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Physical and Functional Interaction between the Human T-Cell Lymphotropic Virus Type 1 Tax_1 Protein and the CCAAT Binding Protein NF-Y

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Tax_1, a potent activator of human T-cell lymphotropic virus type 1 (HTLV-1) transcription, has been shown to modulate expression of many cellular genes. Tax_1 does not bind DNA directly but regulates transcription through protein-protein interactions with sequence-specific transcription factors. Using the yeast two-hybrid system to screen for proteins which interact with Tax_1, we isolated the B subunit of the CCAAT binding protein NF-Y from a HeLa cDNA library. The interaction of Tax_1 with NF-YB was specific in that NF-YB did not interact with a variety of other transcription factors, including human immunodeficiency virus Tat, human papillomavirus E6, and Bicoid, or with the M7 (amino acids ^{29}CP-AS) Tax_1 mutant. However, NF-YB did interact with the C-terminal Tax_1 mutants M22 (^{130}TL-AS) and M47 (^{319}LL-RS). We also show that in vitro-translated NF-YB specifically bound to a glutathione *S*-transferase–Tax_1 fusion protein. Further, Tax_1 coimmunoprecipitated with NF-Y from nuclear extracts of HTLV-1-transformed cells, providing evidence for in vivo interaction of Tax_1 and NF-YB. We further demonstrate that Tax_1 specifically activated the NF-Y-responsive DQβ promoter, as well as a minimal promoter which contains only the Y-box element. In addition, mutation of the Y-box element alone abrogated Tax_1-mediated activation. Taken together, these data indicate that Tax_1 interacts with NF-Y through the B subunit and that this interaction results in activation of the major histocompatibility complex class II promoter. Through activation of this and other NF-Y driven promoters, the Tax_1–NF-Y interaction may play a critical role in causing cellular transformation and HTLV-1 pathogenesis.

Human T-cell lymphotrophic virus type 1 (HTLV-1) is the causative agent of the aggressive, usually fatal adult T-cell leukemia (see references 22, 23, 48 and 86 and references therein). Recent reports have linked HTLV-1 with additional and clinically diverse human diseases (31, 46, 53, 59, 63, 65). The best characterized of these is a progressive demyelinating syndrome termed HTLV-1-associated myelopathy (HAM) or tropical spastic paraparesis (TSP) (see references 22, 23, 48, and 86 and references therein). Because of the long incubation period between viral exposure and disease onset, the mechanism of HTLV-1 pathogenesis is still unclear. It is clear that host cell control of HTLV-1 replication is a primary determinant of virus expression and subsequent disease. Studies on the variation of viral sequences among different infected groups have not revealed any particular determinant which distinguishes onset of a particular HTLV-1 associated disease (18, 44, 60, 74, 87, 88).

In vitro, HTLV-1 has been shown to activate and immortalize human T lymphocytes, resulting in the polyclonal proliferation of infected cells and subsequent oligoclonal or monoclonal growth (26, 43). HTLV-1 contains no known cellular protooncogene, and because there is no preferential site of integration among infected individuals, it is unlikely that insertional mutagenesis is responsible for viral transformation. Several lines of evidence suggest that the viral transcriptional activator Tax contributes to the development of disease (23, 28, 29, 82).

Tax, which is essential for viral replication (22, 23, 48, 86), has recently been shown to transform established rodent fibroblasts to anchorage-independent growth (77, 82, 85). Moreover, Tax can transform primary rat embryo fibroblasts in cooperation with activated *ras* (67) as well as immortalize normal human T lymphocytes when expressed from a herpes simplex virus-based vector (28, 29). Finally, transgenic mice carrying the Tax gene frequently develop mesenchymal tumors and neurofibromas (34, 62). These findings suggest that Tax plays a critical role in HTLV-1-associated leukemogenesis.

Tax is believed to exert its effect by stimulating viral gene expression and by deregulating expression of cellular genes (22, 23, 48, 86). Tax has been shown to activate transcription of a number of cellular genes involved in cell proliferation, such as those for interleukin-3 (IL-3), IL-2, the IL-2 receptor, granulocyte-macrophage colony-stimulating factor, c-Fos, c-Jun, parathyroid-related protein, and the major histocompatibility complex (MHC) class I (see references 22, 23, 48, and 86 and references therein). The aberrant expression of these growth-related genes has been implicated in contributing to the establishment of HTLV-1-associated pathogenesis. While Tax does not bind DNA directly, it appears to stimulate RNA synthesis through protein-protein interactions with host cell transcription factors. The most well studied of these interactions is with the ATF/CREB family of transcription factors, which bind to cyclic AMP-responsive elements in the HTLV-1 long terminal repeats (1, 5, 22, 24, 66, 81, 83, 91). Tax has also been shown to mediate transcription of a variety of cellular genes through interaction with NF-κB-like factors, Ets-1, and serum response

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factor (22, 23, 36, 48, 61, 86). The molecular steps leading to the deregulated expression of cellular genes by Tax are not yet understood; however, recent reports suggest that Tax may activate transcription through enhancement of the DNA binding activities of cellular transcription factors (4, 5, 24, 56, 83, 91). This Tax-dependent enhancement of DNA binding activity is in part due to an increase in basic leucine zipper protein dimerization (4, 7, 10, 83). In addition, Tax has been shown to stimulate transcription of cellular genes which contain NF-κB sites by increasing the nuclear concentration of active NF-κB complexes through protein translocation (36, 57, 61).

