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The trans-activator Tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1

(DNA-binding proteins/protein–protein interaction)

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ABSTRACT **The trans-activator protein Tax of human T-cell leukemia virus type 1 (HTLV-1) activates the viral 21-base-pair (bp) enhancer in the long terminal repeat and has been suggested to associate indirectly with the enhancer DNA. To demonstrate this, we used DNA-affinity precipitation assay and detected the Tax protein in 21-bp DNA–protein complexes isolated from HTLV-1-infected cells. To identify cellular components in the complexes, we tested various 21-bp DNA-binding proteins by gel electrophoretic mobility-shift assay. Each binding protein gave a shifted band of each 21-bp DNA–protein complex, and exogenously added Tax protein further shifted these bands of cAMP-responsive element (CRE) binding protein (CREB) and CRE modulator but did not shift other bands. Anti-Tax antibodies blocked formation of the complex, indicating complex formations of [Tax–CREB(or CRE modulator)–21-bp DNA]. The formations of these complexes paralleled the functional activities of Tax mutants. Furthermore, the Tax–CREB complex was detected in a nuclear extract of HTLV-1-infected cells, and the Tax–CREB–21-bp-DNA complex was demonstrated as a major component of Tax complexes containing the 21-bp DNA probe. These observations indicate that Tax protein binds to CREB and CRE modulator and the complexes then bind to the 21-bp enhancer, suggesting that the complex binding to the enhancer mediates trans-activation of transcription.**

Human T-cell leukemia virus type 1 (HTLV-1) (1, 2) is an etiologic agent of adult T-cell leukemia (3–5) and also of tropical spastic paraparesis (also known as HTLV-1-associated myelopathy) (6, 7). Tax protein ($p40^{tax}$) of HTLV-1 enhances transcription from the viral long terminal repeat (LTR) (8–10) and, thus, is essential for the viral gene expression. Tax also activates expression of certain cellular genes such as those encoding interleukin 2 (IL-2) (11), IL-2 receptor α (11–13), granulocyte/macrophage colony-stimulating factor (14), c-Fos (15), and c-Jun (16). Activations of the IL-2 receptor α gene and some other protooncogenes have been proposed to contribute to the abnormal proliferation of HTLV-1-infected cells (17). The NF-κB-binding site was reported to be essential for trans-activation of the IL-2 receptor α gene (18, 19). On the other hand, Tax requires direct repeats of a 21-base-pair (bp) sequence that contains a consensus of the cAMP-responsive element (CRE) (20–23) for trans-activation of the viral LTR. Thus Tax protein trans-activates at least two nonhomologous enhancers.

Because there is no evidence for direct binding of Tax protein to these enhancers, the participation of cellular proteins was suggested (23–25). Previously we (26) showed that a fusion protein of Tax and the GAL4 DNA-binding domain has to bind to DNA to activate transcription and, thus, proposed that indirect association of Tax protein with the enhancer DNA is required for the transcriptional activation. In fact, Zhao and Giam (27) recently found that the Tax protein associates with the LTR 21-bp sequence only in the presence of nuclear extract, suggesting indirect binding of Tax protein to DNA. Another group (28) also reported that Tax protein was detected in LTR 21-bp DNA–protein complexes in a nuclear extract of Tax-expressing HeLa cells. For identification of cellular components, a number of cellular factors that bind to the LTR 21-bp sequence were studied—namely, Tax-responsive element-binding proteins (TREBs) (29, 30), CRE binding proteins (CREBs) (31), CRE modulator (CREM) (32), activating transcription factors (33), and some others (34). However, their functional significance in Tax-mediated trans-activation has not been shown.

Here, we report evidence that the CREB and CREM proteins interact with Tax protein on the LTR 21-bp enhancer and probably mediate trans-activation of transcriptional initiation.

