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Mutational Analysis of Human T-Cell Leukemia Virus Type 2 Tax

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A mutational analysis of human T-cell leukemia virus type 2 (HTLV-2) Tax (Tax-2) was performed to identify regions within Tax-2 important for activation of promoters through the CREB/ATF or NF-κB/Rel signaling pathway. Tax-2 mutations within the putative zinc-binding region as well as mutations at the carboxy terminus disrupted CREB/ATF transactivation. A single mutation within the central proline-rich region of Tax-2 disrupted the transactivation of the NF-κB/Rel pathway. Surprisingly, this mutation, which is thought to be in a separate activation domain, was suppressed by mutations within or around the putative zinc-binding region, suggesting an interaction between these two regions. These analyses indicate that the functional regions or domains important for transactivation through the CREB/ATF or NF-κB/Rel signaling pathway are similar, but not identical, in Tax-1 and Tax-2. Identification of these distinct Tax-2 mutants should facilitate comparative biological studies of HTLV-1 and HTLV-2 and ultimately lead to the determination of the functional importance of Tax *trans*-acting capacities in T-lymphocyte transformation by HTLV.

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) contain at least two *trans*-regulatory genes, *tax* and *rex* (23, 27, 39, 46). Both Tax and Rex proteins modulate viral gene expression and are essential for viral replication (10, 21, 27, 35, 36, 48). In addition, we have recently reported that Tax is essential for HTLV-mediated transformation of primary human T lymphocytes in culture (37). The Tax proteins of HTLV-1 (Tax-1) and HTLV-2 (Tax-2) are highly similar and function as activators of viral transcription (9, 10, 12, 40, 51). Tax transactivation of the HTLV promoter is mediated primarily by cellular proteins, which include the cyclic AMP (cAMP) response element (CRE) and activating transcription factor (ATF) binding proteins (CREB/ATF) (1, 3, 5, 52, 55, 56). Tax facilitates the binding of these proteins to the nonpalindromic consensus CRE sequences contained within the 21-bp repeats in the HTLV promoter (1, 54). Interestingly, a previous report indicated that Tax-2 could transactivate both the HTLV-1 and HTLV-2 long terminal repeats (LTRs) efficiently but that Tax-1 was restricted in its ability to transactivate the HTLV-2 LTR (8). Similar to its action on the HTLV promoter, Tax stimulates expression of the c-*fos*, c-*jun*, and granulocyte colony-stimulating factor genes through CRE sequences (2, 13, 14, 18, 24, 34). Tax also induces nuclear expression of members of the NF-κB/Rel family of transcription factors, which leads to the expression of many cellular and viral promoters containing NF-κB motifs (7, 25, 26, 29–32). These include interleukin-2 (IL-2), IL-2 receptor α, IL-3, and granulocyte/macrophage colony-stimulating factor, as well as human immunodeficiency virus type 1 (HIV-1) (4, 6, 28, 34, 47, 53). It is hypothesized that aberrant expression of these growth-related genes contributes to the establishment of HTLV-associated cellular transformation and pathogenesis.

Generation of mutant Tax-2 expression constructs. Our objective was to make point mutations in selected regions of Tax-2 for the purpose of comparing and contrasting the functional domains of Tax-1 and Tax-2. Our choice of mutations was based on alignment of the Tax-1 and Tax-2 protein sequences and previously identified structural and functional regions within Tax-1 (1, 13, 16, 43, 45, 49, 54). These regions include activation domains at the amino and carboxy termini, a distinct nuclear localization signal and a zinc-binding region within the first 60 amino acids, and a CREB binding domain (Fig. 1). Twenty mutations were isolated by the QuickChange PCR site-directed mutagenesis method (Stratagene) with the *tax*/*rex* cDNA vector BC20.2 as a template (19). These mutations consisted of 10 single amino acid substitutions, 2 double amino acid substitutions, 4 combinations of single and double amino acid substitutions, and 4 truncation mutations. If applicable, mutations in the *tax* reading frame were designed so that they would not affect the amino acid sequence of the overlapping *rex* gene product. The locations of these mutations are represented schematically in Fig. 1. The predicted amino acid substitutions introduced by each mutation are listed below (see Table 2).

