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SHORT COMMUNICATION

HTLV-I and HTLV-II Tax: Differences in Induction of Micronuclei in Cells and Transcriptional Activation of Viral LTRs

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Human T-cell leukemia virus (HTLV) types I and II are highly related viruses that differ in disease manifestations. HTLV-I has been linked unmistakably to adult T-cell leukemia–lymphoma. On the other hand, there is little evidence that prior infection with HTLV-II increases risk for lymphoproliferative disorders. Both viruses encode homologous transcriptional-activating proteins (respectively designated as Tax1 and Tax2) which have been suggested to be important mediators of viral pathogenesis. Previously, we reported that Tax1 is a potent inducer of micronuclei formation in cells. Here, we present evidence that Tax2 lacks micronuclei inductive ability. We contrast this phenotypic difference between Tax1 and Tax2 at the cellular level with their similarities at the molecular level in transcriptional activation.

Human T-cell leukemia virus (HTLV)-I and HTLV-II share significant antigenic and nucleotide sequence relatedness (*1–3*). Interestingly, the clinical manifestations of the two viruses are different. HTLV-I is linked to the development of ATL and TSP/HAM (*4–7*). HTLV-II, on the other hand, was isolated originally from individuals with hairy cell leukemia (*8, 9*). However, most patients with hairy cell leukemia lack antibodies to or have evidence for infection with an HTLV (*10, 11*). Furthermore, recent studies do not demonstrate a contribution of HTLV-II in lymphoproliferative malignancies (*12–14*).

Both HTLV-I and -II encode a *trans*-activator protein, Tax. Tax has been shown to modulate pleiotropically many aspects of cellular metabolism (*15–22*). The exact link between viral protein and cellular transformation is unclear. However, it has been observed that most adult T-leukemia/lymphoma cells contain extensive karyotypic abnormalities (*4, 5*), and we have suggested that Tax1 expression in cells correlates with increased DNA damage (*23*), similar to that described for other oncogenes (reviewed in ref. *24*).

It is known that damaged cellular chromosomes present as light-microscope-visible micronuclei (*25–29*). We demonstrated previously that Tax1 expression is associated with increased cellular DNA damage that is reflected by an elevation in the prevalence of micronuclei in cell cultures (*30, 30a*). Because Tax1 has a postulated role in transformation (*31–36*) which correlates reasonably with DNA damage/micronuclei induction, and because HTLV-II lacks an association with lymphoproliferative disorders, we wondered if one could better understand micronuclei induction by comparing and contrasting Tax1 and Tax2 functions.

An alignment of Tax2 (CG; ref. *12*) with its Tax1 counterpart reveals extensive conservation (>77% amino acid identity; Fig. 1). For example, within the N-terminal 90 amino acids shown to be involved in CREB binding (*37–39*), the two Tax proteins differ grossly at only 4 amino acids (Fig. 1). Similarly, in a C-terminal region (amino acids 290–330), recently described as a minimal activation domain (AD2; Fig. 1; ref. *40*), the Tax proteins differ in two residues. Elsewhere, there is more divergence, most notably an absence in Tax2 of the 330 to 353 amino acids found in Tax1.

We compared the relative potency of micronuclei induction by Tax1 with two naturally isolated Tax2 cDNAs (JD, CG; Fig. 2A; ref. *12*). Tax2JD and Tax2CG are identical except for six amino acids (Fig. 2A). At these positions, some of the differences between JD and CG actually bring either of the two proteins closer to Tax1. For example, four positions [D(60), Y(116), N(142), Y(157)] in CG are conserved in counterpart