MATERIALS AND METHODS

Construction of Plasmids and DNA Probes. All recombinant proteins were produced in a fused form with six histidine residues at their N terminus (35, 36). The coding sequences of TREB5 (29), TREB7 (29), TREB36 (29), a human counterpart of CREM (unpublished work), Tax (37), and Tax mutants (ref. 38 and unpublished work) were inserted into the *Nde* I–*Bam*HI site of 6HisT-pET11 vector (35), which has the T7 promoter (36). DNA probes were prepared by the PCR of 5-fold repeats of the 21-bp sequence cloned in plasmids (23) by using primers complementary to the junction regions of the vector. For the probe in DNA-affinity precipitation (DNAP) assay, the primer contained a biotinylated nucleotide and, for electrophoretic mobility shift, the PCR was done in the presence of [α-^{32}P]dCTP.

Purification of Histidine Fusion Proteins. *Escherichia coli* BL21 (DE3) cells containing 6HisT-pET11 derivatives were grown and induced by addition of isopropyl β-D-thiogalactoside at a final concentration of 1 mM. The histidine fusion proteins were purified from cell lysates on a (nickel nitrilotriacetate) resin column (Diagen, Düsseldorf, Germany) by step-wise elution with buffer containing 20–100 mM imidazole. Fractions containing histidine fusion proteins were dia-

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Abbreviations: HTLV-1, human T-cell leukemia virus type 1; LTR, long terminal repeat; Tax, transcriptional trans-activating factor of HTLV-1; TREB, Tax-responsive element-binding protein; CRE, cAMP-responsive element; CREB, CRE-binding protein; CREM, CRE modulator; DNAP assay, DNA-affinity precipitation assay; IL-2, interleukin 2.

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lyzed against 20 mM Hepes·KOH buffer, pH 7.9/80 mM KCl/0.2 mM EDTA/0.5 mM dithiothreitol/10% (vol/vol) glycerol/0.1 mM phenylmethylsulfonyl fluoride and used directly for the assays.

DNAP Assay. Nuclear and cytoplasmic extracts were prepared as described (38). Briefly, cells were lysed in a buffer containing 0.5% Nonidet P-40, and nuclei isolated by centrifugation were extracted with 0.4 M NaCl. The biotinylated DNA probe (1 μg) was mixed with cell lysates containing poly(dI·dC) (15 μg) in the presence or absence of the histidine–Tax (40 ng), and the mixture was incubated for 30 min on ice. Then, streptavidin–Dynabeads (Dynal, Great Neck, NY) were added with mixing by rotation for 30 min. The Dynabeads were collected with a magnet and washed twice with buffer. The trapped proteins were analyzed by SDS/PAGE followed by immunoblotting as described (38). The antibodies used were raised in rabbit against the C-terminal peptide of Tax (39) or CREB. The bands were detected with protein A–horse radish peroxidase conjugate and the enhanced chemiluminescence detection system (Amersham).

Gel Electrophoretic Mobility-Shift Assay. Histidine fusion protein (≈3–4 ng) was incubated at 4°C for 1 hr with or without histidine–Tax (≈8 ng) and then mixed with poly(dI·dC) (0.5 μg) and ^{32}P-labeled probe (10^5 cpm) in 20 mM Hepes·KOH buffer, pH 7.9/60 mM KCl/1 mM EDTA/2 mM dithiothreitol/3 mM spermidine in a final 10-μl vol. After 30 min at 4°C, the mixture was directly analyzed on nondenaturing 5% polyacrylamide gel in 90 mM Tris borate, pH 8.0/1 mM EDTA.

Immunoprecipitation. Purified histidine–Tax or histidine–d3 mutant (40 ng) and histidine–CREB (40 ng) were mixed in RIPA buffer and then treated with anti-CREB antibodies. A nuclear extract of HuT 102 cells was also treated similarly. The immunocomplexes isolated with protein A–Sepharose were separated by SDS/acrylamide gel electrophoresis and immunoblotted with anti-Tax antibodies.