The expression of the *tax-2* mutants was determined by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a rabbit anti-Tax peptide antiserum (directed against amino acids 72 to 88) and lysates prepared from transiently transfected and metabolically labeled COS cells. As expected, the wild-type (wt) Tax-2 migrated with a relative molecular mass of 37 kDa. Each of the missense *tax* mutant expression vectors encoded a protein with a mobility comparable to that of wt Tax-2 (Fig. 2). The truncation mutant, Y290Term, encoded a truncated protein with an approximate size consistent with a deletion of 42 amino acids. Interestingly, the Y157Term truncation mutant was not detected (Fig. 2), suggesting either that the protein's conformation was not recognized by the anti-Tax peptide antiserum or that the truncation resulted in a significant reduction in the stability of the polypeptide. We attribute the differences in the levels of the various Tax proteins to differences in immunoprecipitation. These differences were not significant and did not correlate with transactivation phenotypes (see Table 2).

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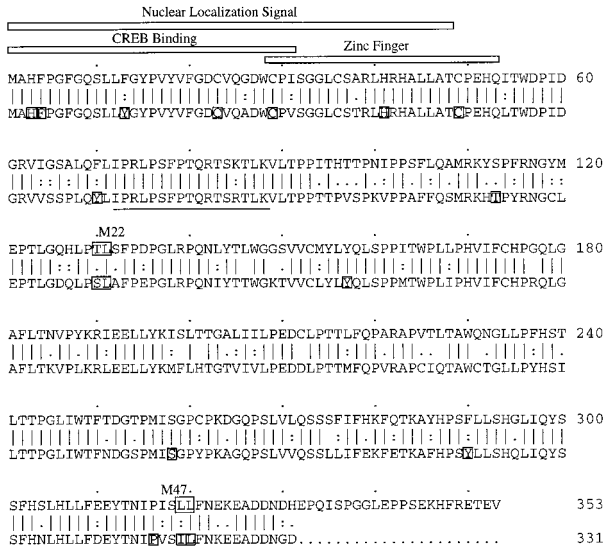

FIG. 1. A comparative amino acid sequence alignment of Tax-1 (top) and Tax-2 (bottom). Identities in amino acids are indicated by vertical lines, similarities in amino acids are indicated by dots, and differences in amino acids are indicated by spaces. Note that Tax-2 is 22 amino acids shorter than Tax-1. Previously described Tax-1 functional regions, including the nuclear localization signal (50), zinc finger (44), and CREB binding domain (1, 16, 54), and the location of transactivator mutants M22 and M47 are indicated (49). The locations of Tax-2 mutations relevant to this study are boxed. The underlined region (amino acid residues 72 to 88) depicts the amino acid residues against which the Tax-specific antiserum used in this study was directed.

