185, 60 (1990).] This pET vector contained a T7 promotor inserted into the Bam HI site of the plasmid pBR322 and a gene encoding ampicillin resistance. The *E. coli* that contained the CksHs2 encoding plasmid were grown in the presence of ampicillin and chloramphenicol, and the T7 promoter was induced by addition of isopropylthiogalactoside. Cells were harvested by centrifugation and lysed by sonication. The CksHs2 protein was then precipitated with ammonium sulfate, resuspended in a buffer solution (50 mM tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.02 percent NaN_3), dialyzed against 3 liters of this same buffer, and eluted over a Sephacryl-200 HR gel filtration column. For crystallization, the purified protein was dialyzed against 5 mM imidazole-malate, pH 6.0, and concentrated to 5 mg/ml. Crystals (up to 1 by 0.4 by 0.4 mm) were grown by the hanging-drop vapor diffusion method with 54 percent saturated ammonium sulfate and 50 mM sodium citrate, pH 5.5, as the precipitant. The space group was determined by precession photography. Diffraction photographs and self-rotation function results confirmed that the threefold axis was noncrystallographic and was located about 20° from the crystallographic *a* axis. On the basis of crystal symmetry, cell dimensions, and molecular mass of 9711 daltons, a V_m [B. W. Matthews, *J. Mol. Biol.* **33**, 491 (1968)] of 2.24 Å^3/dalton and 45 percent solvent content were obtained for three molecules per asymmetric unit.

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HTLV-I Tax Protein Stimulation of DNA Binding of bZIP Proteins by Enhancing Dimerization

Susanne Wagner and Michael R. Green

The Tax protein of human T cell leukemia virus type–1 (HTLV-I) transcriptionally activates the HTLV-I promoter. This activation requires binding sites for activating transcription factor (ATF) proteins, a family of cellular proteins that contain basic region–leucine zipper (bZIP) DNA binding domains. Data are presented showing that Tax increases the in vitro DNA binding activity of multiple ATF proteins. Tax also stimulated DNA binding by other bZIP proteins, but did not affect DNA binding proteins that lack a bZIP domain. The increase in DNA binding occurred because Tax promotes dimerization of the bZIP domain in the absence of DNA, and the elevated concentration of the bZIP homodimer then facilitates the DNA binding reaction. These results help explain how Tax activates viral transcription and transforms cells.

Human T cell leukemia virus type I (HTLV-I) is associated with malignant adult T cell leukemia (ATL) and has been linked to two degenerative neurologic diseases, HTLV-I–associated myelopathy and multiple sclerosis (*1*). Replication of HTLV-I is dependent on the viral Tax protein, which activates transcription of the HTLV-I long terminal repeat (LTR) as well as other cellular and viral promoters (*2*). The ability of HTLV-I to induce malignant transformation of cultured cells requires a functional Tax gene, and Tax itself has the properties of a nuclear oncoprotein (*3*).

Transcriptional activation of the HTLV-I LTR by Tax involves three 21–base pair Tax-response elements (TRE) (*4*). Mutational analysis indicates that the critical region of each 21-bp repeat is an ATF binding site (*5*, *6*). The ATF proteins (ATF) form a family of cellular transcription factors that contain homologous basic region–leucine zipper (bZIP) DNA binding domains (*7*). Indeed, several ATFs have been shown to bind TRE sequences (*8*). These and other observations suggest that Tax, which is not a sequence-specific DNA binding protein (*6*), functions through ATF proteins to stimulate HTLV-I LTR-directed transcription.

Precisely how Tax affects ATFs to activate transcription is not understood. We now demonstrate that Tax increases the DNA binding activity of multiple ATFs and other bZIP proteins. This DNA binding increase occurs by a previously undescribed mechanism involving regulation of dimerization.

Tax increases DNA binding activity of ATF proteins. To investigate the possible effect of Tax on the DNA binding activity of ATF proteins, we purified three ATFs [ATF-1, ATF-2, and cAMP response element–binding protein (CREB)] as glutathione-*S*-transferase (GST) fusion proteins and assayed their binding to a DNA oligonucleotide containing the most distal TRE of the HTLV-I LTR. When the DNA binding reactions were performed at protein concentrations that resulted in a low level of DNA binding in the absence of Tax, the addition of purified Tax increased the DNA binding of each of the ATFs. In contrast, DNA binding was not increased when an irrelevant protein (Fig. 1A) or basic peptides were added (*9*, *10*, *11*). Tax comparably stimulated DNA binding of ATF derivatives lacking a GST moiety, such as H6-ATF2 (Fig. 1A, lanes 2 and 3), and a minimal bZIP peptide, excluding the possibility that the GST moiety is involved in the Tax-mediated DNA binding increase. Consistent with previous studies (*6*), there was no detectable interaction between purified Tax and DNA (Fig. 1A, lane 1).