Because of the critical role that Tax has in HTLV-1 pathogenesis, we utilized the yeast two-hybrid system to identify cellular proteins which interact with Tax. Our studies clearly demonstrate that Tax interacts with the transcription factor NF-Y. NF-Y is an evolutionarily conserved heteromeric DNA binding protein composed of A, B, and C subunits (52, 76). NF-Y (also called CP-1, CBF, or YEBP) recognizes the CCAAT motif and was initially identified by its binding to Y boxes of the MHC class II promoters (20, 89). Our studies demonstrate that the B subunit of NF-Y directly interacts with Tax and that this interaction results in an activation of the MHC class II promoter. Activation of NF-Y by Tax may also increase expression of other cellular genes which contribute to HTLV-1 induced pathogenesis.

MATERIALS AND METHODS

cDNA library and DNA binding domain plasmids. HeLa cDNA library-encoded proteins were expressed by using the 2μm *TRP1* galactose-inducible pJG4-5 vector (kindly provided by Roger Brent), which also contains sequences coding for the influenza virus hemagglutinin epitope tag, the B42 acidic activation domain, and the simian virus 40 nuclear localization signal located upstream of the cDNA (90). Tax genes (wild type, M7, M22, and M47) were cloned downstream from the DNA binding dimerization domains of the LexA bacterial repressor in the 2μm *HIS3* vector pL202PI. pL202PI-based vectors expressing the Tat, E6, or Bicoid protein were also used (16).

Two-hybrid assay. EGY48 (MATa *trp1 ura3 his3 LEU2*::pLexAop6-*LEU2*) (kindly provided by Roger Brent) carrying pJK103 (*URA3*), which directs the expression of a *GAL1-lacZ* gene from two high-affinity ColE1 LexA operators (90), was transformed with pLexA/Tax or pLexA/M16. The resulting strains were transformed with the pJG4-5-based HeLa library (*TRP1*) by the lithium acetate method of Ito et al. (37). A total of 8.5×10^5 primary transformants were selected on Ura^- His^- Trp^- glucose plates, scraped, and pooled. Cells (3×10^6) from this pool were spread on four Ura^- His^- Trp^- Leu^- galactose plates. Colonies appearing 3 days later were streaked onto Ura^- His^- Trp^- glucose plates and Ura^- His^- Trp^- Leu^- X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) galactose plates. Plasmids from blue, Leu^+ colonies were recovered by direct introduction into the *Escherichia coli* K-12 strain KC8 *pyrF*::Tn*5* hsdR leuB600 trpC9830 lacD74 strA galK hisB436 by electroporation as described by Marcil and Higgins (54).

The cDNA clones were sorted into a total of 12 classes based on restriction enzyme digestion patterns. At least one representative cDNA from each class was partially sequenced to establish identity by using the Sequenase system (U.S. Biochemicals). Two identical, independently isolated, full-length NF-YB clones were recovered.

β-Galactosidase assays. Quantitative β-galactosidase assays with *o*-nitrophenyl-β-D-galactosidase were performed on permeabilized cells essentially as described by Ausubel et al. (6). Expression of the pJG4-5 Gal1 promoter was induced by shaking cultures overnight in Ura^- Trp^- His^- Leu^- galactose medium at 30°C. The cells were washed in ultrapure water and suspended to an optical density at 600 nm of 0.2/ml in buffer Z (10 mM KCl, 1 mM $MgSO_4$, 60 mM Na_2PO_4, 40 mM NaH_2PO_4). Cells (1.0 ml) were permeabilized by addition of 15 μl of 0.1% sodium dodecyl sulfate (SDS) and 20 μl of chloroform followed by a 10-s vortex. After incubation of the samples at 30°C for 10 min, 0.2 ml of *o*-nitrophenyl-β-D-galactoside (4 mg/ml in buffer Z) was added, and incubation at 30°C was continued for 15 min. The reactions were terminated by addition of 0.5 ml of 1 M Na_2CO_3. The cells were pelleted, and the optical density at 420 nm of the supernatant was determined.

Cell lines and nuclear extracts. All cell lines were maintained at a density of 1×10^6 to 2×10^6 cells/ml in RPMI 1640 supplemented with 100 mM glutamine and 10% fetal bovine serum. Nuclear extracts were prepared by the method of Schreiber et al. (72) from Jurkat and CEM (American Type Culture Collection) T-cell lines, as well as from the HTLV-1-transformed cell lines C81 and MT2. The cells were washed twice in phosphate-buffered saline (PBS) and suspended in buffer A (10 mM HEPES [pH 7.9], 100 μM EDTA, 100 μM EGTA, 500 μM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], 10 mM KCl) at a density of 10^6 cells per 400 μl. After incubation on ice for 15 min, Nonidet P-40 (NP-40) was added to a final concentration of 0.6%, and the samples were vortexed for 10 s. Nuclei were pelleted at $10,000 \times g$ for 30 s at room temperature. The pellets were suspended in 50 μl of buffer C (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and rocked at 4°C for 15 min to extract proteins. Extracts were centrifuged ($10,000 \times g$) for 5 min at 4°C, the supernatants were transferred to new tubes, and protein concentrations were determined by the Bradford assay (Bio-Rad).

