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Pleiotropic effect of the human T-cell leukemia virus Tax protein on the DNA binding activity of eukaryotic transcription factors

(NF-κB/serum response factor/Fos–Jun/trans-activation)

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ABSTRACT **The Tax protein, encoded by the human T-cell leukemia virus type I, is a potent activator of viral and cellular gene transcription. Tax does not bind DNA directly but appears to trans-activate through an interaction with host-cell transcription factors that recognize sequences within the promoters of Tax-responsive genes. Cellular transcriptional activators implicated in mediating Tax trans-activation include members of the activating transcription factor/cAMP response element binding protein (ATF/CREB) family of proteins, serum response factor, Fos–Jun, and NF-κB. Recent evidence suggests that Tax may stimulate human T-cell leukemia virus type I transcription, at least in part, through enhanced binding of ATF/CREB proteins to their recognition elements within the Tax-responsive 21-bp repeats of the viral promoter. In this report, we demonstrate that Tax also enhances the site-specific DNA binding activity of serum response factor and Fos–Jun and modestly enhances the binding of the NF-κB subunits, p50 and p65. We also show that Tax increases the DNA binding activity of the eukaryotic transcription factors ATF-1, Sp1, and GAL4. These results are consistent with the finding that Tax is highly pleiotropic and suggest that Tax trans-activation may involve enhancement in the DNA binding activity of target transcriptional regulatory proteins. In addition, we show that the mechanism of Tax-enhanced DNA binding activity does not involve an alteration in the redox state of the target protein.**

The Tax protein, encoded by the human T-cell leukemia virus type I (HTLV-I), is essential to the life cycle of the virus as efficient transcription from the viral long terminal repeat (LTR) is strongly dependent upon Tax (1–5). Tax stimulates HTLV-I gene expression through conserved 21-bp repeat enhancer elements located in the promoter of the virus (5–7). Tax also deregulates a wide variety of cellular genes, possibly leading to the pathogenesis associated with the virus. Tax does not efficiently bind DNA, suggesting that host-cell DNA binding proteins may mediate transcriptional activation by Tax (8). Examination of Tax-responsive promoter sequences indicates that multiple cellular transcription factors may be involved in mediating Tax trans-activation. For example, the Tax-responsive 21-bp repeats of HTLV-I contain sequences that serve as recognition elements for the activating transcription factor/cAMP response element binding protein (ATF/CREB) family of transcription factors (9, 10, 35); the human immunodeficiency virus, interleukin 2, and interleukin 2 receptor α promoters contain Tax-responsive elements that correspond with binding sites for NF-κB (11–14); the c-*fos*, *Krox-20*, and *Krox-24* genes are Tax trans-activated through multiple sequences in each promoter that include binding sites for serum response factor (SRF) (15–17); and, finally, transforming growth factor β1 appears to be Tax trans-activated through promoter elements that correspond with activating protein 1 (AP-1) binding sites that are likely recognized by the Fos–Jun heterodimer (18). Thus, Tax appears to utilize a variety of transcription factors to deregulate gene expression.

Recent reports suggest that Tax may deregulate target genes by enhancing the DNA binding activity of the cellular transcription factors that recognize the Tax-responsive promoter elements (19–21). We have recently demonstrated that Tax strongly enhanced the binding of both native and recombinant CREB and ATF-2 to each of the three 21-bp repeats of HTLV-I and that these proteins cooperate with Tax to stimulate HTLV-I transcription *in vitro* (35). In the present work, we extend these studies to other transcription factors implicated in mediating Tax trans-activation. We demonstrate that Tax strongly enhances the site-specific DNA binding activity of SRF and the Fos–Jun heterodimer but only modestly affects the DNA binding activity of the NF-κB subunits. Consistent with the highly pleiotropic effect of Tax on transcription *in vivo*, we show that Tax increases the DNA binding activity of several other eukaryotic transcriptional activator proteins from a variety of structural classes. Although the DNA binding activity of many of these proteins has been shown to be regulated by reduction–oxidation (redox) (22), we show that Tax does not utilize this mechanism to enhance binding.