To define the portion of ATF required for Tax-responsiveness, we analyzed a GST-ATF2 fusion protein containing a minimal 65 amino acid bZIP domain (amino acids 350 to 415) (*7*). This minimal bZIP domain was equally capable of supporting the Tax-mediated DNA binding increase (Fig. 1B, lanes 2 and 3). This result is consistent with the fact that Tax increased DNA binding of multiple ATFs (Fig. 1A), and the bZIP is the only region of significant homology among the ATF proteins (*7*).

Tax-mediated enhancement of DNA binding specific for bZIP proteins. The finding that Tax increased the DNA binding of multiple ATF proteins prompted us

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to analyze other bZIP proteins. The AP-1 family of transcription factors (*12*) contains bZIP domains that are homologous to those of ATFs, and bind to a DNA consensus sequence (5′-TGACTCA-3′), which differs in only one position from an ATF consensus site (5′-TGACGTCA-3′). The mammalian transcription factor c-Jun and the yeast transcription factor GCN4 are prototype AP-1 proteins. The DNA binding activity of a c-Jun derivative, and a peptide comprising the yeast GCN4 bZIP domain were responsive to Tax in a manner similar to that of bacterially expressed ATFs (Fig. 1C). The CCAAT/enhancer binding protein (C/EBP), a member of another bZIP protein family (*13*), was also Tax-responsive.

In contrast, Tax did not increase the DNA binding of either GAL4-AH, a zinc-finger protein, or MyoD, a basic helix-loop-helix (bHLH) protein (Fig. 1D). The DNA binding of another bHLH protein, GST-myogenin, was only modestly affected by Tax. Tax did not significantly increase DNA binding of these proteins when tested over the same concentrations used to test GCN4 (*11*). It appears, therefore, that Tax can stimulate the DNA binding activity only of proteins that contain a bZIP motif.

To investigate the possible role of bZIP concentration in the Tax-mediated DNA binding increase, we measured the effect of Tax at GCN4 concentrations ranging from 0.04 to 40 nM. Maximal stimulation of DNA binding was observed at peptide concentrations below 4 nM (Fig. 1E) and at higher protein concentrations, Tax did not significantly increase DNA binding (*14*). The failure to increase DNA binding at higher bZIP concentrations was not the result of a limiting amount of Tax; DNA binding was also not affected when the concentration of Tax was increased (*15*). These results indicate that Tax overcomes a concentration-dependent step that normally limits the extent of DNA binding.

Tax interacts with the bZIP-DNA complex. Tax increases DNA binding but does not alter the electrophoretic mobility of the bZIP-DNA complex. One explanation for this result is that Tax is initially a component of the bZIP-DNA complex but dissociates during electrophoresis in the nondenaturing gel. Such instability of ternary complexes during native gel electrophoresis has been reported in several other systems (*16–18*).

To address this possibility, we analyzed DNA binding in other nondenaturing gel systems. In tris-glycine buffer, the addition of Tax gave rise to a second DNA-protein complex of reduced electrophoretic mobility (Fig. 2A, lanes 4 and 5) (*19*). However, even under these conditions the complex dissociates and only a part remains stable in the nondenaturing gel.