Transactivation phenotypes of Tax. The human T-cell line JM4 (38) was used in these studies because these cells have a high transfection efficiency and, more importantly, because human T lymphocytes are the actual targets for HTLV transformation. We initially tested the ability of previously characterized Tax-1 mutants designated M22, which is deficient in the activation of the NF-κB/Rel pathway, and M47, which is deficient in the activation of the CREB/ATF pathway (49), to transactivate the HTLV-2 LTR (LTR-2-CAT) (10, 46), the HTLV-1 LTR (pU3R-1-CAT) (51), or a minimal promoter containing two NF-κB binding sites from the HIV-1 enhancer (HIV-κB-CAT) (7). The Tax-1 M22 mutant, in which the threonine at position 130 is replaced by an alanine and the leucine at position 131 is replaced by a serine [T130-A/L131-S]), transactivated both the HTLV-1 LTR and the HTLV-2 LTR with the same efficiency but failed to activate HIV-κB-CAT. The Tax-1 M47 mutant (L319-R/L320-S) did not transactivate either the HTLV-1 or HTLV-2 LTR but displayed greater-than wt levels of activation of HIV-κB-CAT (Table 1). The capacity of these Tax-1 mutants to transactivate the HTLV-1 LTR and HIV-κB-CAT is consistent with a previous report (49) and indicates that in JM4 T cells, M22 and M47 Tax-1 mutants functionally segregate transactivation of CREB/ATF- and NF-κB/Rel-responsive promoters, respectively. Furthermore, the observation that Tax-1 mutants behave similarly with either the HTLV-1 or HTLV-2 LTR suggests that the same mechanism (CREB/ATF activation) is used by Tax-1 to transactivate both the HTLV-1 and HTLV-2 LTRs. Interestingly, we found that wt Tax-1 efficiently transactivated the HTLV-2 LTR. This is in agreement with a recent report (41) but conflicts with another study (8) that indicated that transactivation of the HTLV-2 LTR by Tax-1 is restricted. Possible

TABLE 1. Transactivation phenotypes of HTLV-1 *tax* gene mutants[a]

Tax mutation	% chloramphenicol acetylation with reporter plasmid:		
	pU3R-1-CAT (HTLV-1 LTR)	LTR-2-CAT (HTLV-2 LTR)	HIV-κB-CAT (HIV-1 κB-TATA)
None (wt Tax-1)	100	100	100
Tax-1 M22	46	58	<10
Tax-1 M47	<10	<10	125
None (wt Tax-2)	85	123	92
Vector	<10	<10	<10

[a] JM4 T cells (5×10^6) were cotransfected by electroporation, as described previously (37), with 1 μg of the indicated CAT reporter plasmid and 5 μg of the indicated cDNA expression vector encoding the wt or a mutant form of Tax-1 (49), wt Tax-2, or the BC12 vector (control). After 48 h of growth, cells were harvested and assayed for CAT activity. All CAT reactions were standardized for equivalent levels of protein, and incubation for a period of 1 h resulted in levels of enzymatic activity that were in the linear range. Percentages of [^{14}C]chloramphenicol acetylation were quantified with a Fuji imaging system. The numbers, which represent average percent chloramphenicol acetylation values for three independent experiments, are normalized to the value for wt Tax-1 (set at 100%).

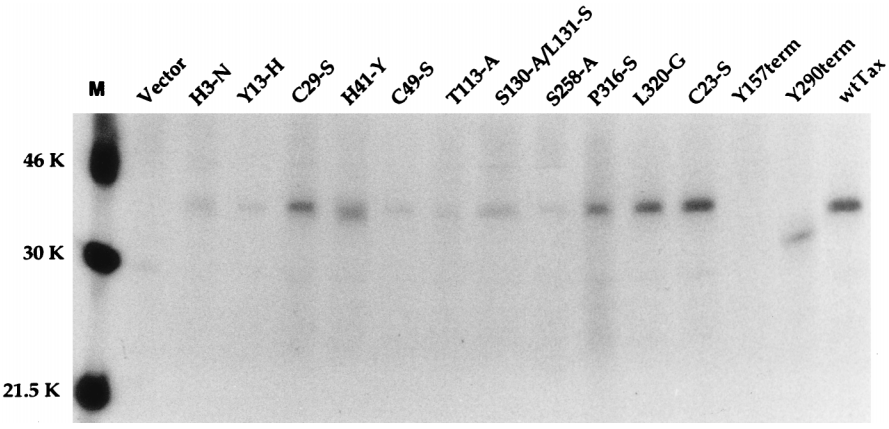

FIG. 2. Detection of Tax-2 in transfected COS cells. COS cells were transfected with 25 μg of wt *tax* gene cDNA expression vector, mutant *tax* gene cDNA expression vector, or vector alone as indicated. After 48 h of growth, cells were labeled for 3 h with [^{35}S]methionine-[^{35}S]cysteine and cell lysates were made as described previously (20). Lysates were immunoprecipitated with rabbit anti-Tax peptide antiserum (prepared against amino acids 72 to 88) (48) in the presence of protein A-Sepharose. Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by autoradiography. The sizes (in kilodaltons) are indicated on the left (lane M).