Protein analysis. Nuclear extracts (300 μg) were immunoprecipitated overnight at 4°C with either NF-YA antibody (10 μg) or immunoglobulin G (IgG) antibody (10 μg) of the same isotype in a final volume of 100 μl. The reaction mixtures were incubated for 1 h at 4°C with protein A-Sepharose beads (Pharmacia) to capture antibody-protein complexes. The samples were washed four times with wash buffer (120 mM NaCl, 1 mM DTT, 1 mM PMSF, 200 μM sodium vanadate, 50 mM NaF, 0.5% NP-40, 50 mM Tris-Cl [pH 7.4], 5 mM EDTA with 1 μg of leupeptin per ml and 18 μg of aprotinin per ml), and the proteins were eluted from the beads by boiling in SDS sample buffer and separated in 4 to 20% polyacrylamide Tris-glycine gels (Novex). Fifty micrograms of nuclear extract was run as a control. The gels were analyzed by immunoblotting as described by Ausubel et al. (6) with antiserum specific for Tax (14), NF-YA (Rockland Inc.), or NF-YB (a kind gift from Robert Mantovani) and visualized by chemiluminescence (Amersham).

GST binding assay. Five hundred nanograms of purified glutathione *S*-transferase (GST) or GST-Tax was incubated with 30 μl of a 50% slurry of glutathione-Sepharose (Pharmacia) for 1 h at 4°C. The beads were washed three times in binding buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) to remove unbound proteins and suspended in 200 μl of binding buffer. Radiolabeled NF-YA, NF-YB, and YB-1 proteins were synthesized in vitro by using the TNT T7-coupled rabbit reticulocyte lysate system (Promega) and radiolabeled with 40 μCi of [^{35}S]methionine (>1,000 Ci/mmol) (Amersham) in a 50-μl volume. The in vitro-translated mixtures (5 μl) were added to either GST or GST-Tax coupled to glutathione-Sepharose beads. The samples were rotated at 4°C for 3 h and washed three times in binding buffer plus 0.5% NP-40, and the proteins were eluted from the beads by boiling in SDS sample buffer. The proteins were separated by electrophoresis on 10% polyacrylamide–SDS gels and analyzed by autoradiography.

Northern blot analysis. mRNAs from human T-cell lines and an osteosarcoma cell line were prepared and transferred to nylon membranes as described by Dittmer et al. (19). Gel-purified oligonucleotide probes (DQβ [5′-GGTCGTG CGGAGCTCCAACTGGTAGTTGTGTCTGCACACCGTGTCCA-3′ and NF-YB [5′-CCGTTTCTCTTGATGGCACCTTTCACTTGC-3′]) were end labeled by using T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]dATP (>3,000 Ci/mmol) as described by the manufacturer.

Electroporations and CAT assays. Jurkat cells cultured to a density of 1×10^6 cells/ml were washed once in PBS and suspended in RPMI 1640 at 2.5×10^8 cells/ml. DNA diluted in 50 μl of distilled water was added to 250 μl of cells and electroporated with the Gibco BRL Cell Porator Electroporation System at 250 V and 800 μF. The cells were chilled on ice for 30 min and suspended in 5 ml of RPMI 1640 supplemented with 10% fetal bovine serum. Cells were harvested at 24 to 36 h posttransfection, washed twice with PBS, suspended in 100 μl of 0.25 M Tris-Cl (pH 7.5), subjected to three cycles of freezing-thawing, and assayed for chloramphenicol acetyltransferase (CAT) activity (6). Conversion was quantitated with a Molecular Dynamics PhosphorImager. All other promoter constructs were derived from pDQβCAT (kindly provided by Elliot P. Cowan), which contains the MHC class II DQβ promoter cloned upstream of the CAT gene in pBLCAT3 (50). pΔH/PCAT, which lacks promoter sequences upstream of the Y box, was designed by deletion of the internal *Hin*dIII-to-*Ppu*MI restriction fragment. pΔB/PCAT with the Y box deleted was constructed by digestion of pDQβCAT with *Ppu*MI and *Bsm*FI, and restriction sites were blunt ended with mung bean nuclease prior to ligation. The Y-box mutant pBclICAT was constructed by digesting pDQβCAT with *Bsm*FI and *Ppu*MI and ligating the complementary oligonucleotide 5′-GTCCTTCAGCTCCAGTGCTGATCATTT CTTTTCCGAGGGACCATCCAATCC-3′. All mutant constructs were confirmed by restriction digestion and sequence analysis.

Gel electrophoretic mobility shift assay. The gel-purified oligonucleotide 5′-TCCAGTGCTGATTGGTTTTCCGAGGGA-3′ (100 ng), which contains the Y-box element, was end labeled by using T4 polynucleotide kinase in the presence of [γ-^{32}P]dATP (>3,000 Ci/mmol) and made double stranded by annealing to 100 ng of the gel-purified antisense oligonucleotide. Nuclear extracts (6 μg) were preincubated for 10 min at 4°C with 1 μg of poly(dI-dC) and 100 ng of unlabeled competitor DNA or 1 μg of antibody in (final concentrations) 12.5 mM HEPES (pH 7.9)–6.25 mM $MgCl_2$–0.05% NP-40–5 mM $ZnSO_4$–50 mM KCl–50 μg of bovine serum albumin per ml–10% glycerol. Following preincubation, 100 pg (4,000 cpm) of radiolabeled DNA was added, and the reaction mixtures were incubated at room temperature for an additional 15 to 30 min. The reaction mixtures were electrophoresed on native 4% polyacrylamide gels containing 0.5× Tris-borate-EDTA and 0.05% NP-40. The gels were dried and quantitated with the Molecular Dynamics PhosphorImager.