EXPERIMENTAL PROCEDURES

Expression and Purification of Tax. Tax was purified from the HB101 strain of *Escherichia coli* using the pTaxH$_6$ expression vector (20) by two rounds of Ni^+ chelate chromatography, dialyzed against Superdex buffer [25 mM Hepes, pH 7.9/12.5 mM $MgCl_2$/10 μM $ZnSO_4$/150 mM KCl/4 mM 2-mercaptoethanol/20% (vol/vol) glycerol/0.1% Nonidet P-40], and stored at −70°C. The purity of Tax was analyzed by SDS/PAGE and Coomassie blue staining. Approximately 400 ng of the purified Tax protein was used in each experiment.

Expression and Purification of DNA Binding Proteins. Recombinant NF-κB subunits, p50 (aa 1–377) and p65 (aa 1–309), were purified by Ni^+ chelate chromatography as described (23). *E. coli*-expressed SRF (24) was purified by 15% (wt/vol) ammonium sulfate precipitation of sonicated lysates and dialyzed against Superdex buffer. CREB was expressed in *E. coli* and purified as described (25). ATF-1 was expressed and purified by the same method. Recombinant

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Abbreviations: SRF, serum response factor; HTLV-I, human T-cell leukemia virus type I; ATF, activating transcription factor; LTR, long terminal repeat; AP-1, activating protein 1; CREB, cAMP response element binding protein; EMSA, electrophoretic mobility shift assay; UAS, upstream activation sequence.

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wild-type and mutated Fos (aa 116–211) and Jun (aa 206–316) were provided by Tom Curran (Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology), Sp1 was obtained from Promega, and GAL4 (aa 1–147) was provided by Steve Triezenberg (Department of Biochemistry, Michigan State University).

Electrophoretic Mobility Shift Assays (EMSAs). Binding reaction mixtures contained the DNA binding protein in Superdex buffer, 0.5 nM ^{32}P-end-labeled double-stranded DNA, and Tax or an equivalent volume of Superdex buffer (with 4 mM fresh 2-mercaptoethanol). To visualize the effect of Tax on DNA binding activity, in some experiments, the DNA binding protein was diluted to a point where only a small percent of the labeled probe was complexed with protein. This allowed more accurate quantitation of the fold enhancement in binding activity in the presence of Tax. Binding reaction mixtures were incubated at room temperature for 15 min and analyzed on 5.2% nondenaturing gels [49:1 (wt/wt), acrylamide/*N*,*N*′-methylenebisacrylamide], dried, and autoradiographed. The electrophoresis buffer contained 0.04 M Tris·HCl, 0.306 M glycine (pH 8.5), and 0.1% Nonidet P-40. All reactions with Fos–Jun, p50, or p65 contained 50 ng of the alternating copolymer poly(dI-dC). Binding was quantitated by PhosphorImage analysis.

DNA Probes. Complimentary double-stranded oligonucleotides were used as probes in the binding assays and in competition assays. The nucleotide sequence of the top strands are as follows: serum response element (SRE) (human c-*fos* promoter), 5′-GATCACAGGATGTCCATATTAGGACATCTGC-3′; AP-1 site, 5′-TTCCGGCTGACTCATCAAGCG-3′; κB recognition site (human immunodeficiency virus), 5′-GATCGCTGGGACTTTCCAGGA-3′; cAMP response element (CRE), 5′-GATCTCCATGACGTCAATTGA-3′; HTLV-I site 3, 5′-GATCTCAGGCGTTGACGACAACCCCTCACCTCA-3′; Sp1 site (GC box, HTLV-I LTR), 5′-GATCTCCACCAAGAACCACCCATTTCCTA-3′; upstream activating sequence (UAS, GAL4 binding site), 5′-GATCGGAGGACTGTCCTCC-3′. The UAS, Sp1, and site 3 oligonucleotides were cloned into the *Bgl* II site of pUC 19, and each *Eco*RI–*Hin*dIII fragment was 5′-end-labeled for use as a probe.

RESULTS

Tax Enhanced the DNA Binding Activity of SRF, Fos–Jun, and NF-κB. We have recently shown that Tax dramatically enhanced the DNA binding activity of CREB and ATF-2 to the three 21-bp repeats in the transcriptional control region of HTLV-I (35). Since the cellular proteins SRF, Fos–Jun, and NF-κB also appear to be major targets for mediating Tax trans-activation, it was of interest to examine the effect of purified recombinant Tax on the binding activity of these proteins *in vitro*. Tax was expressed in *E. coli* using the pTaxH$_6$ expression system (20) and purified to near-homogeneity by Ni$^+$ chelate chromatography. An SDS/polyacrylamide gel of the purified Tax protein is shown in Fig. 1*A*, lane 2.