TABLE 2. Phenotypic analysis of HTLV-2 *tax* gene mutants[a]

Mutation	% Chloramphenicol acetylation with reporter plasmid:			Subcellular localization[b]
	pU3R-1-CAT (HTLV-1 LTR)	LTR-2-CAT (HTLV-2 LTR)	HIV-κB-CAT (HIV-1 κB-TATA)	
None (wt Tax)	100	100	100	N
H3-N	86	71	119	N
Y13-H	52	45	48	
C23-S	110	48	73	
C29-S	<5	<10	67	N
H41-Y	95	90	92	
C49-S	<5	<10	59	N
T113-A	15	<10	<10	N
S130-A/L131-F	48	75	<10	N
S258-A	<5	<10	<10	
P316-S	83	68	112	
I319R/L320S	<10	<10	136	N
L320G	<10	<10	153	
C23-S/S130-A/L131-F	83	48	82	N
C29-S/S130-A/L131-F	<5	<10	84	N
C49-S/S130-A/L131-F	<5	<10	80	
S130-A/L131-F/L320-G	<5	<10	<10	N
F4Term (ΔTax)	<10	<10	<10	−
Y70Term	<10	<10	<10	
Y157Term	<10	<10	<10	−
Y290Term	<10	<10	50	
Vector	<5	<5	<5	−

[a] JM4 T cells were cotransfected by electroporation with 1 μg of the indicated CAT reporter plasmid and 5 μg of either the indicated cDNA expression vector encoding wt or a mutant form of Tax-2 or BC12 vector control. After 48 h of growth, cells were harvested and assayed for CAT activity as described in the footnote to Table 1. The numbers, which represent average percent chloramphenicol acetylation values for three independent experiments, are normalized to the value for wt Tax-2 (set at 100%).

[b] COS and 293T cells were transfected with a select group of *tax-2* expression constructs, and IF microscopy was performed as previously described (42) using an anti-Tax-2 peptide antiserum (prepared against amino acids 72 to 88). All Tax-2 substitution mutants tested localized predominantly to the nucleus (N), whereas the truncation mutants were not detected (−).

explanations for these discrepancies include differences in the cell type and Tax expression construct used in the two studies.

Ability of mutant Tax-2 proteins to transactivate the HTLV-2 LTR, the HTLV-1 LTR, and a κB minimal promoter. Our series of Tax-2 mutants was tested for the ability to activate the HTLV-2 LTR, the HTLV-1 LTR, and a minimal κB promoter. Our analysis yielded four groups of Tax-2 mutants: mutants that were only defective for transactivation of both HTLV-1 LTR-CAT and HTLV-2 LTR-CAT, mutants that were only defective for transactivation of HIV-κB-CAT, mutants which could transactivate neither HTLV-1 LTR-CAT, HTLV-2 LTR-CAT, nor HIV-κB-CAT, and mutants with unaltered transactivation potential. Among the 20 Tax-2 mutants characterized, there were no significant differences between their capacities to transactivate the HTLV-2 LTR and their capacities to transactivate the HTLV-1 LTR (Table 2). These results demonstrate that Tax-2 transactivation of the HTLV-2 LTR and that of the HTLV-1 LTR occur by the same mechanism and suggest that similar domains or regions of Tax-2 are required to activate both LTRs.