RESULTS

Association of Tax with the 21-bp DNA–Protein Complexes. We had proposed that indirect association of Tax protein with the 21-bp enhancer sequence is required for activation of transcription (26). To demonstrate this interaction of Tax protein in HTLV-1-infected cells, we used a DNAP assay (28). Biotinylated DNA probes consisting of five direct repeats of the 21-bp sequence were generated by PCR with biotinylated primers. The biotinylated DNA probe was incubated with a nuclear or cytoplasmic extract of HuT 102 cells, a HTLV-1-infected T-cell line, and the DNA–protein complexes formed were isolated with streptavidin-conjugated magnet beads (Dynabeads). The isolated complexes were examined by immunoblotting for Tax protein (Fig. 1*A*). When the wild-type 21-bp sequence was used, Tax protein was detected in the 21-bp–protein complexes in the nuclear extract but was not detected in the cytoplasmic fraction (lanes 1 and 2). In a parallel experiment, Tax was detected in both the nuclear and cytoplasmic extracts at almost equal levels as reported (39). Therefore, these results clearly indicated that Tax protein complexes that can bind to the 21-bp DNA sequence were present only in the nuclear extract. However, an inactive B1 mutant (23) of the 21 bp that has a mutation in the CRE sequence did not complex (lanes 3 and 4), indicating that specific protein complexes on the 21-bp DNA sequence include the endogenous Tax protein.

We examined whether exogenous Tax can associate with the 21-bp sequence. A recombinant Tax protein (histidine–Tax) carrying six histidine residues at the N terminus was produced in *E. coli* and purified on a metal-chelating column. The histidine–Tax was incubated with nuclear and cytoplasmic extracts of Jurkat cells, an uninfected human T-cell line, and the 21-bp DNA–protein complexes were isolated by the DNAP method. As with endogenous Tax protein, histidine–

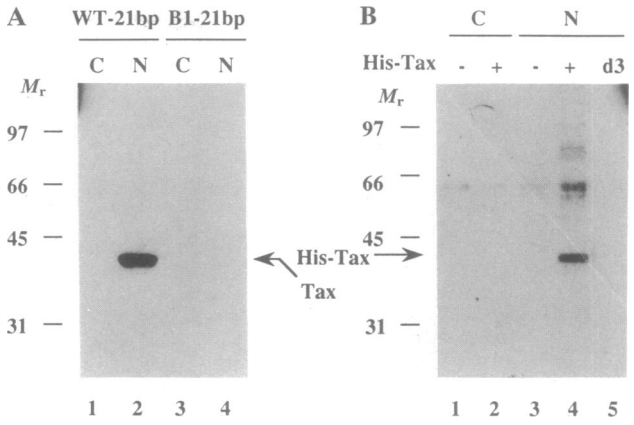

FIG. 1. Formation of a complex of endogenous or exogenous Tax protein with the 21-bp DNA sequence in nuclear extract. (*A*) Detection of Tax protein in the 21-bp DNA–protein complexes in HuT 102 cell extract. A cytoplasmic (C; lanes 1 and 3) or nuclear (N; lanes 2 and 4) extract of HuT 102 cells was incubated with the biotinylated wild type (WT; lanes 1 and 2) or mutant 21-bp probe (B1; lanes 3 and 4), and the isolated DNA–protein complexes were analyzed by immunoblotting with anti-Tax antibodies. (*B*) Nuclear factor-dependent association of exogenous Tax with the 21-bp sequence. A cytoplasmic (lanes 1 and 2) or nuclear (lanes 3 and 4) extract of Jurkat cells was incubated with the wild-type 21-bp probe in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of histidine–Tax. The DNA–protein complexes were analyzed by immunoblotting with anti-Tax. In lane 5, inactive Tax mutant histidine–d3 was used in the presence of Jurkat nuclear extract. M_r ($\times 10^{-3}$) markers are indicated.