SRF binds to an AT-rich recognition sequence called a SRE found in many promoters, including several Tax-responsive immediate early genes (16, 17, 26). To test the effect of Tax on the DNA binding activity of SRF, the recombinant protein was assayed by EMSA in the presence and absence of Tax. Addition of Tax to the binding reaction mixture dramatically increased the amount of protein–DNA complex formed between SRF and its recognition element (40-fold in this experiment; Fig. 1*B*, lanes 9–12). Tax did not alter the mobility of the SRF–DNA complex, suggesting that Tax increases the DNA binding activity of SRF without entering into the complex, consistent with the recently reported weak interaction between SRF and Tax (17). The

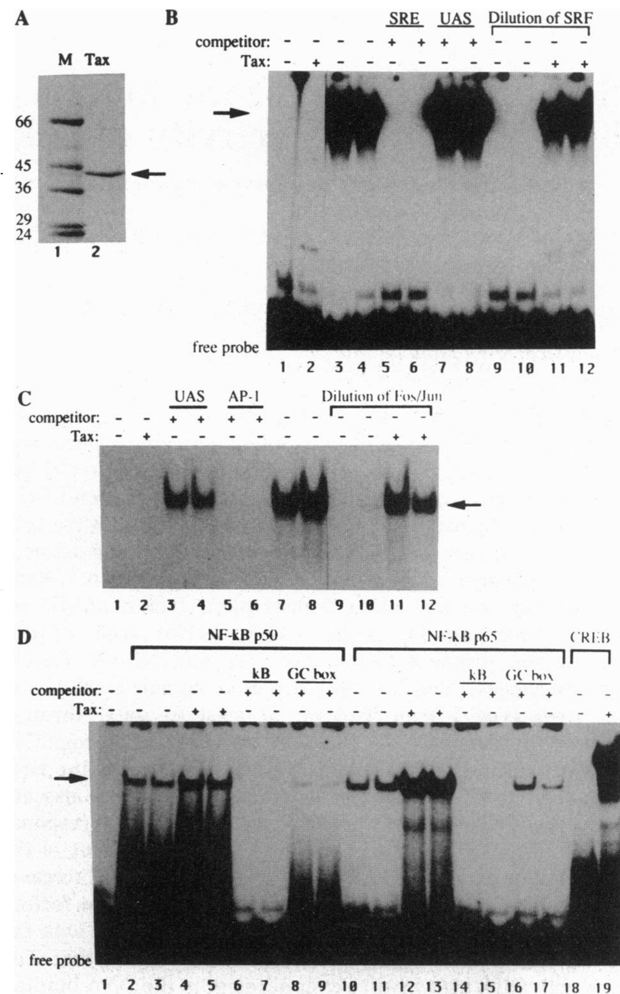

FIG. 1. Recombinant Tax protein enhances the DNA binding activity of SRF, Fos–Jun, and NF-κB. (*A*) Recombinant Tax protein (pTaxH$_6$) was expressed in *E. coli* and purified by Ni$^+$ chelate chromatography. The purified Tax protein (8 μl) was assayed by SDS/PAGE and visualized by Coomassie staining. The size (kDa) of the protein molecular mass standards (lane M) is shown on the left. The position of Tax (40 kDa) is indicated by an arrow. (*B*) EMSA with purified recombinant SRF in the presence and absence of Tax. Approximately 1 ng of SRF was incubated with the SRE oligonucleotide probe in the absence (lanes 3 and 4) or presence of a 250-fold molar excess of specific (SRE, lanes 5 and 6) or nonspecific (UAS, lanes 7 and 8) competitor oligonucleotides. To allow accurate quantitation of the effect of Tax on the binding activity of SRF, a 1:5 dilution of SRF (0.2 ng) was incubated in the absence (lanes 9 and 10) or presence (lanes 11 and 12) of Tax. As a control, the SRE probe was electrophoresed alone (lane 1) or with Tax (lane 2). (*C*) EMSA with purified Fos–Jun heterodimers in the presence and absence of Tax. Fos and Jun (each at 0.1 ng) were incubated with the AP-1 oligonucleotide probe in the absence (lanes 7 and 8) or presence of a 500-fold molar excess of nonspecific (UAS, lanes 3 and 4) or specific (AP-1, lanes 5 and 6) competitor oligonucleotides. The proteins were diluted 1:2.5 and assayed in the absence (lanes 9 and 10) or presence (lanes 11 and 12) of Tax. As a control, the AP-1 probe was electrophoresed alone (lane 1) or with Tax (lane 2). The gel was truncated to show only the protein–DNA complexes. (*D*) EMSA with purified NF-κB in the presence and absence of Tax. The p50 (6.5 ng) or p65 (33 ng) subunits of NF-κB were incubated with the κB oligonucleotide probe in the absence (lanes 2, 3, 10, and 11) or presence (lanes 4, 5, 12, and 13) of Tax. Specificity of binding was measured by adding a 500-fold molar excess of specific (κB, lanes 6, 7, 14, and 15) or nonspecific [Sp1 binding site (GC box), lanes 8, 9, 16, and 17] competitor oligonucleotides. Lane 1 shows the κB probe electrophoresed in the absence of protein. As a control, 2 ng of CREB were incubated in the presence of the HTLV-I site 3 oligonucleotide in the absence (lane 18) or presence (lane 19) of Tax.