We performed immuno-depletion experiments to establish the existence of a complex of Tax, bZIP and DNA. Reaction mixtures containing GST-ATF2 were incubated with various antisera, antibody-bound DNA-protein complexes were removed by absorption on Pansorbin cells, and the remaining DNA-protein complexes were detected after native gel electrophoresis. In the presence of Tax, antiserum directed against either GST, which reacts with GST-ATF2, or Tax, depleted >95

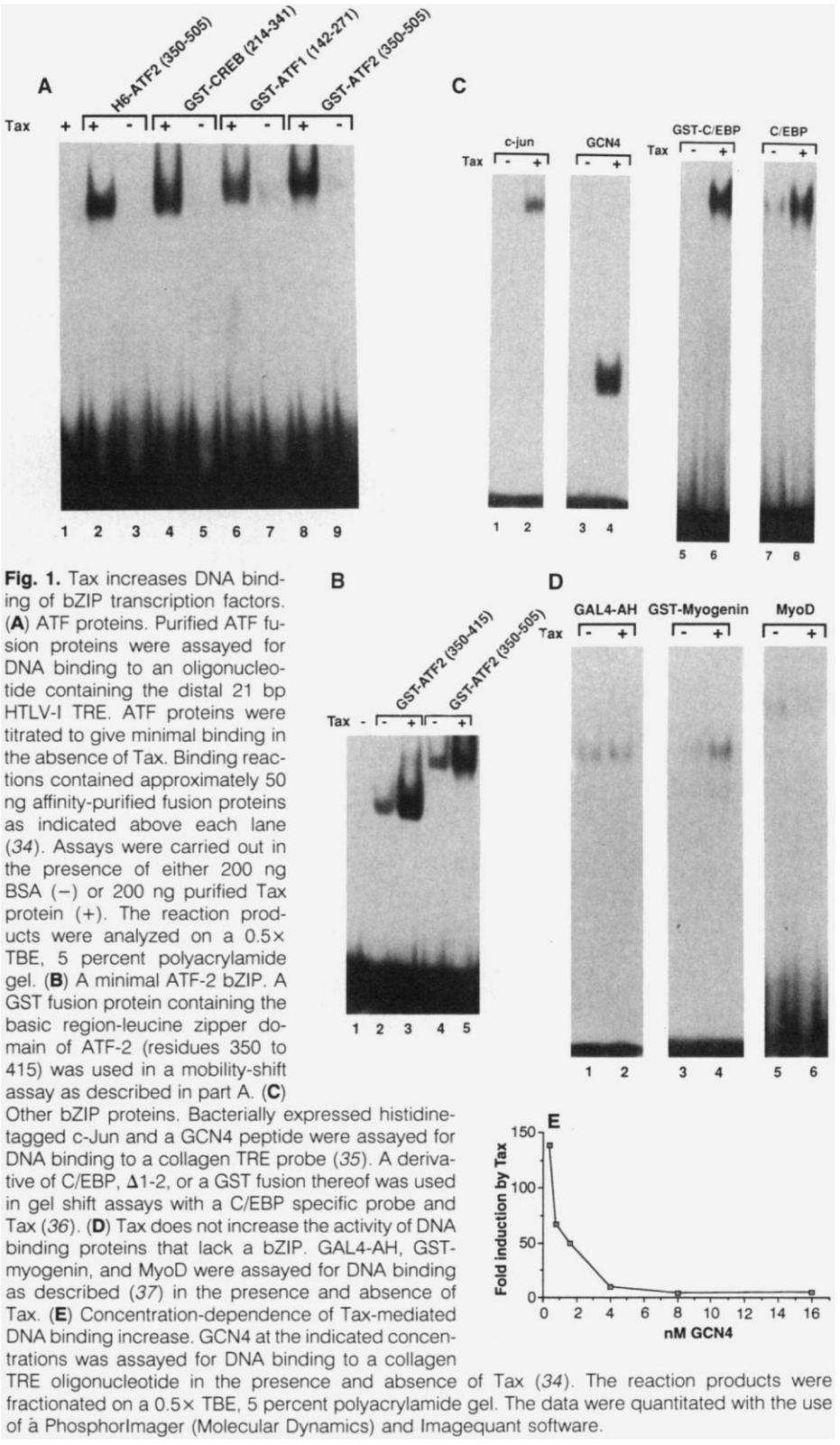

Fig. 1. Tax increases DNA binding of bZIP transcription factors. (**A**) ATF proteins. Purified ATF fusion proteins were assayed for DNA binding to an oligonucleotide containing the distal 21 bp HTLV-I TRE. ATF proteins were titrated to give minimal binding in the absence of Tax. Binding reactions contained approximately 50 ng affinity-purified fusion proteins as indicated above each lane (*34*). Assays were carried out in the presence of either 200 ng BSA (−) or 200 ng purified Tax protein (+). The reaction products were analyzed on a 0.5× TBE, 5 percent polyacrylamide gel. (**B**) A minimal ATF-2 bZIP. A GST fusion protein containing the basic region-leucine zipper domain of ATF-2 (residues 350 to 415) was used in a mobility-shift assay as described in part A. (**C**) Other bZIP proteins. Bacterially expressed histidine-tagged c-Jun and a GCN4 peptide were assayed for DNA binding to a collagen TRE probe (*35*). A derivative of C/EBP, Δ1-2, or a GST fusion thereof was used in gel shift assays with a C/EBP specific probe and Tax (*36*). (**D**) Tax does not increase the activity of DNA binding proteins that lack a bZIP. GAL4-AH, GST-myogenin, and MyoD were assayed for DNA binding as described (*37*) in the presence and absence of Tax. (**E**) Concentration-dependence of Tax-mediated DNA binding increase. GCN4 at the indicated concentrations was assayed for DNA binding to a collagen TRE oligonucleotide in the presence and absence of Tax (*34*). The reaction products were fractionated on a 0.5× TBE, 5 percent polyacrylamide gel. The data were quantitated with the use of a PhosphorImager (Molecular Dynamics) and Imagequant software.