Tax was detected in the 21-bp DNA–protein complexes formed in the nuclear extract but was not detected in cytoplasm (Fig. 1*B*, lanes 2 and 4). Histidine–Tax alone could not bind to the 21-bp sequence (data not shown). Thus, a nuclear factor(s) appears essential for forming the complex of histidine–Tax with the 21-bp sequence.

We next tested a mutant of Tax protein, d3, that has a deletion of three amino acids at the N terminus. This mutant was almost completely inactive in activation of the LTR but was almost fully active in activation of the NF-κB site (38). The histidine–d3 mutant did not associate with the 21-bp DNA–protein complexes (lane 5). This observation on Tax mutant strongly indicates that complex formation of Tax protein–21-bp DNA is specific to the functional Tax protein and, thus, suggest the significance of the complexes in the trans-activation of the 21-bp enhancer by Tax protein.

CREB Mediates Association of Tax with 21-bp DNA. To identify the nuclear proteins that mediate the binding of Tax protein to the 21-bp DNA sequence, we tested the histidine–Tax protein and various 21-bp binding proteins by gel electrophoretic mobility-shift assay; bacterially produced and purified histidine–TREB5, –TREB7 (CREBP-1), –TREB36 (ATF-1), and –CREB proteins were separately incubated with histidine–Tax and DNA probe containing five copies of the 21-bp sequence, and the mixture was analyzed by nondenaturing gel electrophoresis. Typical results are shown in Fig. 2. Each 21-bp binding protein shifted the band without histidine–Tax (lanes 1 and 5). Addition of histidine–Tax to the reaction mixture decreased mobility (super-shift) of the histidine–CREB–21-bp complex (lane 2) but did not decrease that of TREB36 (lane 6). TREB5 and TREB7 gave similar results to those with TREB36 (data not shown). Treatment of the reaction mixture with anti-Tax resulted in a band with the original mobility, but normal rabbit serum had no effect (lanes 3 and 4), indicating that the anti-Tax inhibits formation of the Tax–CREB–21-bp complex and dissociates Tax from the complex.

Correlation of Tax–CREB–21-bp Complex with Trans-Activation. To ascertain the functional significance of Tax

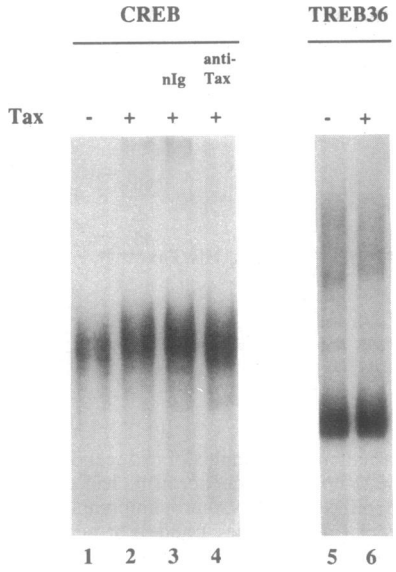

FIG. 2. Specific association of Tax protein with the CREB–21-bp complex. Histidine–CREB (lanes 1–4) or histidine–TREB36 (lanes 5 and 6) was incubated with (lanes 2–4, 6) or without (lanes 1 and 5) histidine–Tax. Normal rabbit IgG (nIg) and anti-Tax antibodies were added for lanes 3 and 4, respectively. Then ^{32}P-labeled 21-bp DNA probe was added, and mixtures were analyzed by nondenaturing gel electrophoresis.

binding to the CREB–21-bp DNA complex, we examined mutants of Tax protein. The four mutants used, d3 (38), d17/5 (38), d7/16 (38), and d320 (unpublished work), had deletions of amino acids 1–3, 112–115, 233–246, and 320–324, respectively. None of the four mutants activated the 21-bp sequence, but two of them, d3 and d320, could still activate NF-κB site. The effect of these mutants on interaction with CREB–21-bp complex was examined by gel-shift assay. Mutants did not induce super-shift of the band of the CREB–21-bp complex, except mutant d320 (Fig. 3). These results suggest that complex formation of Tax–CREB–21-bp DNA correlates with the trans-activating function of the Tax protein. The exceptional case of an inactive mutant d320 can be explained reasonably by assuming activation domain and CREB-binding domain in Tax protein, as to be described in *Discussion*.