TABLE 1. β-Galactosidase activities of NF-YB-expressing strains

DNA binding (LexA) fusion	Activator fusion	β-Galactosidase activity[a] (U)	Fold increase[b]
NF-YB	Vector	7.1 ± 1.7	
	Tax	304 ± 17.3	42.8
Tax	Vector	17.3 ± 2.4	
	NF-YB	458 ± 19.5	26.5
Tax M7 (^{29}CP-AS)	Vector	6.8 ± 1.8	
	NF-YB	5.3 ± 1.6	0.8
Tax M22 (^{130}TL-AS)	Vector	15.9 ± 3.8	
	NF-YB	693 ± 17.6	43.6
Tax M47 (^{319}LL-RS)	Vector	18.6 ± 2.9	
	NF-YB	438 ± 22.5	23.5
Bicoid	Vector	18.2 ± 1.5	
	NF-YB	18.2 ± 1.0	1.0
Tat	Vector	7.7 ± 2.2	
	NF-YB	4.7 ± 1.7	0.6
E6	Vector	14.9 ± 1.0	
	NF-YB	16.0 ± .73	1.1

[a] The β-galactosidase activities of four independent clones for each strain were measured as described in Materials and Methods.
[b] Fold increase in activity compared with vector control.

RESULTS

Isolation of NF-YB in a yeast two-hybrid library screen. Tax, expressed as a fusion to LexA, was used as bait to screen a conditionally expressed cDNA library by using the yeast two-hybrid system. Two identical independent clones of the B subunit of the human CCAAT binding protein NF-Y were isolated and chosen for further analysis.

We examined the specificity of the NF-YB–Tax interaction by testing the ability of NF-YB fused to an acidic activation domain to interact with a panel of different LexA fusion proteins (Table 1). A 27-fold increase over background β-galactosidase activity was produced in strains which contained LexA-Tax and NF-YB. In reciprocal assays in which NF-YB was expressed as the bait and Tax was expressed as the prey, β-galactosidase levels were 43-fold above the background level, supporting the initial observation of Tax–NF-YB interaction. M22 (^{130}TL-AS) and M47 (^{319}LL-RS), mutant derivatives of Tax that have differential abilities to activate the HTLV-1 and human immunodeficiency virus (HIV) long terminal repeats (78), interact with NF-YB at levels equivalent to or greater than what is observed with wild-type Tax (Table 1). NF-YB did not interact with LexA or with the transactivators Bicoid, Tat, and E6. Interestingly, NF-YB failed to interact with the Tax mutant M7 (^{29}CP-AS) (78), which is defective in activating transcription from the HTLV-1 or human immunodeficiency virus long terminal repeats in mammalian cells. Although the M7 mutation does not allow Tax to localize to the nucleus, when fused to the LexA DNA binding domain this mutant was able to enter the nucleus as determined by repression assays (6) and thus to provide information on Tax–NF-YB interaction. Together these results demonstrate that NF-YB specifically interacts with Tax in the two-hybrid system.

Interaction of Tax with NF-YB. We next examined the interaction of Tax with NF-YB through capture by GST-Tax fusion proteins. In vitro-synthesized NF-YA, (Fig. 1A, lanes 1, 7, and 8), NF-YB (lanes 2, 4, and 5), or YB-1 (lanes 3, 6, and 7) was incubated with purified GST or GST-Tax proteins bound to glutathione-Sepharose (Fig. 1B). GST-Tax, but not GST, allowed the efficient capture (15%) of NF-YB (Fig. 1A, compare lanes 4 and 5). In contrast, transcription factor YB-1, which has also been shown to bind to the Y box of the MHC class II promoter (58, 68, 70, 84), failed to bind to either GST or GST-Tax (Fig. 1A, lanes 6 and 7). Interestingly, the A subunit of NF-Y (Fig. 1A, lanes 1, 8, and 9) also failed to bind to GST-Tax over background GST binding, demonstrating specificity of Tax–NF-YB interaction.

We next analyzed the intracellular association of NF-YB and Tax by coimmunoprecipitation analysis with Tax-expressing HTLV-1-transformed T cells. Nuclear extracts were prepared (see Materials and Methods) from both Jurkat and C81 (HTLV-1-transformed) T-cell lines. Although C81 cells do not produce virus, they constitutively express Tax protein from an integrated copy of the HTLV-1 genome. The relative amounts of NF-YA, NF-YB, and Tax detected in 50 μg of nuclear extract from the two cell lines are shown in Fig. 2A. NF-YA and NF-YB proteins are detected in both cell lines. The NF-Y protein levels appear to be slightly higher in nuclear extracts prepared from C81 cells. Tax is detected only in C81 cells, and equivalent levels of the general transcription factor Sp1 are detected in both cell lines. Antibodies directed against the NF-YA subunit were used for testing the Tax–NF-YB interaction in vivo since the antibody to NF-YB fails to immunoprecipitate NF-Y complexes. Nuclear extracts immunoprecipitated with NF-YA antibody coprecipitated Tax, while a control IgG antibody of the same isotype failed to coprecipitate Tax (Fig. 2B). In Western blot analysis (as in Fig. 2A), NF-YA antibody did not cross-react with Tax, indicating that Tax is not directly immunoprecipitated by anti-NF-YA. These same results were observed with the HTLV-1-transformed cell line MT2 (data not shown). NF-Y–Tax complexes could not be detected when anti-Tax antibodies were used. However, this may be due to disruption of the complex upon antibody binding, or the antibodies we have used may block the interaction. Since we observed an interaction with Tax and NF-YB in the two-hybrid and GST binding assays, the interaction of Tax with the NF-Y complex by coimmunoprecipitation is probably through its interaction with the NF-YB subunit. Consistent with this notion, the A subunit of NF-Y, although present in our cDNA library as determined by PCR analysis (data not shown), was not identified in the interactive screen. In addition, NF-YA did not bind to GST-Tax. The fact that Tax