percent of the total DNA-protein complexes (Fig. 2B, compare lane 1 to lanes 2 and 3). In the absence of Tax, DNA-protein complexes were depleted by the antibody to GST (anti-GST) (compare lanes 4 and 5) but not by the antibody to Tax (anti-Tax) (compare lanes 4 and 6). Thus, the depletion of DNA-protein complexes in lane 3 is not a nonspecific effect of the anti-Tax. Together these results indicate that in solution the vast majority of the protein-bound DNA is in the form of a Tax, bZIP, and DNA complex; during the course of gel electrophoresis either all (Fig. 1A; tris, borate, EDTA buffer) or most (Fig. 2A; tris, glycine, EDTA buffer) of the Tax, bZIP, and DNA complex dissociates into a bZIP-DNA complex.

We performed a DNA-coimmunoprecipitation assay (*20*) to complement the results of Fig. 2B. Binding reactions contained a ^{32}P-labeled DNA probe and a minimal ATF-2 bZIP domain (Fig. 2C, upper) or a CREB derivative (Fig. 2C, lower). After incubation in the presence or absence of Tax, anti-Tax was added to immunoprecipitate the ternary complex. The anti-Tax immunoprecipitated the ^{32}P-labeled DNA probe only if Tax and the bZIP were both present (Fig. 2C). In contrast, the ^{32}P-labeled DNA probe was not immunoprecipitated by the control serum. These combined results confirm that there is a ternary complex containing the DNA-bound bZIP protein and Tax. Deoxyribonuclease (DNase) I protection and ultraviolet light crosslinking experiments confirmed an increased DNA binding by ATF, but failed to reveal an interaction between Tax and DNA (*21*). These results indicate that in the ternary complex Tax interacts primarily (or exclusively) with the minimal bZIP domain.