Tax-2 mutants that only fail to transactivate the HTLV LTR. Tax-2 mutations, which resulted in a specific defect in HTLV transactivation only, mapped to two distinct locations: one region at the amino terminus and the other at the carboxy terminus. At the amino terminus, Tax-2 C29-S and C49-S both failed to transactivate either HTLV LTR (Table 2). A previous study demonstrated that a potential zinc finger motif utilizing amino acids C29, C36, C49, and H52 is necessary for Tax-1 to form a secondary structure compatible for zinc binding (44). In addition, this configuration correlated with Tax-1 transactivation of the HTLV-1 LTR (44). This cysteine-rich region is highly conserved (96% identical) between Tax-1 and Tax-2 (Fig. 1). Although Tax-2 binding of zinc has not been demonstrated, these results strongly suggest that Tax-2 forms a zinc finger utilizing the same amino acids. These results also suggest that the ability to bind zinc is necessary for the secondary structure required to transactivate the HTLV LTR. Tax-2 mutants located at the carboxy terminus that disrupted activation of the HTLV LTR while preserving the capacity to transactivate HIV-κB-CAT included Y290Term, L320-G, and I319-R/L320-S. These results are in agreement with data from the Tax-1 studies and are consistent with the hypothesis that this region of Tax is important for interaction with effector proteins of the CREB/ATF activation pathway. In addition, electrophoretic mobility shift assays indicated that the Tax-2 mutations that disrupted transactivation of only the HTLV LTR failed to enhance the binding of CREB/ATF proteins to CRE sites contained within the HTLV enhancer but were capable of inducing NF-κB transport to the nucleus (Fig. 3 and data not shown).

A Tax-2 mutant that only fails to transactivate HIV-κB-CAT. We identified a single mutation in Tax-2 (S130-A/L131-F) leading to defective transactivation of HIV-κB-CAT only (Table 2). Gel shift analysis also indicated that this mutant failed to induce nuclear expression of NF-κB but retained the ability to enhance the binding of CREB/ATF proteins to CRE sites contained within the HTLV enhancer (Fig. 3). This phenotype is similar to that of the M22 Tax-1 mutant (T130-A/L131-S). This portion of the protein (amino acids 60 to 180) is rich in proline (20%), suggesting that it forms several β-turns. In contrast, few prolines are contained within either the amino or carboxy terminus, suggesting that Tax-2 contains a compact center with two relatively exposed termini. Our transactivation data, in conjunction with protein folding models (11, 15) (data

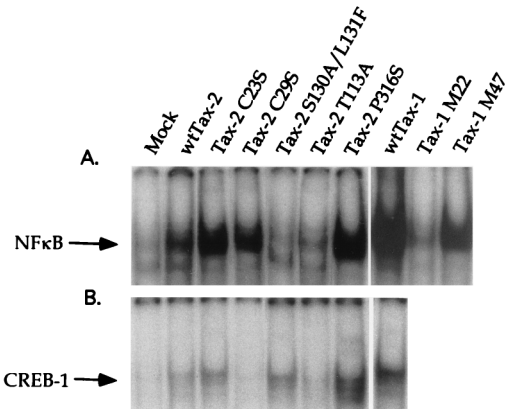

FIG. 3. Induction of nuclear NF-κB and CREB binding activity. Jurkat T cells were transfected by electroporation with 10 μg of the indicated expression vector encoding either wt Tax-2, wt Tax-1, a mutant form of Tax-2, or a mutant form of Tax-1. After 48 h of growth, nuclear extracts were prepared as described previously (7). Five micrograms of Jurkat nuclear extract, 2 μg of double-stranded poly(dI-dC), and 10 μg of bovine serum albumin buffered in 20 mM HEPES (pH 7.9)–5% glycerol–1 mM EDTA–1% Nonidet P-40–5 mM dithiothreitol (20-μl total volume) were incubated with either a ^{32}P-labeled probe specific for the κB motif derived from the κB enhancer sequences in the IL-2 receptor α promoter (5′CAACGGCAGGGGAATTCCCCTCTCCTT3′) (32) (A) or the CRE motif derived from the HTLV-1 21-bp repeat III (TCGACG TCCTCAGGCGTTGACGACAACCCCTCAC) of the HTLV-1 LTR (54) (B) for 20 min. Resultant nucleoprotein complexes were resolved on native 5% polyacrylamide gels and detected by autoradiography. Arrows indicate the positions of the NF-κB and CREB nucleoprotein complexes.