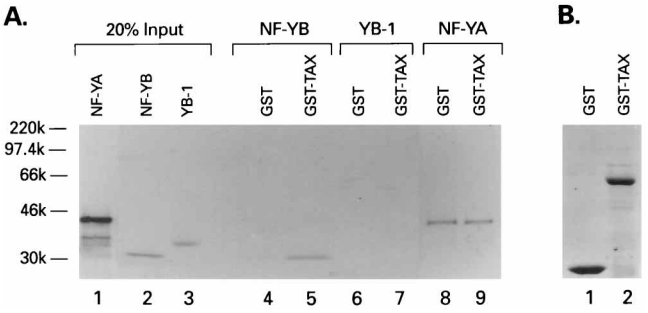

FIG. 1. NF-YB binds to GST-TAX. (A) Radiolabeled, in vitro-translated proteins (NF-YA, NF-YB, and YB-1) were coprecipitated with GST (lanes 4, 6, and 8) or GST-TAX (lanes 5, 7, and 9). The translated proteins retained on the glutathione-Sepharose matrix were resolved on 10% polyacrylamide–SDS gels, dried, and autoradiographed. Lanes 1, 2, and 3 contain 20% of the total protein used in the binding assays. (B) Coomassie blue-stained 10% polyacrylamide–SDS gel containing 500 ng of GST (lane 1) and GST-TAX (lane 2) used in panel A.

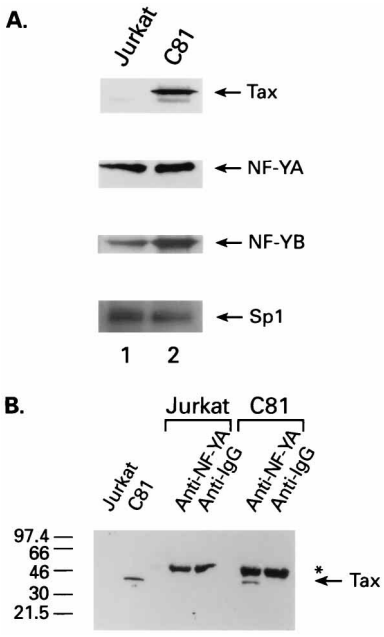

FIG. 2. Tax binds to NF-Y in HTLV-1-transformed cells. (A) Immunoblot analysis of NF-YA, NF-YB, Tax, and Sp1 (used as a loading control) expressed in 50 μg of nuclear extracts from Jurkat (lane 1) or C81 (lane 2) T-cell lines. (B) The same extracts (300 μg) were subjected to immunoprecipitation with either NF-YA or mouse anti-rabbit IgG antibody. The captured proteins were compared to 50 μg of nuclear extracts of Jurkat and C81 cells by immunoblot analysis with Tax antibody after separation on 4 to 20% polyacrylamide Tris-glycine gels. The band marked with an asterisk indicates the IgG heavy chain. Numbers on the left are molecular weights in thousands.

coimmunoprecipitated with NF-YA demonstrates that the binding of Tax to NF-YB does not dissociate NF-YB from the NF-Y heterotrimer, and thus the complex could still be capable of binding DNA.

Increased expression of MHC class II DQ. Previous studies have established that NF-Y is a critical factor in MHC class II gene expression and that MHC class II gene expression is increased in HTLV-1-infected cells (3, 25, 38, 47, 51, 64). Therefore, we sought to determine what effect the interaction of Tax with NF-Y has on the expression of MHC class II genes. Since the HTLV-1 activation of the MHC class II DQ promoter has not been examined at the RNA level, we first determined the relative levels of MHC class II DQβ (HLA-DQβ, one of the three class II alleles) mRNA from T-cell lines which were either HTLV-1 negative (Jurkat and CEM) or HTLV-1 positive (HUT-102, MT-2, and C81). A dramatic increase in MHC class II transcription was seen in HTLV-1-transformed cell lines compared to Jurkat and CEM cell lines (Fig. 3A). In contrast, the levels of NF-YB message (Fig. 3B) were approximately equal in all cell lines examined.