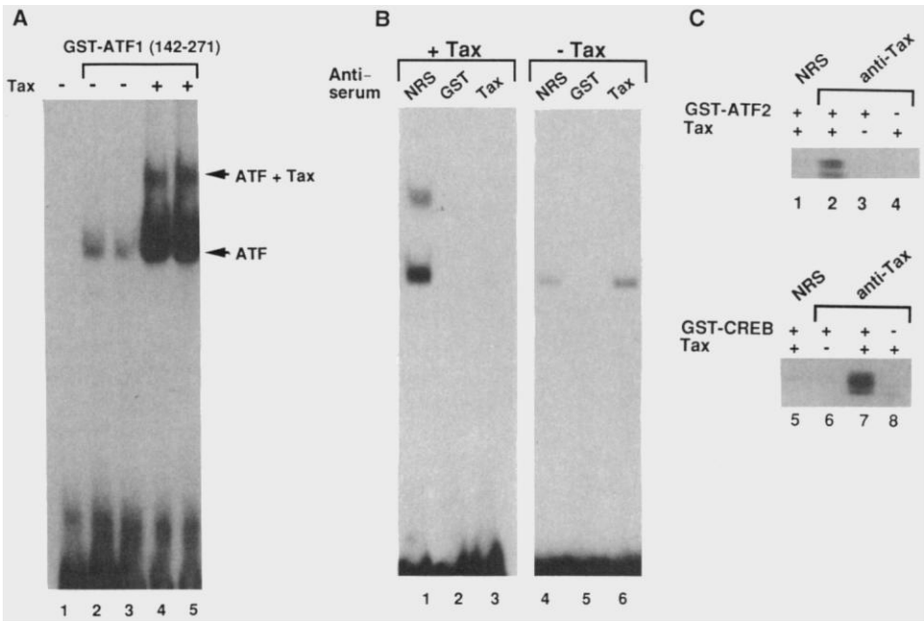

Fig. 2. Tax is a component of the bZIP-DNA complex. (**A**) Electrophoretic-mobility shift. Reaction mixtures containing purified GST-ATF1 (amino acids 142 to 271) and Tax were assayed for DNA binding as described in Fig. 1A. The reaction products were separated on a 5 percent native polyacrylamide gel in tris, glycine, EDTA buffer (*19*). (**B**) Immuno-depletion. Reaction mixtures containing GST-ATF2 (amino acids 350 to 415) in the presence (lanes 1–4) or absence (5–8) of Tax were incubated 15 minutes at room temperature with the indicated antiserum followed by absorption on Pansorbin cells (Calbiochem). The supernatant was analyzed by native gel electrophoresis as in Fig. 2A. Normal rabbit serum, NRS. (**C**) DNA coimmunoprecipitation assay. Protein complexes assembled on a ^{32}P-labeled DNA probe were immunoprecipitated with an anti-Tax (*38*) or normal rabbit serum (NRS), and bound DNA was analyzed by SDS–polyacrylamide gel electrophoresis. The GST fusion proteins contained either the ATF-2 minimal bZIP domain (amino acids 350 to 415; lanes 1 to 4) or CREB (amino acids 214 to 341; lanes 5 to 8) (*34*).

Tax increases formation of bZIP homodimers in the absence of DNA. Dimerization of bZIP proteins occurs in the absence of DNA and is a prerequisite for DNA binding (*13, 22–25*). Tax could therefore stimulate DNA binding by increasing either dimerization or the subsequent interaction between the bZIP homodimer and DNA. Using a chemical crosslinking assay, we measured the effect of Tax on bZIP dimerization. The two subunits of bZIP dimers can be crosslinked to one another with glutaraldehyde, a bifunctional crosslinking reagent (*13, 24*). The GST-ATF2 fusion protein was incubated in the presence or absence of Tax, and after the addition of glutaraldehyde the products were fractionated on an SDS–polyacrylamide gel and analyzed by immunoblotting (Fig. 3). At a concentration of 0.1 μM, GST-ATF2 is predominantly a monomer (Fig. 3, lanes 2 and 3) and as the concentration of GST-ATF2 was raised to 1.0 μM, homodimer formation increased (Fig. 3, lanes 6 and 7). Addition of Tax greatly increased the amount of ATF-2 homodimer (Fig. 3; compare lane 3 to lanes 4 and 5). Prolonged treatment with the crosslinking agent resulted in slower mobility complexes containing both ATF-2 and Tax (*26*).

To investigate how Tax affects the kinetics of DNA binding, we measured the association and dissociation rates of the bZIP-DNA complex in the presence and absence of Tax. In the absence of Tax, binding of GST-ATF2 to DNA reached a maximal level by 15 minutes (Fig. 4). In the presence of Tax, total DNA binding increased, as expected, and equilibrium was achieved after only 1 minute. Similar results were observed when the GCN4 peptide was used (Fig. 4, middle). Thus, Tax increases the on-rate of DNA binding.

For determining the dissociation rate of the bZIP-DNA complex, a reaction mixture was allowed to reach equilibrium, excess specific competitor DNA was added,