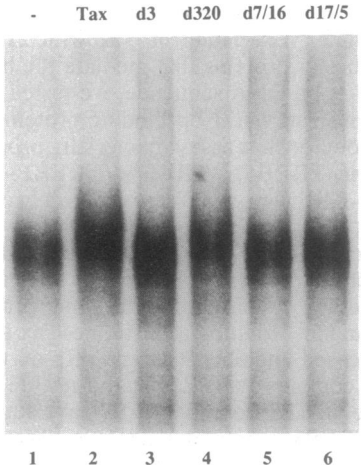

FIG. 3. Complex formation of Tax–CREB–21-bp DNA with mutant Tax protein. Histidine–CREB was incubated alone (lane 1), with wild-type histidine–Tax, or with mutant histidine–d3, –d320, –d7/16, or –d17/5 (lanes 2–6, respectively). Then ^{32}P-labeled 21-bp DNA probe was added, and mixtures were analyzed by nondenaturing gel electrophoresis.

Tax Binding to CREB Is Independent of DNA. We examined whether Tax protein can bind to CREB in the absence of 21-bp DNA; a mixture of purified histidine–Tax and histidine–CREB was treated with anti-CREB antibodies, and histidine–Tax was detected in the immunoprecipitates by immunoblot analysis (Fig. 4*A*). The inactive mutant d3 did not form a complex with histidine–CREB, clearly indicating specific binding of Tax to the CREB protein in the absence of DNA. Furthermore, the complex of endogenous Tax with CREB was also shown in a nuclear extract of HuT 102 cells by immunoprecipitation analysis (Fig. 4*B*). Thus, the Tax–CREB protein complex is formed under natural conditions.

Tax–CREB–21-bp Complex in Nuclear Extract of HTLV-1-Infected Cells. As described above, Tax and CREB form a complex and can bind to the 21-bp enhancer sequence. To demonstrate the significance of this complex in a cell extract, we tried to deplete a nuclear extract of CREB protein to test its effect on the amount of the Tax protein–21-bp complexes formed. For this, a nuclear extract of HuT 102, an infected T-cell line, was treated with anti-CREB antibodies immobilized on protein A–Sepharose, and the supernatant was subjected to DNAP assay (Fig. 5*A*). Treatment with anti-CREB antibodies, but not with normal antibodies, reduced the amount of CREB in the 21-bp DNA–protein complexes, confirming specific depletion of CREB. The amount of Tax protein in the 21-bp DNA–protein complexes was also significantly reduced. These results clearly indicated that CREB in the nuclear extract of infected cell line complexed with endogenous Tax protein and could bind to the 21-bp sequence. The decrease of Tax protein was significant, indicating that CREB contributes to a significant part of the Tax protein–21-bp complexes. CREB depletion gave a similar effect on the histidine–Tax protein–21-bp complexes in a nuclear extract of Jurkat cells (Fig. 5*B*). In Jurkat cells, virtually all CREB was depleted, but a significant amount of Tax protein–21-bp complexes was detected, suggesting the existence of additional 21-bp DNA-binding proteins that interact with Tax protein.