not shown), suggest that the free ends of the Tax polypeptide are necessary for interaction with CREB/ATF proteins and the basal transcription complex whereas the compact proline-rich region is critical for Tax interaction with the NF-κB/Rel activation pathway.

Tax-2 mutants that fail to transactivate either HTLV-1 LTR-CAT, HTLV-2 LTR-CAT, or HIV-κB-CAT. Several Tax-2 mutants, including three of the four truncation mutants (F4Term, Y70Term, and Y157Term) and point mutants T113-A and S258-A, lost all transactivation capacity (Table 2). It is important to note that Tax-2 mutant T113-A does consistently maintain the capacity to transactivate the HTLV-1 LTR slightly (15% activity) (Table 2). Consistent with the reporter assays, these mutants also failed to enhance the binding of CREB/ATF proteins to CRE sites contained within the HTLV enhancer and were incapable of inducing NF-κB/Rel migration to the nucleus (Fig. 3 and data not shown). The total loss of transactivating function suggests that these mutations affect either the structure and stability of the protein (likely for Y157Term [Fig. 2]) or they affect a common region necessary for inducing both activation pathways. Interestingly, similar point mutations in Tax-1 (S113-A and S258-A) did not affect the capacity of the protein to transactivate the HTLV-1 LTR but completely abolished transactivation of the HIV-1 LTR (45). Our results suggest that the two residues in Tax-2 may be more critical for proper folding. In contrast to Tax-1, Tax-2 has a threonine residue at position 113 but maintains a serine at position 258. One possible explanation for the importance of residue 113 is that both Tax-1 and Tax-2 contain a perfect consensus site (RKYS/T) for cAMP-dependent phosphorylation of amino acid 113. Both Tax-1 and Tax-2 are phosphoproteins; however, it is not known whether phosphorylation of Tax-1 or Tax-2 is critical for transactivation function or transformation by HTLV.

Tax-2 mutants displaying wt levels of transactivation. Several Tax-2 mutations, including H3-N, Y13-H, C23-S, H41-Y, and P316-S, had little effect on the protein's ability to transactivate either the HTLV-1 LTR-CAT, HTLV-2 LTR-CAT, or HIV-κB-CAT (Table 2). With the exception of Y13-H, the transactivation phenotypes of these Tax-2 mutants were distinct from those of similar Tax-1 mutants, suggesting that differences in the two polypeptides exist. Smith and Greene identified a Tax-1 mutant (H3-S) defective for transactivation of HIV-1-CAT which retained a partial ability (55%) to transactivate the HTLV-1 LTR (49). Two possibilities for differences in the Tax-1 and Tax-2 phenotypes resulting from amino acid substitutions at histidine residue at amino acid 3 exist. First, this residue might be critical for activation of NF-κB by both Tax-1 and Tax-2 but sensitive to specific amino acid substitutions. Our ultimate goal is to transfer these mutations into a full-length infectious proviral clone. Therefore, since Tax-2 H3-S would have altered the essential Rex protein amino acid sequence, it was not assessed. The second possibility is that the amino termini of Tax-1 and Tax-2 differ with respect to interaction with the NF-κB activation pathway and that Tax-1 is more sensitive to a change at this position. It was shown previously that the cysteine residue at amino acid 23 in Tax-1 was outside the predicted zinc-binding region and that a C23-S mutant retained the capacity to transactivate the HTLV-1 LTR but failed to activate the HIV-1 LTR (44). In contrast, the Tax-2 C23-S mutant maintained the capacity to activate both CREB/ATF- and NF-κB/Rel-responsive promoters. Mutations surrounding or including histidine residue 41 of Tax-1 resulted in the inability of the protein to transactivate the HIV-1 LTR but did not affect HTLV-1 LTR transactivation. The Tax-2 H41-Y mutant displayed a transactivation profile similar to that of wt Tax-2. Finally, the Tax-2 mutant P316-S displayed near-wt levels of transactivation of HTLV-1 LTR-CAT, HTLV-2 LTR-CAT, and HIV-κB-CAT, whereas the identical mutation in Tax-1 (44) led to failure of the protein to transactivate the HTLV-1 LTR but did not affect transactivation of the HIV-1 LTR. These results suggest that there are subtle differences in the carboxy termini of Tax-2 and Tax-1. One explanation for this difference might be the smaller size of Tax-2 (331 amino acids) compared to Tax-1 (353 amino acids).