magnitude of the Tax enhancement on SRF binding is similar to that typically observed for the ATF/CREB proteins binding to their recognition element (35). The binding of SRF was specific, as an excess of unlabeled SRE DNA competed for binding, whereas an equivalent amount of an unrelated site did not (Fig. 1*B*, lanes 3–8).

The nuclear factors Fos and Jun preferentially associate to form heterodimers that bind the AP-1 recognition element and modulate transcription. To test the effect of Tax on the DNA binding activity of the Fos–Jun heterodimer, we assayed truncated forms of Fos and Jun proteins, containing principally their DNA binding/dimerization domains (27). Tax produced a dramatic increase in the binding activity of the Fos–Jun heterodimer to an AP-1 oligonucleotide probe (Fig. 1*C*, lanes 9–12). The specificity of Fos–Jun binding was shown by competition assay (Fig. 1*C*, lanes 3–8). Parallel binding experiments with full-length Fos–Jun heterodimers and Jun homodimers showed similar enhancement in DNA binding activity in the presence of Tax (data not shown). Tax enhancement of the truncated forms of Fos–Jun suggests that the amino acids essential for the effect of Tax on DNA binding activity reside in or near their conserved DNA binding and dimerization domains. This observation is consistent with results we have recently obtained with similarly truncated forms of the related CREB and ATF-2 proteins (35). Tax did not enhance the DNA binding activity of homodimers formed with high concentrations of Fos (data not shown).

We next tested whether Tax could affect the DNA binding activity of the transcriptional activator protein NF-κB. Cellular NF-κB is a heterodimer of two subunits, designated p50 and p65. The DNA binding activities of truncated p50 and p65, containing principally their *rel* DNA binding/dimerization domains (23), were separately assayed in the absence and presence of Tax. Tax enhanced the binding activity of both p50 and p65 to a κB recognition element from the human immunodeficiency virus LTR (Fig. 1*D*, lanes 2–5 and 10–13; 2-fold and 6-fold, respectively). However, the magnitude of the enhanced binding, especially for p50, was considerably less than that observed for CREB binding to the third 21-bp repeat of HTLV-I in the same experiment (Fig. 1*D*, lanes 18 and 19; 65-fold). The binding of both p50 and p65 was κB-specific, as shown by competition assay (Fig. 1*D*, lanes 6–9 and 14–17). In the experiment shown, the high concentration of nonspecific competitor partially competed for p50 binding. Other experiments, using lower concentrations of competitor, clearly show p50 binding is specific for the κB element. These data indicate that Tax trans-activation through NF-κB recognition elements may occur, in part, through a modest enhancement in NF-κB binding activity (see *Discussion*).