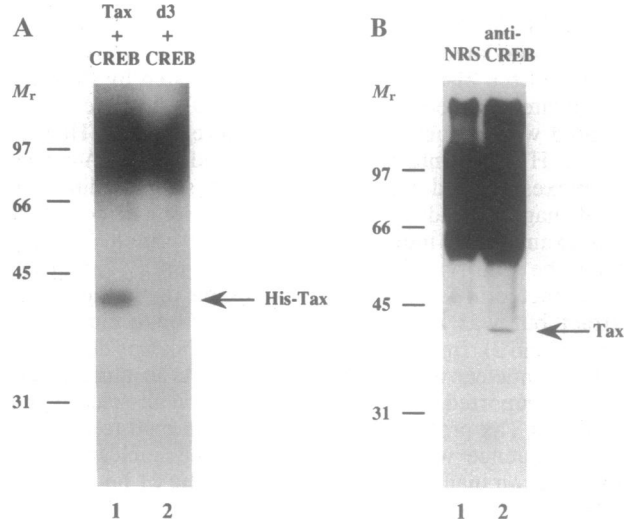

FIG. 4. Binding of Tax protein to CREB in the absence of the 21-bp DNA. (*A*) Formation of Tax–CREB complex between purified proteins. Purified histidine–CREB and wild-type histidine–Tax (lane 1) or mutant histidine–d3 (lane 2) were incubated and then treated with anti-CREB. The immunoprecipitates were subjected to immunoblot analysis with anti-Tax antibodies. (*B*) Tax–CREB complex in a nuclear extract of HuT 102 cells. A nuclear extract of HuT 102 cells was treated with normal rabbit IgG (NRS, lane 1) or anti-CREB antibodies (lane 2), and the immunoprecipitates were analyzed as in *A*. M_r markers ($\times 10^{-3}$) are indicated.

