Interaction of the human T-cell lymphotrophic virus type I (HTLV-I) transcriptional activator Tax with cellular factors that bind specifically to the 21-base-pair repeats in the HTLV-I enhancer

(protein–protein interaction/protein–DNA interaction/trans-activation/Tax activation factor/Tax purification)

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ABSTRACT   The human T-cell lymphotrophic virus type I (HTLV-I) Tax protein activates transcription from three 21-base-pair (bp) repeat sequences in the viral enhancer. Using gel electrophoretic mobility-shift assays, we now show that Tax interacts directly with the nuclear proteins, Tax activation factors (TAFs), that bind the 21-bp repeats. This interaction is demonstrated by decreased electrophoretic mobilities of the TAFs–21-bp-repeats complexes upon supply of Tax exogenously. Formation of the TAFs–21-bp-repeats and Tax–TAFs–21-bp-repeats complexes correlates with in vivo trans-activation by Tax. Furthermore, interaction of Tax with TAFs enhances their binding to the 21-bp repeats. These data indicate that trans-activation by Tax is most likely mediated by interaction of Tax with TAFs.

Human T-cell lymphotrophic virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia/lymphoma (ATL) (1). Most recently HTLV-I has been associated with a neurological disorder called tropical spastic paraparesis (also known as HTLV-I-associated myelopathy) (2–5). Similar to other viruses such as simian virus 40 (Tag), adenovirus (E1a), herpes simplex virus (VP16), and human immunodeficiency virus (HIV) (Tat), HTLV-I encodes a 40-kDa nuclear protein, Tax, which stimulates transcription from three 21-base-pair (bp) repeat sequences in the viral enhancer (6–14). The key element in the 21-bp repeat responsible for Tax activation is a sequence motif TGACG(T/A) (15–19), which appears in the enhancer/promoter region of numerous cellular and viral genes (20–22). Tax has also been shown to activate expression of interleukin 2, interleukin 2 receptor α-chain genes, and HIV enhancer via the NF-κB binding site (23–31). As Tax does not bind DNA directly, it has been proposed that trans-activation by Tax is mediated by NF-κB-like factors and cellular transcription factors that interact with the 21-bp repeats (15–19). The molecular mechanisms through which Tax affects these transcriptional factors remain unknown largely due to the lack of an in vitro system for dissecting the biochemical events orchestrated by Tax. We now report evidence showing that Tax interacts with the cellular factors that specifically bind the 21-bp repeats.

MATERIALS AND METHODS

Construction of Plasmids Expressing TaxH₆. Plasmid pX5 contains the *tax* open reading frame under control of bacteriophage T5 P21 promoter (32). This plasmid was linearized with *Hind*III and used as the template for PCR with primers 1 (ATGAGCCCCAAATATC) and 2 (TTGAGCCATATGT-TATTAATGGTGGTGATGGTGGTGGACTTCTGTTTC-

TCGGAA) (Fig. 1A). The PCR-amplified DNA was digested with *Xma* I and ligated to the pX5 plasmid digested with *Xma* I and *Eco*RV to generate pTaxH₆. The *Acc* I–*Bam*HI fragment of pTaxH₆ was inserted into a mammalian Tax expression plasmid, HTLV-*tat* I (34) at the *Acc* I and *Bgl* II sites to generate pLTR-TaxH₆ for transient expression in mammalian cells.

Purification of TaxH₆ Fusion Protein. *Escherichia coli* HB101 cells containing pTaxH₆ were grown, harvested, treated with EDTA/lysozyme, and sonicated. The high-speed supernatant was then precipitated with ammonium sulfate at 25% saturation (32). The precipitate was dissolved in buffer A (50 mM sodium phosphate, pH 7.8/0.5 M NaCl/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/5 mM imidazole), loaded on a Pharmacia chelating Sepharose 6B column precharged with Ni^{2+}, and eluted with an imidazole gradient of 0–0.4 M in buffer B [50 mM sodium phosphate, pH 7.2/0.3 M NaCl/10% (vol/vol) glycerol]. Fractions containing TaxH₆ were dialyzed against buffer C [50 mM Hepes, pH 7.9/50 mM KCl/0.5 mM $MgCl_2$/0.1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.25 mM dithiothreitol/20% (vol/vol) glycerol], aliquoted, and frozen in −70°C.