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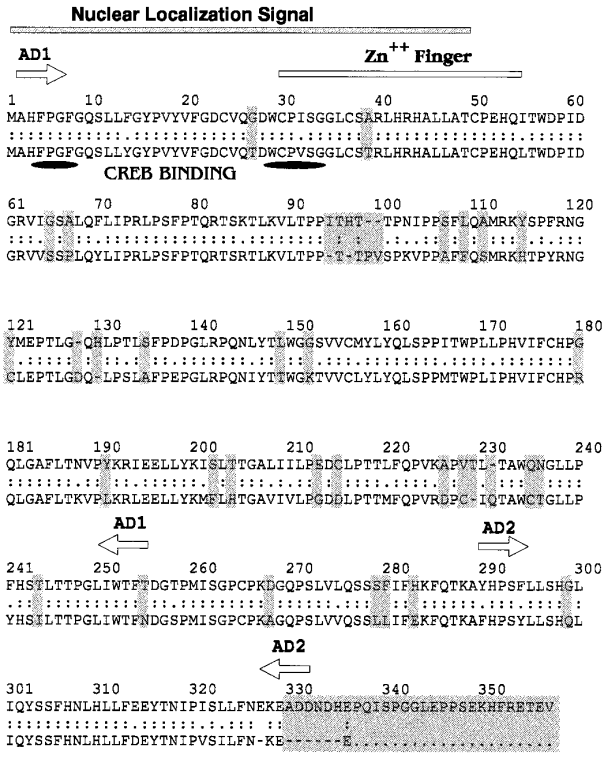


FIG. 1. A comparative protein alignment between Tax1 (top) and Tax2 (isolate CG; bottom). Identities and similarities in amino acids are indicated by dots; differences in amino acid sequences are highlighted by shading. Previously described structural and functional regions within Tax1 such as activation domain 1 (AD1; ref. *45*), activation domain 2 (AD2; ref. *40*), nuclear localization signal (46), Zn^{2+} finger (*47*), and CREB binding stretches (*37–39*) are designated. Note that Tax2 is shorter than Tax1 in that amino acids beyond position 330 in the latter are absent from the former.

amino acids in Tax1 while E(210) in JD is maintained in Tax1 (see Fig. 1). Thus, in using both Tax2CG and Tax2JD for comparison with Tax1, if the micronuclei-inducing domain within Tax should be segregated by the six changes, one would expect divergent phenotypes for Tax2CG and Tax2JD.

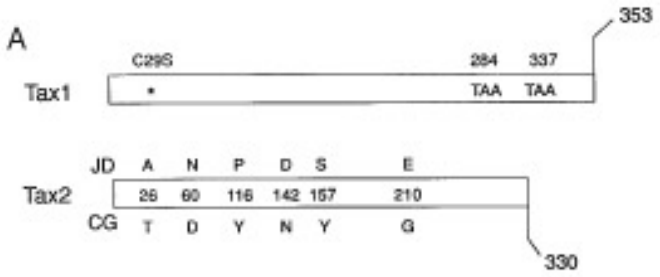
We performed cellular assays using Tax expression vectors without (Fig. 2B) or with (Fig. 2C) a clastogenic agent, mitomycin C (MMC). For each assay point, Cos cells were transfected as previously described (*30*) and 3000 nuclei with associated micronuclei were counted. Each assay point was repeated for a total of three times. From these experiments, we found that Tax2JD and Tax2CG were similar to each other and different from Tax1 in inducing micronuclei. We observed a background 0.3% frequency of micronuclei (see also Mock; Fig. 2B) in Tax2JD and Tax2CG (Figs. 2B and 2D) transfected cells while parallel Tax1-expressing cells had a 1.4% prevalence (Figs. 2B and 2D). The differences were statistically significant ($P < 0.001$; *G* test; ref. *52*), and as expected, simultaneous treatment with clastogenic agent, MMC (Fig. 2C), increased the absolute numbers of micronuclei in all cultures. Nonetheless, the relative differences between Tax1 and Tax2 were maintained. These findings suggested that protein sequence differences between Tax1 and Tax2 (beyond the intratypic changes seen between Tax2JD and Tax2CG) dictated micronuclei phenotype. We note that the numbers derived from transient transfections underestimate the frequency of micronuclei induction. This is because <25% of these transfected cells (Semmes, unpublished observation) take up DNA and express Tax protein. Hence, as discussed previously (*30*), the frequency of Tax-induced micronuclei is much higher, if normalized to a cell population in which every cell expresses Tax. DNA damage is, in fact, ubiquitous in ATL cells (*4, 5, 23*).