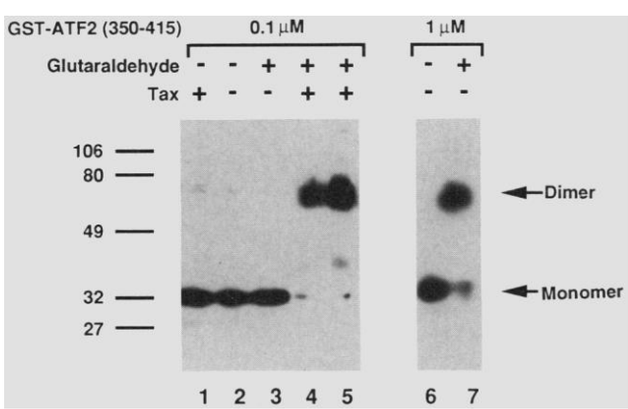

Fig. 3. Tax stimulates dimerization of the bZIP domain. Chemical crosslinking of GST-ATF2 was performed in the presence of 200 ng of bovine serum albumin (lanes 2, 3, and 6) or 100 ng (lane 4) and 200 ng of Tax (lanes 1 and 5), with a final concentration of 0.02 percent glutaraldehyde (lanes 3, 4, 5, and 7). Incubation was for 15 minutes at room temperature after which the reaction products were fractionated on a 12 percent SDS–polyacrylamide gel and detected by immunoblotting with a GST-specific polyclonal antiserum (*39*).

and the amount of remaining bZIP-DNA complex was measured as a function of time. The data (Fig. 4, bottom) indicate that bound ATF-2 dissociates from DNA at a comparable rate in the presence and absence of Tax. This result provides additional evidence that Tax does not interact directly with DNA in the ternary complex. We conclude that Tax increases the rate of formation of the bZIP-DNA complex, without affecting the off-rate, accounting for the observed DNA binding increase.

Our data indicate that Tax increases the DNA binding of several bZIP proteins. This stimulation of DNA binding requires only a minimal bZIP domain and appears to be specific for proteins containing this DNA binding motif. All bZIP domains contain a highly basic region used for contacting DNA, a leucine repeat that serves as a dimerization interface, and other conserved residues (*27*). These sequence and structural elements may provide the basis for recognition by Tax.

Previous studies have shown that a bZIP protein binds DNA in a two-step reaction (*13, 22–25*). In the first step, the bZIP dimerizes followed by a second step in which the homodimer binds to DNA. We have now shown that Tax increases dimerization in the absence of DNA and have provided evidence that Tax can interact with a minimal bZIP domain. The simplest interpretation of these data is that Tax binds preferentially to the dimer, thereby shifting the monomer-dimer equilibrium toward dimer formation (Fig. 5). The resulting increased concentration of the bZIP homodimer would account for the increased extent and rate of DNA binding.

Our model fits in well with several previous studies on bZIP proteins. For example, dimerization of a minimal bZIP peptide has an apparent dissociation constant in the micromolar range whereas the apparent dissociation constant for the overall DNA binding reaction is in the nanomolar range (*23, 28*). Thus, at the nanomolar concentrations typically used in DNA binding experiments, only a small fraction of the bZIP is in the dimer form. Under these conditions, therefore, the dimerization step, and not the subsequent DNA-protein interaction, limits DNA binding. A regulatory protein, such as Tax, that increases dimerization would therefore stimulate DNA binding.

Regulation of DNA binding is a means for controlling the activity of transcription factors, and several mechanisms for regulating DNA binding have been described (*29*). The Tax-mediated DNA binding increase reported here appears to be distinct from the previously described mechanisms. For example, the DNA binding activity of some AP-1 proteins such as Jun and Fos can be regulated by a redox (reduction-oxidation) reaction involving a conserved cysteine residue within the basic region of the bZIP domain (*30*). Accordingly, reducing agents, such as DTT, enhance the DNA binding of *E. coli*-derived Jun and Fos proteins. We found that the Tax-mediated stimulation of DNA binding is indistinguishable in the absence or presence of DTT (*31*). Moreover, the GCN4 peptide used here lacks a cysteine residue, ruling out redox regulation. Likewise, Tax increased DNA binding in the absence of Mg^{2+} and ATP, indicating that phosphorylation was not involved.

Auxiliary factors can also modulate DNA binding of several mammalian transcription factors. For example, dimerization of the homeodomain protein hepatocyte nuclear factor–1α (HNF-1α) is promoted by a tissue-specific cofactor, DCoH (dimerization cofactor of HNF-1α). However, unlike the case reported here, DCoH does not affect the DNA binding activity of HNF-1α, but rather enhances its ability to stimulate transcription (*16*).