Construction of 21-bp-Repeat Mutants. The sequence of the *Xho* I–*Nco* I fragment in plasmid p13 (18) used for gel electrophoretic mobility-shift assays is as follows: 5' (*Xho* I)-TCGAGAAGGCCCTGACGTCTCCCCCAGATCTGG-GCGTTGACGACAACCCCTCACCTCAAAAAACTT-TCCATG-(*Nco* I) 3'; TGACG(T/A) motif is in boldface letters. In a derivative of p13, p12, the first thymine of the promoter distal copy of the TGACG(T/A) motif was mutated to guanine. This base substitution abrogates in vivo trans-activation of p12 by Tax (18). An identical mutation in the promoter proximal TGACG(T/A) motif of p12 was created by replacing the *Bgl* II–*Nco* I fragment with a double-stranded oligonucleotide containing the base substitution to yield plasmid p221.

Gel Electrophoretic Mobility-Shift Assay. *Xho* I–*Nco* I fragments (70 bp) containing two copies of wild-type (p13) or mutant (p221) 21-bp repeats were excised from the respective parent plasmids and labeled with ^{32}P by filling in the 3'-recessed ends. DNA-binding reaction was as described (18). After incubation for 30 min at 37°C with or without 0.1 μg of purified TaxH₆, the reaction mixtures were chilled on ice and electrophoresed in a 4% polyacrylamide gel in 25 mM Tris, pH 8.5/192 mM glycine/1 mM EDTA buffer at 4°C until the tracking dye xylene cyanol migrated ≈12 cm from the origin.

FIG. 1.   Expression of TaxH$_6$ fusion protein. (A) Addition of six histidine codons to the 3' end of the *tax* open reading frame. pX5 plasmid (32) containing the *tax* open reading frame was used as template in PCR with primers 1 and 2. The PCR-amplified DNA fragment containing six histidine codons (solid box) was inserted at *Xma* I–*Eco*RV sites of pX5 to produce pTaxH$_6$. pLTR-TaxH$_6$ was derived by inserting the *Acc* I–*Bam*HI fragment of pTaxH$_6$ to the *Acc* I–*Bgl* II sites of HTLV-*tat* I, a mammalian expression vector for Tax (33). (B) Purification of TaxH$_6$ fusion protein from metal-ion-chelating Sepharose column. TaxH$_6$ was bound to a metal-ion-chelating Sepharose 6B column (Pharmacia) charged with Ni^{2+} and eluted with an imidazole gradient. Column fractions were analyzed by SDS/PAGE and Coomassie blue staining. TaxH$_6$ eluted at ≈0.3 M imidazole (fractions 14–16). (C) *In vivo* trans-activation of HTLV-LTR by TaxH$_6$ fusion protein. pLTR-TaxH$_6$ (lane TaxH$_6$) or HTLV-*tat* I (lane Tax) was cotransfected into Jurkat cells with pU3RCAT (6) using the DEAE-dextran method (18). A chloramphenicol acetyltransferase assay was done 50 hr after transfection. Lane − shows chloramphenicol acetyltransferase activity of control cells transfected with pU3RCAT alone.

RESULTS

Expression and Rapid Purification of TaxH$_6$. To understand the mechanism of Tax action, we previously constructed a plasmid pX5 that expresses biologically active Tax (32). To facilitate purification of Tax, we introduced six histidine residues at the COOH terminus of Tax by a PCR approach (Fig. 1A). The histidine extension chelates transition metal ions, such as Ni^{2+} or Zn^{2+} via coordination chemistry and allows rapid purification of recombinant proteins by metal ion-chelating Sepharose (34). The TaxH$_6$ recombinant protein expressed from pTaxH$_6$ was purified on a metal-chelating Sepharose column charged with Ni^{2+} (Fig. 1B). TaxH$_6$ eluate peaked at 0.3 M imidazole (lane 15) and remained soluble after dialysis. When the TaxH$_6$ coding sequence was placed under control of the HTLV-I long terminal repeat (LTR), the resultant plasmid, pLTR-TaxH$_6$ (Fig. 1A), trans-activated the appropriate HTLV-I LTR chloramphenicol acetyltransferase construct (6), pU3RCAT, as the wild-type Tax construct, HTLV-*tat* I (34), upon cotransfection into Jurkat cells (Fig. 1C). Hence, TaxH$_6$ is functionally indistinguishable from the wild-type Tax.