To better characterize the effect of Tax on the HLA-DQβ promoter, we tested the ability of Tax to transactivate the wild-type and mutant DQβ promoters in Jurkat T cells by using transient-transfection assays (Fig. 4A). Tax caused an increase in expression of the wild-type DQβ construct in a dose-dependent manner (Fig. 4C). When the mutant constructs were compared, the level of Tax expression was similar in each promoter construct transfection (Fig. 5B); however, differences in CAT activity were observed. Tax had no effect on expression from the vector alone, whereas a fivefold increase in CAT activity from the MHC class II promoter (pDQCAT) was observed (Fig. 5A). A threefold increase in activity was seen when the Y-box and CCAAT sequences were the only elements directing CAT expression (pΔH/PCAT). Deletion of both the Y box and the CCAAT element (pΔB/PCAT) (Fig. 5A) abolished promoter activity. When we mutated the Y box in the context of the full-length promoter (pBclICAT), the basal promoter was active; however, Tax-mediated transactivation was abrogated. To determine whether this mutation prevented NF-Y binding to the Y box, gel shift assays were performed. As shown in Fig. 5C, lane 4, an oligonucleotide containing the BclI mutation was unable to compete with the wild-type Y-box element for NF-Y binding. The wild-type Y-box oligonucleotide did compete for NF-Y binding, and anti-NF-YA antibody supershifted the NF-Y complex to the well (Fig. 5C, lanes 2 and 5, respectively). These results support the hypothesis that the Y box is the Tax-responsive element in the MHC class II promoter.

Tax mutants M22 and M47 activated transcription of the MHC class II promoter (Fig. 5A). This is consistent with the data obtained with the two-hybrid system, in which both M22 and M47 were capable of interacting with NF-YB (Table 1). The Tax mutant M22 is capable of interacting with CREB and thus can activate CREB-driven promoters such as the HTLV-1 long terminal repeat. However, M22 does not interact with NF-κB and, unlike wild-type Tax, cannot activate NF-κB promoters (78). The M47 mutant has the reverse phenotype from that of M22 (78). Since M22 and M47 are capable of activating the DQβ promoter, these data argue that NF-κB and CREB factors are not involved in the Tax-mediated activation of the MHC class II promoter. Since the Tax mutant M7 does not localize to the nucleus (78), it was not included in these studies. Taken together, these data indicate that Tax can increase expression of the MHC class II promoter and that this increase is a consequence of its interaction with NF-Y.

DISCUSSION

We have demonstrated that Tax can interact with the CCAAT binding factor NF-Y and activate transcription of the MHC class II DQβ gene, a gene involved in cellular differentiation and activation (20, 89). It has previously been shown that the transcription of other genes involved in cellular acti-

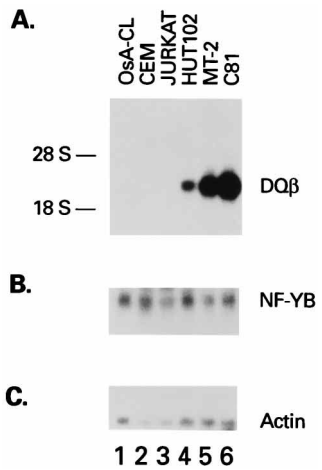

FIG. 3. Expression of MHC class II mRNA. mRNAs (10 μg) from OsA-CL, CEM, Jurkat, HUT102, MT-2, and C81 cells were electrophoretically separated on a 1% agarose gel and blotted onto a nylon membrane. The blot was sequentially hybridized with ^{32}P-labeled oligonucleotide probes specific for DQβ (A), NF-YB (B), and α-actin (C). The positions of the 28S and 18S RNA bands are indicated.

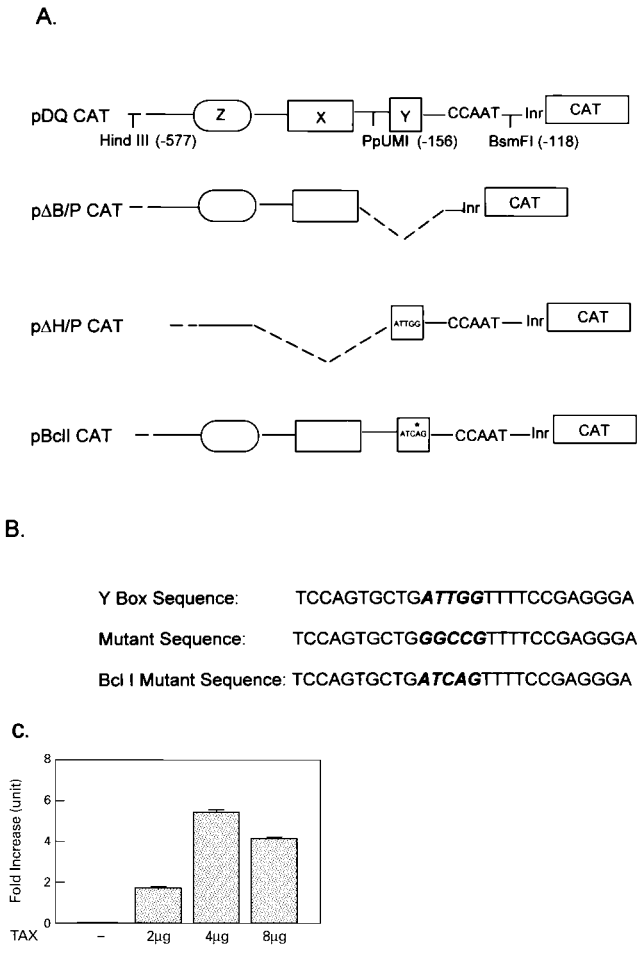

FIG. 4. MHC class II promoter constructs. (A) Diagram of the DQβ promoter constructs used in the CAT assays. The restriction sites and nucleotide positions relative to the transcriptional start site are indicated. (B) Sequences of the wild-type Y-box element and two mutant constructs used in gel shift assays. (C) Increasing amounts of pcTax (2, 4, or 8 μg) or the vector alone (4 μg) was cotransfected with 5 μg of pDQβCAT into Jurkat T cells. Cell lysates were collected 24 h later and assayed for CAT activity. The bars represent the average values from three or more independent experiments.