The effect of Tax on ATF binding is in some respects reminiscent of the induction of DNA binding of SRF (serum response factor) by Phox1 (paired-like homeobox) (*17*). However, there is no evidence that Phox1 increases dimerization of SRF. Another important difference concerns the effects of Tax on association and dissociation rates. Apparently Phox1 increases both the association and the dissociation

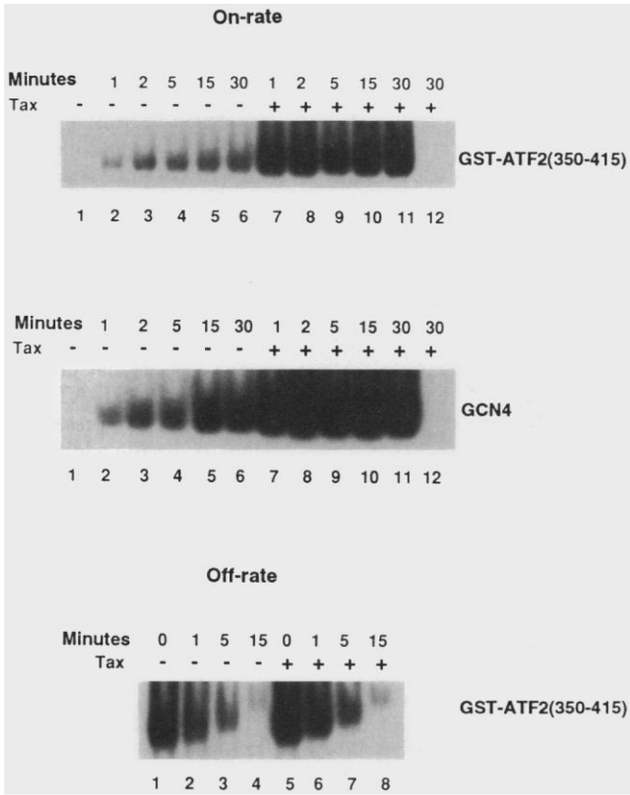

Fig. 4. Effect of Tax on the rate of DNA binding. (**Top**) Association rate of bacterially expressed GST-ATF2 with a HTLV-I LTR binding site. Reaction mixtures containing 50 ng of purified GST-ATF2 protein (lanes 2 to 11) were incubated for the indicated amount of time in the absence (lanes 1 to 6) or presence of 200 ng of Tax (lanes 7 to 12) and analyzed on a ×0.5 TBE, 5 percent polyacrylamide gel. (**Middle**) Association rate of a GCN4 peptide with a TPA response element. Like part A except that reaction mixtures contained 1 ng GCN4 of peptide. (**Bottom**) Dissociation rate of ATF2-DNA complex. Reaction mixtures were incubated for 15 minutes followed by addition of a 50-fold excess of unlabeled LTR oligonucleotide. Portions were removed at the indicated times and analyzed on a ×0.5 TBE (tris, borate, EDTA) 5 percent polyacrylamide gel.

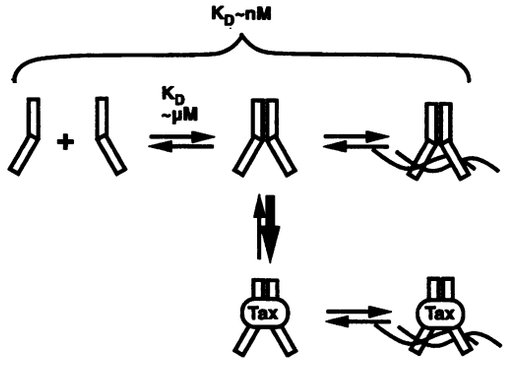

Fig. 5. Model for Tax-mediated stimulation of DNA binding. Efficient DNA binding of bZIP proteins requires that free monomers associate into dimers (*22, 25*). In solution, bZIP peptides form dimers when present at micromolar concentrations (*23*), whereas DNA binding can be detected at nanomolar concentrations (*28*). By interacting with the dimer, Tax shifts the monomer-dimer equilibrium towards the dimer. The elevated concentration of the dimer leads to an increase in DNA binding.

rate of the SRF-SRE complex, and it has been proposed that an increased exchange of SRF on its binding site could allow for a faster response to transient mitogenic signals. Tax increases the association rate of DNA binding without affecting the dissociation rate of the bZIP-DNA complex. The sum of these effects accounts for the overall increase in DNA binding.