Conversion of Specific Tax Activation Factors (TAFs)–21-bp-Repeats Complexes into Lower Mobility Forms by Tax. Protein–protein interaction has been shown to be the mechanism of transcriptional activation of several viral trans-activators such as herpes simplex virus VP16 (35, 36), adenovirus E1a (37), and, more recently, hepatitis B virus pX (38); however, to date, it is not known whether analogous mechanisms are used by the HTLV-I Tax in trans-activation. Because at least two copies of the HTLV-I 21-bp repeats are required for optimal Tax action, we carried out gel electrophoretic mobility-shift assays using Jurkat nuclear extract

and a 70-bp *Xho* I–*Nco* I fragment (18) containing two copies of the 21-bp repeats. Fig. 2A shows that Jurkat nuclear extract yielded mainly three protein–DNA complexes (I–III; lane 1). TaxH$_6$ addition decreased the electrophoretic mobilities (super-shift) of complexes I and II (lane 2), yielding complexes I$_x$ and II$_x$. TaxH$_6$ alone without nuclear extracts produced no observable bands (Fig. 3A, lane 4), indicating that TaxH$_6$ has no affinity or low affinity for DNA, as has been reported (18). Complex III was not altered by TaxH$_6$ and served as a useful internal control for monitoring the effect of Tax on complexes I and II. We also noticed protein–DNA complexes with slower mobilities than complexes I–III; their formation was enhanced when Tax was added. We think these complexes are probably other higher-order forms of complexes I–III or of complexes I$_x$ and II$_x$.

To show that the observed effect of TaxH$_6$ on mobilities of complexes I and II was not from the histidine residues at the COOH terminus, we added to the assay the same amount of a recombinant HIV-1 reverse transcriptase modified to contain six histidine residues at the NH$_2$ terminus (from Stuart Le Grice, Case Western Reserve University). Fig. 2A lane 3 indicates this HIV-1 reverse transcriptase did not change complexes I–III. Furthermore, wild-type Tax partially purified from *E. coli* harboring pX5 (18) altered the mobility of complexes I and II similarly as TaxH$_6$ (lane 4). That conversion of complexes I and II to I$_x$ and II$_x$ was due entirely to exogenously added TaxH$_6$ was further shown by the depletion of TaxH$_6$ by Tax antibodies and the subsequent loss of TaxH$_6$ effects on mobility changes of complexes I and II. As shown, treatment of TaxH$_6$ with Tax antibody (Fig. 2B, lane 2) or Tax-C antibody (prepared against the COOH-terminal 33 amino acid residues of Tax, provided by Bryan Cullen,