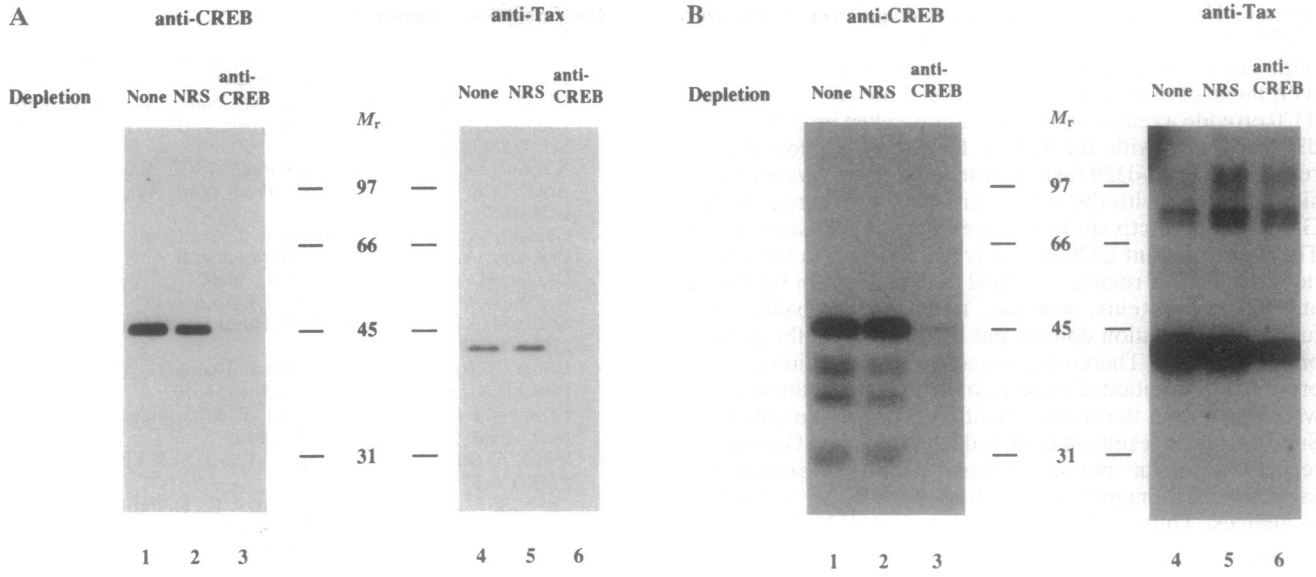

FIG. 5. Effect of depletion of nuclear extracts of CREB on formation of the Tax–CREB–21-bp complex. Nuclear extracts of HuT 102 cells (*A*) and Jurkat cells (*B*) were not treated (lanes 1 and 4) or were treated with normal rabbit IgG (NRS; lanes 2 and 5) or anti-CREB antibodies (lanes 3 and 6) immobilized on protein A–Sepharose. For *A*, the supernatants were incubated with biotinylated 21-bp DNA probe. The 21-bp DNA–protein complexes isolated with magnet beads were analyzed by immunoblotting with either anti-CREB (lanes 1–3) or anti-Tax (lanes 4–6). For *B*, the supernatant from Jurkat cells was mixed with histidine–Tax and then treated as in *A*. M_r markers ($\times 10^{-3}$) are indicated.

Detection of Tax–CREM–21-bp Complex. To identify other 21-bp DNA-binding proteins that interact with Tax protein, we tested an available protein, a human CREM protein, that is homologous to CREB (unpublished results). Purified histidine–CREM protein was tested by electrophoretic mobility-shift assay with and without Tax protein (Fig. 6). Like the CREB protein, histidine–CREM gave a shifted band, and addition of histidine–Tax induced super-shift of the band, indicating the formation of a complex of CREM protein with Tax on the 21-bp sequence. Furthermore, the Tax mutant d3 did not induce the super-shift, but another mutant d320 did. These results with mutant Tax were exactly the same as those with CREB. Therefore, CREM protein is one of the other 21-bp binding proteins that can interact with the Tax protein.

DISCUSSION

A trans-activator Tax of HTLV-1 activates structurally unrelated enhancers, the LTR 21-bp and NF-κB-binding sequences, and thus enhances viral gene expression and is proposed to contribute to development of adult T-cell leukemia and HTLV-1-associated myelopathy/tropic spastic paraparesis through activation of various growth-associated genes and cytokine genes. In this report, we showed that Tax binds to CREB in the absence of DNA and that the complex binds to the 21-bp enhancer forming a Tax–CREB–21-bp complex. The CREM protein can also form a similar complex. No other factor is required for formation of the complex. Thus the CREB and CREM proteins contribute as the nuclear factor to the indirect association of Tax with the 21-bp sequence.

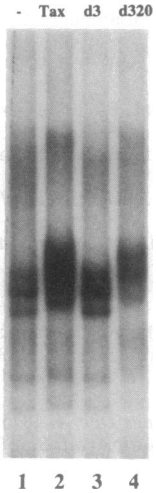

FIG. 6. Formation of a complex of Tax–CREM–21-bp DNA. Histidine–CREM was incubated alone (lane 1) or with wild-type histidine–Tax (lane 2), histidine–mutant d3 (lane 3), or histidine–mutant d320 (lane 4) and then treated with ^{32}P-labeled 21-bp DNA probe. The mixture was analyzed by nondenaturing gel electrophoresis.

The Tax–CREB–21-bp complex was also demonstrated in a nuclear extract of HTLV-1-infected cells. Therefore, this complex is not just an artifact formed when the purified proteins are used at high concentration but is actually formed under natural conditions. Furthermore, results on the effect of depletion of CREB suggested that CREB contributes to formation of a major proportion of the Tax protein–21-bp complexes in HuT 102 cells, but similar studies with Jurkat cells suggested that other factors also contribute to formation of the Tax protein–21-bp complexes. Thus, the contributions of CREB, CREM, and other factors, if involved, to the complex formation may vary in different cell lines or tissues.

The Tax–CREB complex was demonstrated with anti-CREB antibodies in a nuclear extract of HTLV-1-infected cells. However, this complex was not detected with anti-Tax antibodies that identified the NF-κB p105–Tax protein complex (38). These results were initially puzzling. But in this study, we found that anti-Tax antibodies against the C-terminal peptide of Tax protein dissociated the Tax–CREB–21-bp complex. This property of the antibody seems to explain why the Tax–CREB complex was detected with anti-CREB but was not detected with anti-Tax.