Tax Enhanced the DNA Binding Activity of a Broad Range of Eukaryotic Transcription Factors. The reportedly pleiotropic effects of Tax on transcription *in vivo* led us to further test the effect of Tax on the DNA binding activity of other eukaryotic transcriptional activator proteins. ATF-1, a member of the ATF/CREB family of transcription factors, binds to each of the three 21-bp repeats of HTLV-I (data not shown and ref. 21). Because ATF-1 may play a role in HTLV-I gene expression, it was of interest to test the effect of Tax on the binding of ATF-1 to a 21-bp repeat element. Incubation of recombinant purified ATF-1 in the presence of Tax showed enhanced binding activity of ATF-1 to the HTLV-I third 21-bp repeat (Fig. 2*A*, lanes 3–6). The binding of ATF-1 was specific, as shown by competition assay (Fig. 2*A*, lanes 1 and 2). Tax produced a similar enhancement in ATF-1 binding at the first and second 21-bp repeats of HTLV-I (data not shown).

We next tested whether Tax could affect the DNA binding activity of the zinc finger protein Sp1. Sp1 binds to a GC-rich recognition sequence found in many promoters, including the HTLV-I promoter (28). The Sp1 binding site in the HTLV-I promoter is located in a region previously shown to be Tax responsive *in vivo* (29). Tax increased the amount of protein–DNA complex formed between purified Sp1 and a probe containing the Sp1 recognition site from the HTLV-I LTR (Fig. 2*B*, lanes 3–6). The binding of Sp1 was specific, as an excess of an unlabeled Sp1 site competed for binding,