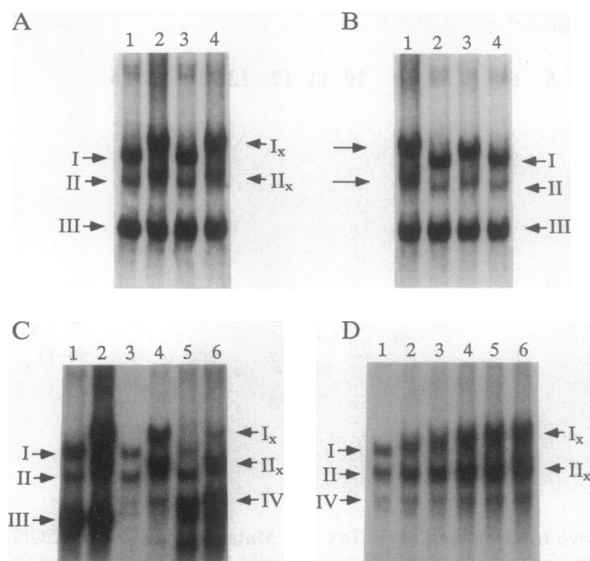Biochemistry: Zhao and Giam

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FIG. 2. Decreased electrophoretic mobilities of TAFs–21-bp-repeats complexes with Tax. (*A*) Conversion of TAFs–21-bp-repeats complexes to lower electrophoretic mobility forms by Tax. Gel electrophoretic mobility-shift assays were done as described with unfractionated Jurkat nuclear extract and radiolabeled *Xho* I–*Nco* I fragment of p13. Lanes: 1, no $TaxH_6$; 2, addition of 0.1 μg of $TaxH_6$; 3, addition of 0.1 μg of HIV reverse transcriptase with six-histidine extension; 4, addition of 0.5 μg of Tax partially purified from *E. coli* (18). (*B*) Loss of Tax-induced mobility shifts upon removal of $TaxH_6$ by anti-Tax-IgG-protein A-Sepharose beads. Ten micrograms of each IgG was charged onto 5 mg of protein A-Sepharose beads (Pharmacia) and then incubated with 0.5 μg of $TaxH_6$ for 1 hr at 4°C followed by brief centrifugation to pellet beads. The supernatant equivalent to 0.1 μg of original $TaxH_6$ preparation was added in electrophoretic mobility-shift assays. Lanes: 1, assay with 0.1 μg of untreated $TaxH_6$; 2–4, assays with $TaxH_6$ pretreated with Sepharose beads charged with anti-Tax IgG, anti-β-galactosidase IgG, or anti-Tax-C IgG (generated against COOH-terminal 33 amino acid residues of Tax), respectively. (*C*) Partial purification of TAFs on DNA cellulose column and their presence in HeLa cells. Gel electrophoretic mobility-shift assays were as in *A*, except with 0.4 M KCl eluate of Jurkat nuclear extract from a calf thymus DNA cellulose column (referred to as 0.4 M fraction) (lanes 3 and 4) or with unfractionated Jurkat (lanes 1 and 2) or HeLa (lanes 5 and 6) nuclear extracts. One-tenth microgram of $TaxH_6$ was added to lanes 2, 4, and 6. (*D*) Titration of $TaxH_6$. The 0.4 M KCl eluate of Jurkat nuclear extract and the same radiolabeled DNA as in *A* were used in the assays. Poly(dI·dC) at 250 μg/ml was used to reduce nonspecific DNA binding. Lanes 1–6 contain 0, 12.5, 25, 50, 75, and 100 ng of $TaxH_6$, respectively.

Duke University, Durham, NC; ref. 39; lane 4) resulted in loss of super-shift by $TaxH_6$, whereas treatment of $TaxH_6$ with anti-β-galactosidase antibody (lane 3) did not affect the ability of $TaxH_6$ to super-shift. The effect of $TaxH_6$ can also be heat-inactivated (Fig. 3*A*, lane 3). From these results, we concluded that the super-shift of complexes I and II by $TaxH_6$ was due entirely to $TaxH_6$. The nuclear factors that gave rise to complexes I and II and interacted with Tax are referred to as TAFs.

We observed (40) that the 21-bp-repeat-binding proteins from Jurkat nuclear extract could be retained on DNA-cellulose column and eluted with 0.4 M KCl. When the 0.4 M KCl eluate was used in the gel-shift assay, bands I and II were again seen (Fig. 2*C*, lane 3), and incubation with purified $TaxH_6$ resulted in super-shift (lane 4; compare with the pattern of unfractionated nuclear extract in lane 1 without $TaxH_6$ and in lane 2 with $TaxH_6$). The 0.4 M KCl eluate also contained an activity that gave rise to complex IV, the electrophoretic mobility of which was apparently unaffected by $TaxH_6$. Tax trans-activation has been shown to occur in

many cell types including HeLa cells. We, therefore, tested the ability of HeLa nuclear extract for TAFs. Fig. 2*C*, lane 5, shows that HeLa nuclear extract also gave rise to bands I–IV and several other additional bands. When $TaxH_6$ was added (lane 6), again mobilities of complexes I and II but not others shifted specifically. These data lend further support to the conclusion that complexes I and II are the specific targets of Tax.