vation and proliferation is also transcriptionally activated by Tax (22, 23, 48, 86). Abnormal expression of such factors or receptors involved in cell growth may be one of the key events that ultimately leads to the development of adult T-cell leukemia or TSP/HAM.

MHC class II genes are comprised of a large family of highly polymorphic heterodimeric glycoproteins. The functional consequence of this variation is the regulation and restriction of the cellular immune response, since the structure of each class II molecule determines which peptide sequences are presented to lymphoid cells (58, 68, 70). The MHC class II-peptide complexes determine the repertoire of mature T cells and the activation of $CD4^+$ T cells and thus play a fundamental role in the homeostasis of the immune response. Quantitative and qualitative changes in the expression of MHC class II molecules on the cell surface dramatically affect the onset of the immune response and may be the basis of a wide variety of disease states (51). By cell surface staining, the MHC class II molecule has been shown to be elevated in peripheral blood lymphocytes isolated from HTLV-1-infected patients as compared to normal donors (3, 47, 64, 92). In addition, HTLV-1 infection of neuroblastoma cells or transfection of a glial cell line with Tax demonstrated that Tax activated the expression of MHC class II in these cells (47). Similarly, infection by bovine leukemia virus, a member of the same family of oncogenic retroviruses as HTLV-1 and -2, causes an increase in MHC class II expression by infected B and T lymphocytes (79). Iwakura et al. (38) reported that as a consequence of overexpression of MHC genes and inflammatory cytokines, transgenic mice which express the HTLV-1 *pX* and *env* regions develop an autoimmune chronic inflammatory arthropathy resembling rheumatoid arthritis. In addition, evidence suggests that certain class II MHC molecules are associated with human rheumatoid arthritis (30). Further, Hasunuma et al. (33) have recently reported the presence of specific cytotoxic T cells against Tax-bearing synoviocytes and augmentation of HLA class II molecules on the cell surface of HTLV-1 synoviocytes. Although latent viral replication in circulating infected T lymphocytes is a common feature of HTLV infection, the percentage of circulating HTLV-infected T lymphocytes is significantly increased in HAM/TSP patients (27, 39, 45) as compared to in asymptomatic carriers. In addition, HTLV-1-specific $CD4^+$ HLA class II-restricted and $CD8^+$ class I-restricted cytotoxic T lymphocytes have been demonstrated in peripheral blood lymphocytes of HAM/TSP patients (39, 40). This increase in cytotoxic T lymphocytes is hypothesized to induce pathogenesis not only in HAM/TSP patients but also in patients with arthritis, uveitis, and Sjorgen's disease (41). Therefore, Tax greatly contributes not only to cellular proliferation but also to induction of an immune response through deregulation of MHC expression. Our observation that Tax interacts with and activates transcription through NF-Y provides a mechanism for this HTLV-1-induced expression of class II molecules.

The primary regulation of class II molecule expression is at the level of transcription, which is both cell type and development stage specific (11, 17, 58, 68, 70). Our work demonstrates that the increase in MHC class II expression on HTLV-1-transformed T cells is due to an increase in the level of MHC class II mRNA. The MHC class II promoters share conserved sequence motifs, which include the W, X, X2, and Y boxes. Nuclear factors which bind to these sequences have been identified, and some have been cloned (17, 68, 70, 84). Reports have shown that binding of the factors which control the class II promoter is cooperative and that NF-Y binding facilitates in vivo recruitment of upstream transcription factors (69, 84). In this paper, we clearly demonstrate that Tax can activate transcription of the DQβ promoter through the NF-Y element. In addition, we find that Tax is able to directly interact with the NF-YB subunit. Recently, Matsuzaki et al. (55) have shown that Tax can interact with a CREB-like factor, TREB5, which binds to the X region of the MHC class II promoter, suggesting that Tax can activate transcription through this element. In our hands, the X box (ΔH/PCAT) is dispensable for Tax transcriptional activation of the DQ promoter. However, transactivation of the class II promoter by Tax is elevated when the X box is present, suggesting a possible cooperative effect of Tax–NF-Y and an upstream factor(s).