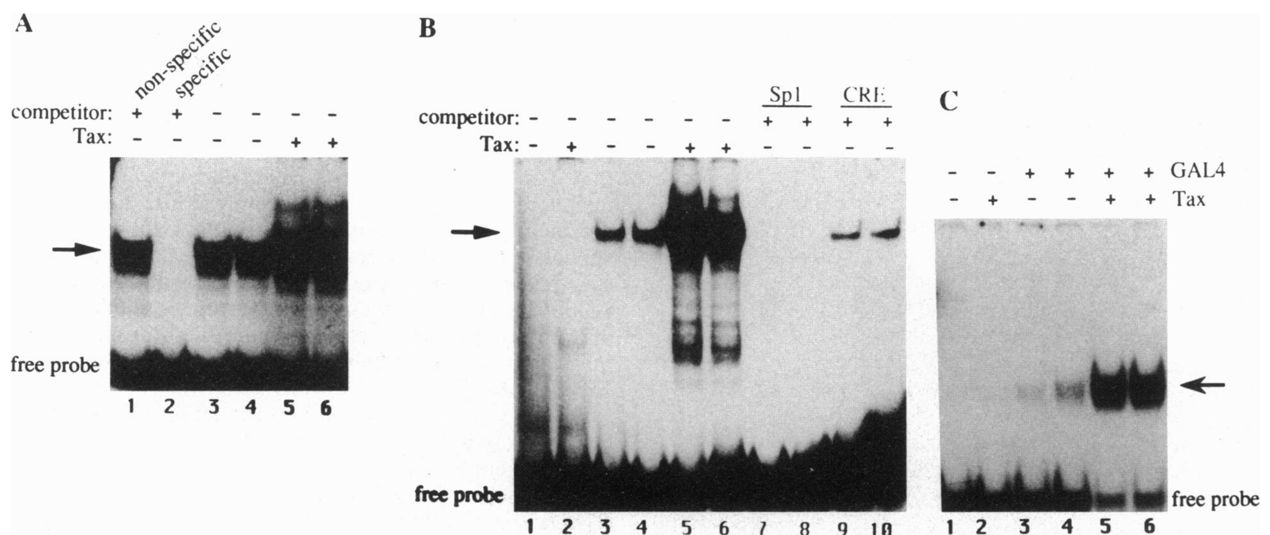

FIG. 2. Tax enhances the DNA binding activity of a broad range of DNA binding proteins. DNA binding activities of ATF-1, Sp1, and GAL4 were assayed by EMSA in the presence or absence of Tax. (*A*) Purified recombinant ATF-1 (2.5 ng) was incubated with a probe representing the third HTLV-I 21-bp repeat in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of Tax. As a control for specificity of binding, ATF-1 was incubated with a 250-fold molar excess of nonspecific (UAS, lane 1) or specific (CRE, lane 2) competitor oligonucleotides. (*B*) Purified transcription factor Sp1 (1 ng) was incubated with a probe representing the HTLV-I Sp1 site. Binding reactions were done in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of Tax. To demonstrate specificity of binding, Sp1 was incubated in the presence of a 250-fold molar excess of specific (Sp1 site, lanes 7 and 8) or nonspecific (CRE, lanes 9 and 10) competitor oligonucleotides. As a control, the probe was electrophoresed alone (lane 1) or with Tax (lane 2). (*C*) The purified DNA binding region of GAL4 (aa 1–147) was incubated with a labeled UAS fragment in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of Tax. As a control, the UAS probe was electrophoresed alone (lane 1) or with Tax (lane 2).

whereas an equivalent amount of an unrelated site did not (Fig. 2*B*, lanes 7–10). The same Tax enhancement of Sp1 binding was also obtained with a truncated form of the protein containing only the zinc finger DNA binding domain (data not shown).

Finally, we were interested in testing whether Tax could enhance the DNA binding activity of a transcription factor that has not been implicated in HTLV-I regulation or Tax trans-activation. We chose the DNA binding domain of the yeast transcriptional activator protein GAL4. GAL4 binds as a dimer to a 17-bp recognition sequence present in the promoters of galactose-regulated genes in yeast. Fig. 2*C* shows that Tax increased the amount of complex formed between the GAL4 DNA binding domain and its recognition site. Similar results were obtained with several fusion proteins containing the GAL4 DNA binding domain; these included GAL4–VP16 and glutathione-*S*-transferase–GAL4 (aa 1–147) (data not shown).

Tax Does Not Enhance DNA Binding Activity Through a Redox Mechanism. The DNA binding activity of certain proteins has been shown to be modulated by redox (22). For example, Fos and Jun each contain a conserved redox-sensitive cysteine residue in their DNA binding domains (30) that is also conserved in the DNA binding domains of many of the ATF/CREB proteins, including CREB, ATF-1, and ATF-2. Recently, cellular redox factor 1 (Ref-1), was shown to enhance DNA binding activity through reduction of a basic domain cysteine residue in both Fos and Jun (22). Ref-1 increased the DNA binding activity of the Fos–Jun heterodimer but had no effect on the Fos homodimer. Furthermore, Ref-1 modulated the DNA binding activity of a wide range of proteins, including NF-κB and members of the ATF/CREB family (CREB, ATF-1, and ATF-2), without altering the mobilities of the protein–DNA complexes (22). Because of the striking similarity between Ref-1 and Tax, we considered whether the two proteins may be functionally analogous and, therefore, whether Tax and Ref-1 might enhance DNA binding activity in mechanistically similar ways. To test this hypothesis, we obtained mutant polypeptides of both Fos and Jun that contained a serine in place of the conserved redox-sensitive cysteine residue in the DNA binding domain of each protein. This single amino acid substitution in both Fos and Jun has been shown to produce proteins that bind DNA constitutively and abolish redox and Ref-1 regulation of binding *in vitro* (30). Tax enhanced the DNA binding activity of the redox-insensitive Fos–Jun proteins (Fig. 3, lanes 9–12). This enhancement in binding activity is nearly identical to that obtained with the wild-type proteins (compare Figs. 1*C* with 3). Again, the binding was specific, as demonstrated by the competition assay (Fig. 3, lanes 3–6). This result indicates that Tax enhances DNA binding activity in a manner distinct from the redox mechanism utilized by the Ref-1 protein.