In binding reactions containing lower amounts of $TaxH_6$, at least one complex migrating at an intermediate position between bands I and I_x appeared (Fig. 2*D*). With 50 ng of $TaxH_6$, three bands were discernible (lane 4). Higher amounts of $TaxH_6$ shifted complex I to the position of I_x (lanes 5 and 6). In our assays, 100 ng of $TaxH_6$ preparation seemed sufficient for complete conversion of complex I to I_x. It is possible that the stoichiometry of Tax may vary as amounts of $TaxH_6$ in the assay alter. We noted that with increased amounts of $TaxH_6$, intensities of bands I_x and II_x increase, suggesting that Tax enhances binding of TAFs to the 21-bp repeats (see below).

Formation of Complexes I, II, I_x, and II_x Correlates with Tax Trans-activation *in Vivo*. Previous studies showed that mutations in the HTLV-I 21-bp repeat that abolish trans-activation by Tax *in vivo* lie primarily in a CRE (cAMP responsive element) -like motif [TGACG(T/A)] (18). To correlate the formation of complexes I, II, I_x, and II_x with biological functions, a DNA construct containing point mutations in both copies of the 21-bp repeat was made. Each mutant repeat contains a point mutation that alters the TGACGT motif to GGACGT, a base substitution shown (18) to abolish Tax trans-activation *in vivo*. Fig. 3*A* shows that mutations in the TGACG(T/A) motif completely abolished formation of complexes I, II, and IV in the absence of $TaxH_6$ (lane 5) and complexes I_x and II_x in the presence of $TaxH_6$ (lane 6). Even in reactions where three times more mutant DNA fragment was used (lanes 7 and 8), no complexes I, II, and IV (lane 7) or I_x and II_x (lane 8) were seen. These data showed that the *in vitro* formation of complexes I, II, I_x, and II_x can be correlated with *in vivo* trans-activation by Tax. Although complex IV was apparently not affected by Tax, its formation was also abolished by the mutation in TGACG(T/A) motifs. To further demonstrate specificity of complexes I and II, competition experiments were done. Fig. 3*B* shows that complexes I, II, IV, I_x, and II_x were competed against efficiently by increased amounts of unlabeled wild-type HTLV-I 21-bp repeats (lanes 1–8) but not by the mutant repeats (lanes 9–16).

