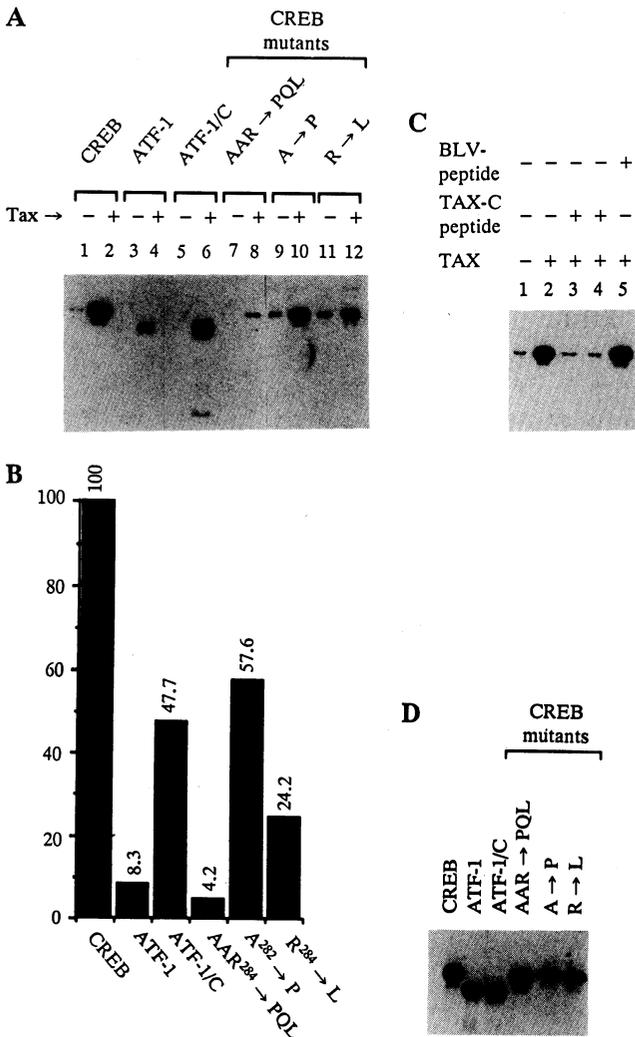
In this paper, we show the amino acid residues AAR<sup>284</sup> of CREB to be critical for strong interaction with the HTLV-I transactivator, Tax. This study extends our previous results showing that Tax increases the DNA recognition specificity of CREB and facilitates the formation of ternary complexes containing Tax, CREB, and CRE motifs flanked with long runs of G-rich and C-rich sequences. Apparently, by interacting with the AAR<sup>284</sup> residues in the proximity of the conserved DNA binding domain of CREB, Tax alters the DNA recognition characteristics of CREB. Via this interaction Tax recruits CREB into a multiprotein complex that assembles preferentially on the viral 21-bp repeats to augment transcription (Fig. 5).

Recently, a model to explain the mechanism of HTLV-I Tax action was proposed (12). According to this model, HTLV-I Tax protein facilitates the dimerization of a wide variety of proteins of the bZip family. It was suggested that the increase in dimerization resulting from Tax interaction caused the bZip transcription factors to bind their cognate DNA binding motifs with increased efficiency, which led to augmentation of transcription by Tax. Whereas Tax may interact with many bZip proteins under specific *in vitro* conditions (12), our results to date differ from this model. We found that under conditions where bZip proteins were limiting, Tax interacted specifically with CREB but only weakly with other bZip proteins such as ATF-1 or ATF-3. The Tax/CREB interaction is through a

unique region adjacent to CREB's DNA-binding domain. This interaction between Tax and CREB has two consequences. First, a Tax/CREB protein complex with altered DNA-binding specificity is formed. This multiprotein complex assembles specifically on the viral Tax-responsive 21-bp repeats. Second, the stable assembly of the ternary complex allows Tax to be anchored indirectly on DNA. This most likely results in the tethering of the transcriptional activation domain present in Tax on the Tax-responsive element. This conclusion is consistent with observations that only a subset of CRE elements are targets for Tax activation (9) and with results showing that Tax contains a transactivating domain at its carboxyl-terminal end (13, 14).