The studies described here were performed in vitro. The observation that extracts prepared from cells containing Tax have increased TRE binding activity (*32*) supports the possibility that Tax also increases DNA binding on the HTLV-I LTR in vivo. Whereas previous studies suggested that CREB was the target of Tax action (*33*), we have found that Tax increases DNA binding of multiple ATFs, AP-1 proteins, and other bZIP proteins. Because Tax can act upon multiple bZIP proteins, Tax may preferentially promote the formation of certain combinations of bZIP homo- or heterodimers. For example, our model would predict that Tax will have a greater effect on homo- and heterodimer combinations whose dimerization constants are relatively weak.

Like other nuclear oncoproteins, Tax can immortalize cells in culture and transform cells in cooperation with other oncogene proteins, such as Ras (*3*). By altering the activity of cellular bZIP proteins, Tax will affect cellular gene expression. In particular, increasing the activity of known oncoproteins, such as c-Jun and c-Fos, may be the mechanism by which Tax transforms cells.

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34. GST fusion proteins were constructed by inserting cDNA sequences for ATF-1 (amino acids 142 to 271), ATF-2 (amino acids 350 to 505), ATF-2 (amino acids 350 to 415), and CREB (amino acids 214 to 341) in frame into pGEX vectors. Proteins were expressed in E. coli and purified on glutathione-agarose (Sigma) as described [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. Histidine-tagged ATF-2 was produced by cloning cDNA sequences of ATF-2 from amino acids 350 to 505 into pRsetB (Invitrogen). Fusion protein was purified by Ni-chelate chromatography on Ni^{2+}-NTA-agarose (Qiagen). Electrophoretic-mobility shift assays were performed with the use of a double-stranded oligonucleotide of the most distal HLTV-I LTR TRE sequence under conditions previously described (*7*). Reactions were analyzed on a 5 percent polyacrylamide- 0.5× TBE gel. Tax protein was expressed and purified as histidine-tagged fusion protein [L.-J. Zhao and C.-Z. Giam, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11445 (1991)]. Oligonucleotide sequence of the LTR probe: 5′-GGGATCCAGGCTCTGACGTCTCCCCCCAGATCTCC-3′.
35. Binding reactions with purified histidine-tagged Jun (residues 225 to 334) [L. Patel *et al.*, *Nature* **347**, 572 (1990)] were performed as described [W. J. Boyle *et al.*, *Cell* **64**, 573 (1991)]. Purified GCN4K58 (amino acids 226 to 281) [T. E. Ellenberger *et al.*, *ibid.* **71**, 1223 (1992)] was assayed under the same conditions as described for ATF proteins (*34*). The DNA probe was a double-stranded oligonucleotide of the collagen TRE 5′-AGCTTGGTGACTCATCCG-3′.
36. C/EBP Δ1-2 was expressed in BL21 cells from a T7 expression system. For gel shift assays partially purified protein from the pellet fraction was incubated with a C/EBP specific probe under conditions published elsewhere [A. D. Friedman and S. L. McKnight, *Genes Dev.* **4**, 1416 (1990)]. GST-C/EBP was constructed by inserting the Δ1-2 cDNA into pGEX2T and purified as described [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)].
37. Purified GAL4-AH and GST-myogenin were used in gel shift assays with a GAL4 oligonucleotide and a MCK right E box probe respectively under conditions published elsewhere [Y.-S. Lin *et al.*, *Cell* **54**, 659 (1988); T. J. Brennan and E. N. Olson, *Genes Dev.* **4**, 582 (1990)]. Purified MyoD, expressed from a T7 expression system, was bound to a MyoD binding site of the MCK enhancer as described [R. L. Davis *et al.*, *Cell* **60**, 733 (1990)].
38. Anti-Tax was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [S. M. Hanly *et al.*, *Genes Dev.* **3**, 1534 (1989)].
39. For chemical cross-linking, 100 ng GST-ATF2 (amino acids 350 to 415) and 100 ng or 200 ng Tax were preincubated in binding buffer (*34*) for 5 minutes at room temperature. Glutaraldehyde was added to a final concentration of 0.02 percent for 10 minutes at room temperature. Proteins were resolved on a 12 percent SDS–polyacrylamide gel. Immunoblotting on nitrocellulose (Schleicher & Schuell) was performed according to standard procedures with an antibody raised against GST and the ECL detection system (Amersham).

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