In a previous report, we (26) proposed that indirect association of the Tax protein with the 21-bp enhancer is required for transcriptional trans-activation. Thus the Tax–CREB (or CREM)–21-bp complex is suggested to play a critical role in trans-activation. Although direct evidence for its function is not yet available, results with mutants of Tax protein suggested a close relationship of the complex with trans-activation; inactive mutants did not form the complex, including a mutant d3. This mutant d3 did not form a complex with either CREB or CREM, although it could bind to the NF-κB precursor p105 (38), and these results are consistent with the functional properties of mutant d3, which cannot activate the 21-bp sequence but activates the NF-κB se-

quence. However, another mutant, d320, complexes with CREB and CREM, although it was inactive in 21-bp activation. The result of mutant d320 is in apparent contradiction with those of mutant d3, but additional properties of mutant d320 provide a reasonable explanation: when mutant d320 or d3 was fused with the GAL4 DNA-binding domain, the resulting GAL4–d320 fusion protein did not activate expression of a gene with the GAL4-binding site, whereas mutant GAL4–d3 did activate this expression (unpublished work). Therefore, mutant d320 probably has a defect in the activation domain but retains a normal binding domain for CREB and CREM proteins, whereas, mutant d3 probably has a functional activation domain but has a defect in the protein-binding domain. Therefore, mutant d320 could bind to these proteins, but mutant d3 could not bind them, although both were inactive in the trans-activation of the 21-bp enhancer. Supporting the results of GAL4–d320, Smith and Greene (40) reported a similar mutant, suggesting that Tax protein may use different domains for activation of the 21-bp and NF-κB sequences. Thus, the results with mutant d320 do not necessarily conflict with the conclusion that complex formation of Tax–CREB(or CREM)–21-bp DNA is important for trans-activation and indicate that Tax binding to CREB or CREM is required but is not sufficient alone for trans-activation of the 21-bp sequence.

However, the mechanism by which the interaction of Tax with CREB or CREM trans-activates transcription is not understood. Tax bound to CREB or CREM proteins on the 21-bp enhancer may provide an efficient activation domain for transcriptional initiation. This possibility is consistent with the *Results* and *Discussion* on Tax mutant d3. Alternatively, association of Tax with these proteins may simply stabilize the complexes of these proteins with the 21-bp enhancer. In either case, the present findings are not sufficient to explain our previous observations that the CRE in the 21-bp enhancer is essential for the Tax-mediated trans-activation, but the CRE in the vasoactive intestinal polypeptide (VIP) promoter is not activated by Tax (23). Various explanations are possible; for example, DNA-dependent interaction of additional factors with Tax–CREB(or CREM)–21-bp complex might be involved in the trans-activation.

After completion of this manuscript, a paper by Zhao and Giam (41) appeared, reporting that Tax protein enhances the binding of CREB protein to the 21-bp enhancer. Formation of a Tax–CREB–21-bp complex is consistent with our results; however, we did not observe reproducible and significant stabilization of CREB binding to the 21-bp DNA by Tax (Figs. 2 and 4). The effect of Tax protein on the degree of super-shift of the bands was less than they observed. The reason for these differences is not known but could be from a difference in the copy numbers of the 21-bp DNA used as probe (five copies by us and two by them) or a sequence difference of the 21-bp enhancer (the middle copy of three 21-bp units in the LTR by us and a dimer of the middle and proximal copies by them). Further comparative studies are required for a final conclusion.

Tax protein also activates the NF-κB sequence. In this connection, we have recently reported that Tax can bind to the NF-κB precursor p105 (38). The NF-κB p105 and CREB or CREM proteins are not related, but they interact with Tax protein. Analyses of the binding domains for CREB, CREM, and Tax protein should provide an insight into the mechanism of trans-activation by Tax protein.

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