NF-Y is an evolutionarily conserved heteromeric CCAAT binding protein composed of A, B, and C subunits, all of which are required for DNA binding activity. Recently it has been shown that the subunits of NF-Y associate in a 1:1:1 ratio and that association occurs in an ordered fashion (75). The B and C subunits must bind each other before the A subunit can bind. Interestingly, NF-YB and NF-YC were identified as having H2B and H2A histone-fold motifs, respectively (8). This implies that the mechanism by which the B and C subunits interact with each other to form dimers is similar to that of H2A

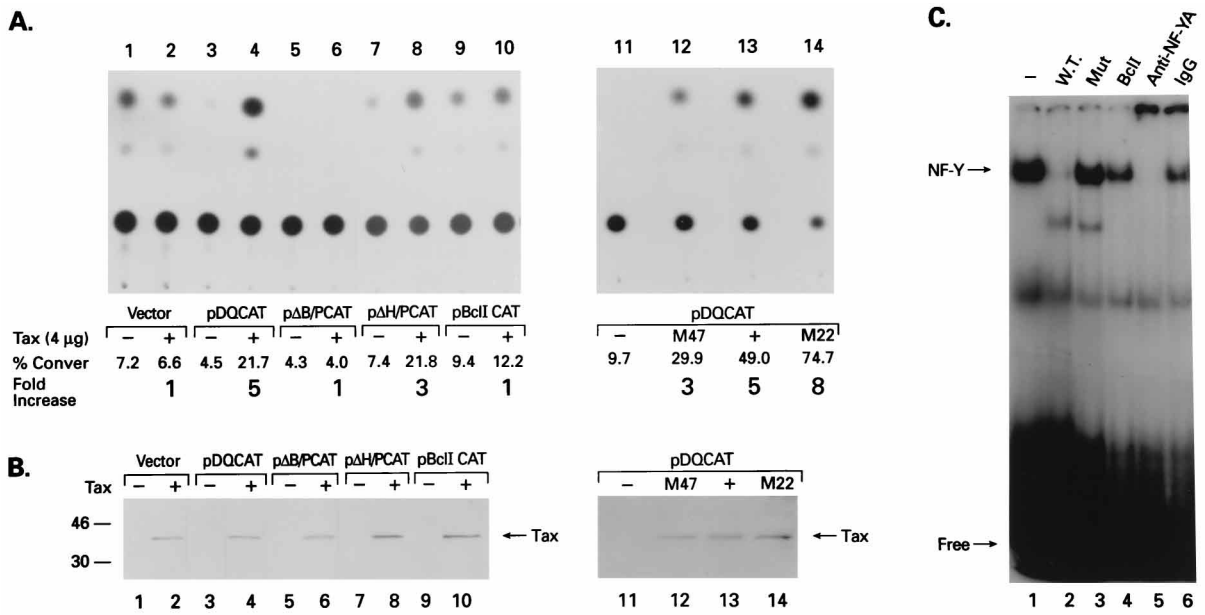

FIG. 5. Tax activation of the DQ promoter. Jurkat T cells were cotransfected with 5 μg of the indicated CAT reporter plasmid and 4 μg of either pCMV vector (−) or pcTax (+) and assayed 24 h later for CAT activity. Shown is a representative assay, with the percent conversion (Conver) of chloramphenicol and fold increase by Tax listed below the lanes. (B) Samples from each transfection were assayed by immunoblot analysis to determine the level of Tax expressed. Numbers on the left are molecular weights in thousands. (C) Nuclear extracts of Jurkat T cells (6 μg) were incubated with 1 ng of ^{32}P-labeled Y-box sequence either alone (lane 1) or in the presence of 1 μg of wild-type (W.T.) (lane 2), mutant (Mut) (lane 3), or BclI mutant (BclI) (lane 4) competitor DNA. In addition, 6 μg of extracts was preincubated with either 1 μg of NF-YA antibody or 1 μg of IgG antibody of the same isotype (lanes 5 and 6, respectively). The NF-Y-bound complex and free probe are indicated.

and H2B. Tax could facilitate complex formation between NF-YB and NF-YC, driving the formation of an A-B-C complex and thus elevating the level of active NF-Y DNA binding factors.

Alternatively, Tax could be stabilizing NF-Y complexes through its interaction with NF-YB and/or could increase the nuclear localization of NF-Y, similar to what is seen for NF-κB (35, 42). To date there has been no evidence of an inhibitor of NF-Y, but the possibility exists. In a report by Schmidt and Schilber (71), describing a difference in regulation between NF-YA and NF-YB, it was suggested that the levels of NF-YB are regulated posttranslationally. This could again be achieved by nuclear localization, protein stabilization, or both. There is no obvious nuclear localization signal in NF-YB, yet the majority of the protein is found in the nucleus (9). Since NF-Y is a heteromeric complex, the stoichiometric levels of the A, B, and C subunits may be maintained in a manner analogous to that which makes stability of IκB dependent on levels of NF-κB (12, 73, 80).

NF-Y has recently been shown to bind to CCAAT motifs in the promoters of several eukaryotic and viral genes. Several of these genes are involved in cellular regulation, such as those for human thymidine kinase, IL-4, fibroblast growth factor-4, and ribonucleotide reductase R2 (13, 14, 21, 32, 49). NF-Y binding sites have also been reported in the cell cycle regulators CDC25C and CDC2 (15, 93) both of which are important for progression through G_2/M. Recently, another important regulator of the cell cycle, the tumor suppressor protein p53, has been shown to bind to NF-Y on the hsp70 promoter, causing a repression in transcription (2). The interaction of Tax with NF-Y in HTLV-1-transformed cells suggests that Tax not only transcriptionally activates MHC class II genes but may also activate other cellular genes regulated by NF-Y. This interaction may also have an important impact on regulation of the cell cycle. Therefore, the interaction that we observe between Tax and NF-Y may play a critical role in cellular transformation and ultimately contribute to HTLV-1 disease.

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