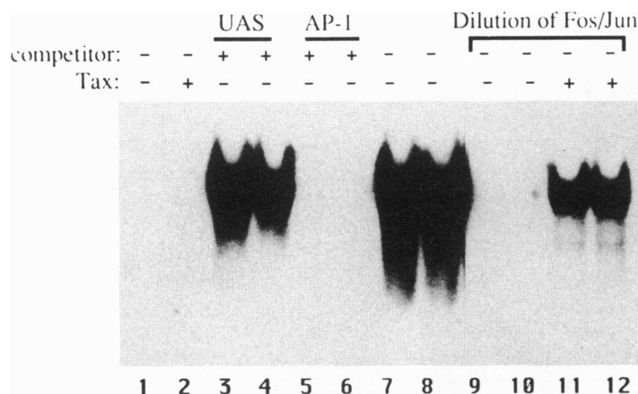

FIG. 3. Tax enhances the DNA binding activity of the redox-insensitive Fos–Jun heterodimer. Heterodimers formed between mutated (see text) Fos and Jun (each at 1.3 ng) were incubated with the AP-1 oligonucleotide probe in the absence (lanes 7 and 8) or presence of a 500-fold molar excess of unlabeled nonspecific (UAS, lanes 3 and 4) or specific (AP-1, lanes 5 and 6) competitor oligonucleotides. The proteins were diluted 1:4 and assayed in the absence (lanes 9 and 10) or presence (lanes 11 and 12) of Tax. As a control, the AP-1 probe was electrophoresed alone (lane 1) or with Tax (lane 2). Protein–DNA complexes were analyzed by EMSA. Free probe was not shown.

DISCUSSION

Tax enhances the DNA binding activity of cellular proteins that bind the HTLV-I 21-bp repeats, including CREB and ATF-2 (19–21, 35). These observations have led to the hypothesis that Tax activation of HTLV-I transcription may be mediated, at least in part, through increased binding of CREB and ATF-2 to the Tax-responsive 21-bp repeats, resulting in a higher concentration of these transcription factors at the HTLV-I promoter. In this report, we consider the possibility that Tax may similarly enhance the DNA binding activity of the other characterized mediators of Tax trans-activation *in vivo*. We demonstrate that Tax strongly enhances the DNA binding activity of SRF and the Fos–Jun heterodimer and modestly enhances the binding of the NF-κB p50 and p65 subunits. These results suggest that Tax may mediate transcriptional activation through these target proteins by enhanced binding at their Tax-responsive recognition elements. The unexpected observation that Tax enhances the DNA binding activity of Sp1 and GAL4 suggests that the targets for Tax trans-activation may extend beyond the previously identified factors and include a variety of additional cellular transcription factors from diverse structural classes. The identification of Sp1 and GAL4 as functional targets for Tax *in vivo* will be essential to confirming this hypothesis and establishing these proteins as true mediators of Tax trans-activation.

Tax trans-activation through NF-κB has been shown to occur via both pre- and post-translational mechanisms (12, 13, 31, 32). Two recent reports have identified Tax mutants that functionally segregate transcriptional activation by the ATF/CREB and NF-κB pathways, indicating that trans-activation through NF-κB may differ mechanistically from the other target activator proteins (33, 34). Our observations that Tax strongly increases the DNA binding activity of members of the ATF/CREB family while only modestly enhancing the DNA binding activity of the p50 and p65 subunits of NF-κB are consistent with the possibility that Tax may trans-activate through two functionally distinct pathways. We cannot, however, exclude the possibility that Tax may have a greater effect on the DNA binding activity of the full-length NF-κB proteins or heterodimers formed between p50 and p65.

Because of the highly pleiotropic effect of Tax on DNA binding activity, we have been careful to ensure that this enhancement is a property intrinsic to the Tax protein. The enhancement of DNA binding activity coelutes with the 40-kDa Tax protein from both gel-filtration and metal-chelate chromatography, and the activity is present in highly purified Tax preparations from both insect and bacterial cells (data not shown and ref. 35). Furthermore, a mock preparation of Tax, prepared from *E. coli* cells transformed with a control plasmid not encoding the Tax gene, does not enhance the binding activity of target proteins (35). The mock Tax was expressed and purified in parallel with authentic Tax. Finally, as a control the addition of bovine serum albumin to binding reaction mixtures has no detectable effect on the DNA binding activity of the proteins reported in this study.

The mechanism by which Tax enhances the DNA binding activity of such a wide range of proteins is not understood. The proteins affected by Tax do not appear to share any obvious structural similarities, and although the DNA binding activity of many of these proteins is redox-sensitive, Tax does not appear to utilize a redox mechanism to enhance DNA binding activity. In a recent study, the homeodomain protein Phox was shown to enhance the DNA binding activity of SRF by accelerating the rate of exchange between SRF and its recognition element (24). Alternatively, Tax may directly stabilize protein–DNA complexes through the formation of a ternary complex. Although we have been consistently unable to detect ternary complexes in our EMSA, they may be transient and elude detection by our methods.

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