Tax-2 mutations with suppressive effects. We further tested the transactivation phenotypes of Tax-2 mutants with several mutations in combination. One might expect that combining a mutation that resulted in failure of the protein to activate the HTLV LTR with a mutation causing defective transactivation of HIV-κB-CAT would result in a protein defective for transactivation of both. This was the case for Tax mutant S130-A/L131F/L320G (Table 2). However, two such combination mutants, C29-S/S130-A/L131-F and C49-S/S130-A/L131-F, failed to transactivate HTLV-1 LTR-CAT and HTLV-2 LTR-CAT but retained the capacity to transactivate HIV-κB-CAT (Table 2). Interestingly, the C23-S/S130-A/L131-F mutant maintained the capacity to activate both CREB/ATF- and NF-κB/Rel-responsive promoters (Table 2). These surprising results indicate that mutations near or within the putative zinc-binding domain suppress the effect of the S130-A/L131-F mutations, thereby restoring the protein's ability to induce the NF-κB/Rel activation pathway. The mechanism of this suppression is not clear from these studies but likely involves alterations in the conformations of the proteins. Determination of the Tax crystal structure might reveal the mechanism of this suppression.

Subcellular localization of Tax. Tax has been shown to localize predominantly to the nuclei of expressing cells (45, 49). Indirect immunofluorescence (IF) microscopy of both COS and 293T cells transfected with *tax* expression constructs indi-

cated that wt Tax-2 and all Tax-2 amino acid substitution mutants tested localized to the nucleus (Table 2 and data not shown). The two truncation mutants tested, F4Term and Y157Term, were not detected by IF microscopy, which correlates with the failure to detect these proteins by SDS-PAGE analysis. The predominantly nuclear localization of the Tax-2 mutants is consistent with that reported for Tax-1 amino acid substitution mutants (45) and indicates that the loss of activity seen with these Tax-2 mutants cannot be explained by drastic changes in subcellular localization. However, the anti-Tax-2 antibodies did not allow us to localize Tax-2 to subnuclear compartments that overlap with interchromatin speckles, as has been recently reported for Tax-1 (42).

In conclusion, these results indicate that Tax transactivation of the HTLV-1 LTR and that of the HTLV-2 LTR occur by the same mechanism. In addition, our data indicate that the domains important for transactivation of the CREB/ATF and the NF-κB/Rel signaling pathways are similar in Tax-1 and Tax-2 but that differences exist. These differences may be important to the distinct biological and pathogenic properties of HTLV-1 and HTLV-2. The role of Tax in transformation of human T cells remains unclear. However, several reports indicate that Tax is necessary for HTLV-mediated transformation (17, 22, 33, 37). Functional analysis of these Tax mutations in a transformation-competent HTLV strain that replicates by a Tax-independent mechanism (37) will lead to a better understanding of the role that Tax plays in the transformation process.

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