Binding of Tax to TAFs. The simplest explanation for the Tax-induced decrease in electrophoretic mobilities of complexes I and II is that Tax is physically bound to TAFs–DNA complexes I and II and converts them to complex I_x and II_x. To test this possibility, anti-Tax-C IgG-purified from protein A–Sepharose column was directly added to the binding reactions. Fig. 4 lane 4 shows that addition of anti-Tax-C IgG to the reaction containing $TaxH_6$ resulted in the formation of an additional large-molecular-size DNA–protein complex (indicated by triangle). This complex is specific for the anti-Tax-C IgG because β-galactosidase IgG added to the reaction containing $TaxH_6$ did not induce any changes (lane 5). The large-molecular-size species forms only when the binding reaction contains $TaxH_6$. Addition of anti-Tax-C IgG to the reaction not containing $TaxH_6$ had no effect (lane 3). These data indicate that Tax is directly present in complexes I_x and II_x, and binding of anti-Tax-C IgG converts complexes I_x and II_x to the large-molecular-size complex(es), causing the decreased intensities of complexes I_x and II_x. When increased amounts of anti-Tax-C IgG were added to the reactions (lanes 7 and 10), larger complexes formed that remained at the origin of sample application. These complexes were most likely due to oligomerization of the IgG–
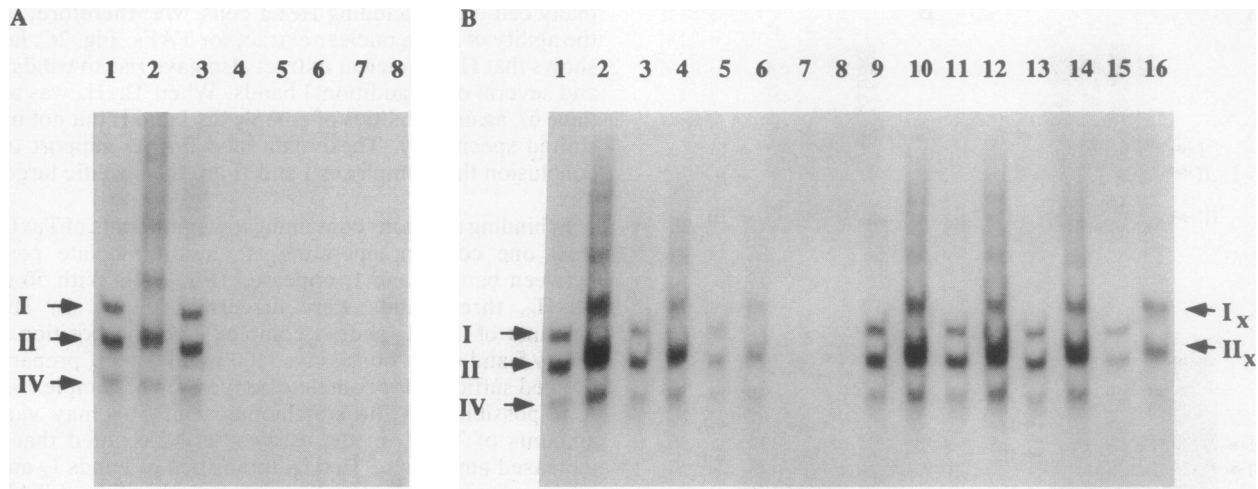
FIG. 3. Formation of TAFs–21-bp-repeats complexes correlates with *in vivo* trans-activation by Tax. (*A*) Mutations in the TGACG(T/A) motif abolish complexes I, I_x and II, II_x formation. Gel electrophoretic mobility-shift assays used radiolabeled wild-type (p13) or mutant (p221) *Xho* I–*Nco* I fragment and the 0.4 M KCl fraction, except for lane 4, where no extract was added. Poly(dI·dC) at 250 μg/ml was included in each assay. For lanes 5, 6 and 7, 8 1.5 and 3 times more mutant DNA was used, respectively. (Lanes 2, 4, 6, and 8) Assay with 0.1 μg of TaxH6. (Lane 3) Assay with 0.1 μg of TaxH6 treated at 65°C for 5 min. (Lanes 1, 5, and 7) Assays without Tax. (*B*) Competition of the TAFs–21-bp-repeats complexes by unlabeled wild-type but not by the mutant 21-bp repeats. The 0.4 M KCl column eluate of Jurkat nuclear extract, radioactively labeled wild-type *Xho* I–*Nco* I fragment and poly(dI·dC) at 250 μg/ml were used in the assays. (Odd-numbered lanes) Assays without Tax. (Even-numbered lanes) Assays with 0.1 μg of TaxH6. For competition, amount of unlabeled wild-type (lanes 1–8) or mutant (lanes 9–16) 21-bp repeats is 0.5 ng (lanes 3, 4, 11, and 12), 2.5 ng (lanes 5, 6, 13, and 14), or 10 ng (lanes 7, 8, 15, and 16).

TaxH6–TAFs–21-bp-repeats complexes. Furthermore, complexes migrating at intermediate positions between bands I and I_x appeared, a pattern consistent with the titration experiment of Fig. 2*D*. We think the interaction of anti-Tax-C IgG with TaxH6 may partially perturb and reverse the interaction of TaxH6 with the TAFs–21-bp-repeats complexes.

Enhanced Binding of TAFs to the 21-bp Repeats by Tax. We repeatedly noticed that when TaxH6 is added to the binding reactions the levels of complexes I_x and II_x significantly increase relative to control (Fig. 2 *C* and *D*); the level of complex IV was also increased. To further investigate the relative stability of complexes I and II vs. I_x and II_x, binding reactions were done with increased amounts of competing poly(dI·dC). Fig. 5 shows that high concentrations of

poly(dI·dC) more efficiently reduced the levels of complexes I and II than those of I_x and II_x. These results suggest that with Tax the affinity of TAFs for the 21-bp repeats is increased such that TAFs become more resistant to the competing nonspecific DNA. The stabilization of complexes I_x and II_x by Tax has interesting implications for the *in vivo* trans-activation by Tax where the Tax-responsive cis regulatory sequences constitute only a minuscule portion of the chromosomal DNA.