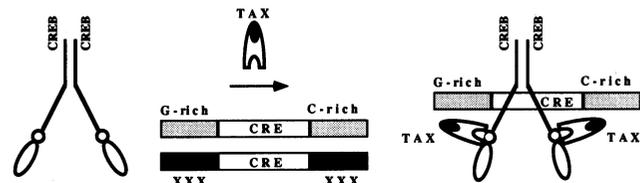
Although there is no evidence to show that Tax interacts directly with AAR<sup>284</sup> of CREB, the segregation of Tax binding with the presence of these residues as shown with the GST-bZip fusion proteins and the various CREB-ATF-1 mutants is strongly suggestive of a direct interaction. Several proteins called CREM, with amino acid sequences highly homologous to CREB, have been cloned (15). Consistent with recent data showing a direct interaction between CREM and Tax (16), the AAR<sup>284</sup> residues critical for Tax/CREB interaction are conserved in CREM.

The Tax/CREB complex preferentially recognizes a 21-bp sequence, highly homologous to the HTLV-I 21-bp repeats (9). Most intriguingly, Tax/CREB and CREB produced essentially the same footprints on the CRE of the 21-bp repeats (9). Although we favor a role of these flanking G or C sequences in altering DNA conformation in or around the CRE motif, the nature of their interaction with Tax/CREB complex and CRE remains to be determined. The DNA-



**FIG. 4.** Coimmunoprecipitation of CREB, ATF-1, and their mutants with Tax. (A) CREB/ATF-1 proteins were translated and labeled *in vitro*. *In vitro* translated proteins (8  $\mu$ l) were incubated with (lanes 2, 4, 6, 8, 10, and 12) or without (lanes 1, 3, 5, 7, 9, and 11) HTLV-I Tax (400 ng) and precipitated by Tax-C antibody. (B) Relative affinities of various CREB/ATF-1 proteins for Tax. Quantitation was by PhosphorImager (Molecular Dynamics). The amount of <sup>35</sup>S-labeled CREB bound to Tax was set to 100%. The affinities of other CREB/ATF-1 proteins for Tax were normalized against that of CREB and the number of methionine residues in the labeled proteins (six in ATF-1 and seven in CREB). (C) Coimmunoprecipitation of CREB and HTLV-I Tax is blocked by specific Tax-C peptide. Coimmunoprecipitation was as in A. Lanes 1 and 2 were without competing peptides. Tax-C peptide (10  $\mu$ g), the peptide against which the Tax-C antibody was generated (lanes 3 and 4), or bovine leukemia virus (BLV) control peptide (10  $\mu$ g) (lane 5) was used to block the binding of Tax-C antibody to Tax/CREB complex. In lane 3, the Tax-C peptide was included while Tax-C antibody was being bound to the protein A-Sepharose. In lane 4, Tax-C peptide was added together with Tax/CREB complex to the antibody binding mixture. (D) *In vitro* translation yielded similar amounts of [<sup>35</sup>S]methionine-labeled products.

binding domains of Fos/Jun (17) and GCN4 (18) convert from random-coil to  $\alpha$ -helical structure upon DNA binding, indicating that flexibility in the local conformation of the basic region is important for defining the DNA-binding properties of bZip proteins. Further, in addition to the residues that make direct contacts with DNA, nonconserved residues in the basic domain have also been shown to play a crucial role in sequence recognition by the bZip proteins (19). It is possible that by binding in close proximity to the DNA-



**FIG. 5.** A model for the mechanism of Tax action. Tax interacts specifically with the AAR<sup>284</sup> residues near the conserved DNA-binding domain of CREB to form a multiprotein complex which assembles preferentially on the viral 21-bp repeats containing the CRE motifs flanked by G- and C-rich sequences.

binding domain of CREB, Tax can induce a conformational change in CREB, resulting in preferential binding to the HTLV-I 21-bp repeats.

Similar mechanisms as described here may be employed by eukaryotic cells to modulate the DNA recognition specificity and transcriptional activity of other CREB/ATF- and c-Jun/c-Fos-like proteins. The transcriptional regulation of the related retrovirus BLV is also mediated by a viral transactivator, BLV Tax, and three 21-bp repeats in the BLV U3 region (20). The BLV repeats specify exclusive transactivation response to BLV Tax and, most interestingly, contain CRE motifs with distinct flanking sequences. We think BLV offers another example of the regulatory mechanism described here.

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