DISCUSSION

In this paper, we demonstrate that HTLV-I Tax directly interacts with the cellular factors, TAFs, that bind the 21-bp repeat elements in the viral enhancer. Tax binding to the TAFs–21-bp-repeats complexes I and II decreased electro-
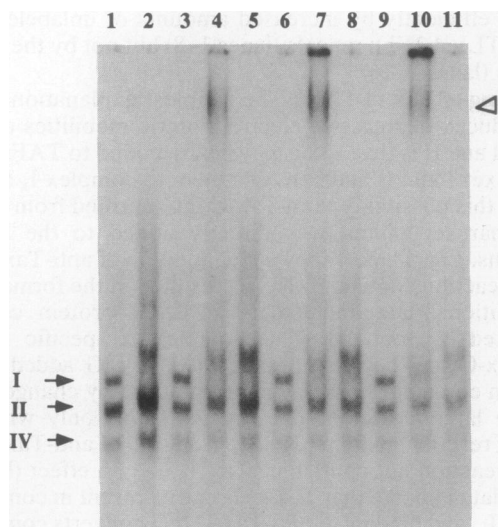

FIG. 4. Complexes I_x and II_x contain Tax. Gel electrophoretic mobility-shift assays were done as for Fig. 3. (Lanes 1, 3, 6, and 9) Assay without Tax. (Lanes 2, 4, 5, 7, 8, 10, and 11) Assay with 0.1 μg of TaxH6. In addition, anti-Tax-C IgG (lanes 3 and 4, 1 μg; lanes 6 and 7, 1.5 μg; lanes 9 and 10, 2 μg) or anti-β-galactosidase IgG (lane 5, 1 μg; lane 8, 1.5 μg; lane 11, 2 μg) was added.
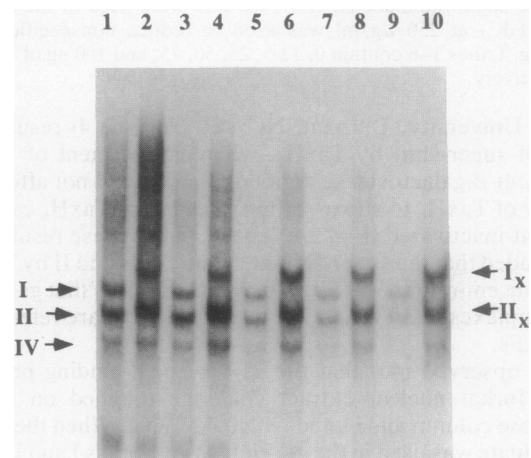

FIG. 5. Enhanced binding of TAFs to 21-bp repeats by Tax. Assays were done by using radiolabeled wild-type 21-bp-repeat DNA and the 0.4 M KCl fraction. (Odd-numbered lanes) Assays without Tax. (Even-numbered lanes) Assays with 0.1 μg of TaxH6. Concentration of poly(dI·dC) is 25 μg/ml (lanes 1 and 2), 50 μg/ml (lanes 3 and 4), 100 μg/ml (lanes 5 and 6), 150 μg/ml (lanes 7 and 8), and 250 μg/ml (lanes 9 and 10).

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phoretic mobilities of these complexes. Furthermore, direct interaction of Tax with TAFs enhances their binding to the 21-bp repeat element. Mutation and competition experiments showed that complexes I, II, and IV are specific for the TGACG(T/A) motif in the 21-bp repeat element. Mutations that abolish *in vivo* trans-activation by Tax also abolished the formation of all three complexes.

A number of cellular factors (ATF, TREB, CREB, CRE-BP1) that bind to the CRE and/or the 21-bp element have been cloned and sequenced recently (41–46). These cellular factors all contain leucine zipper domains at their COOH termini, some of which can engage in heterodimer or homodimer interactions (43). Possibly TAFs are among these cloned factors. The assay described in this report should facilitate their identification. Because the interleukin 2, interleukin 2 receptor α-chain genes, and HIV enhancer are also activated by Tax via the NF-κB binding sites, determining whether similar interactions can also occur between Tax and the NF-κB like factors would be of interest.

In summary, our results provide biochemical evidence that HTLV-I Tax interacts with the cellular factors, TAFs, that bind the 21-bp repeats. This interaction alters the TAFs-DNA complexes I and II qualitatively and enhances TAFs binding to the 21-bp repeats quantitatively. These changes most likely are responsible for the transcriptional activation mediated by Tax. An *in vitro* assay for Tax function described in this study should help reveal the molecular details of Tax action.

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