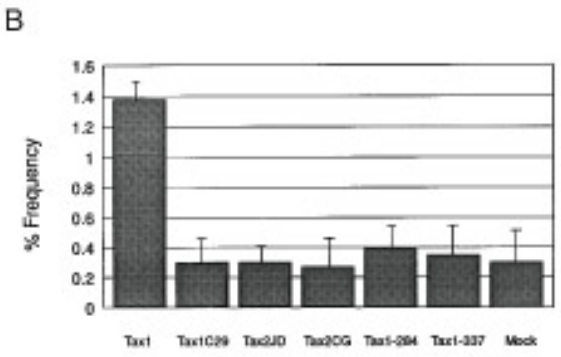
To understand better the micronuclei-inducing "domain," we additionally surveyed three Tax1 mutants (TaxC29S, Tax1-284, Tax1-337; Fig. 2A). In all three cases, the mutations abolished micronuclei induction (Fig. 2B). When the transcriptional phenotypes of the Tax1 mutants (Fig. 2E) were examined, we found that Tax1-337, although unable to induce micronuclei, was very active. Thus the Tax1-337 activity differed from that of TaxC29S and Tax1-284 and suggested a segregation of a domain(s) that specifies the two functions (transactivation of LTR versus micronuclei induction). It was intriguing that the carboxyl terminus of Tax1-337 resembles that of a Tax2 protein. This suggested that an absence of the Tax1 carboxyl tail (amino acids 330–353) could contribute to a different micronuclei phenotype.

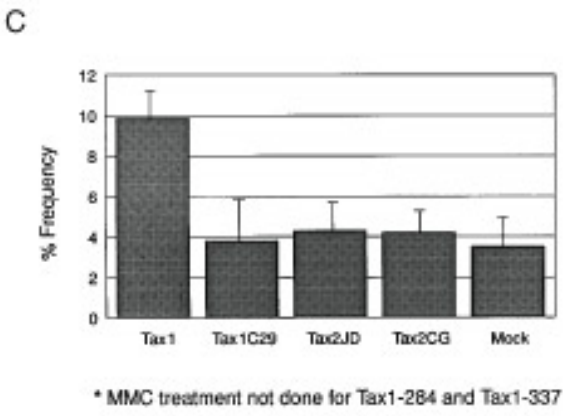
The above experiments demonstrated that Tax1-337 was similar to Tax1 in transcription (Fig. 2E) and to Tax2 (Fig. 2B) in micronuclei induction. Previously, it had been suggested that Tax1 differed from Tax2 in transcription. The former was restricted in being unable to activate the HTLV-II LTR, while the latter showed unrestricted activation of both HTLV-I and -II LTRs (*41, 42*). Based on those suggestions, we wondered if the explanation for why Tax1-337 had a Tax2 micronuclei phenotype was because this truncated Tax1 protein was transcriptionally a Tax2 protein. Hence, we investigated this possibility in detailed reciprocal *trans*-activation assays of the HTLV-I and -II LTRs (Figs. 3 and 4) using various forms of Tax protein.

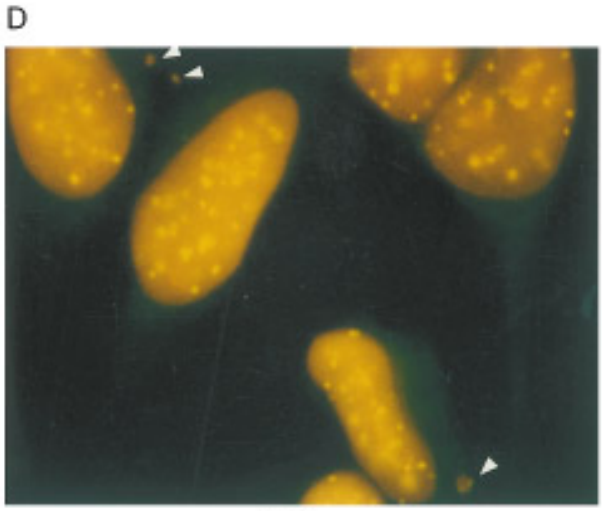
We constructed HTLV-I and HTLV-II LTR reporters such that the downstream CAT genes were identically spaced from the respective +1 positions (Fig. 3A). The U3 segment of each LTR was derived from the pU3RCAT (*43*) and the pH6 (*2*) constructs. In each case the three copies of wild-type Tax-responsive 21-bp elements were maintained (Fig. 3A). Both constructions were sequenced to verify correctness.

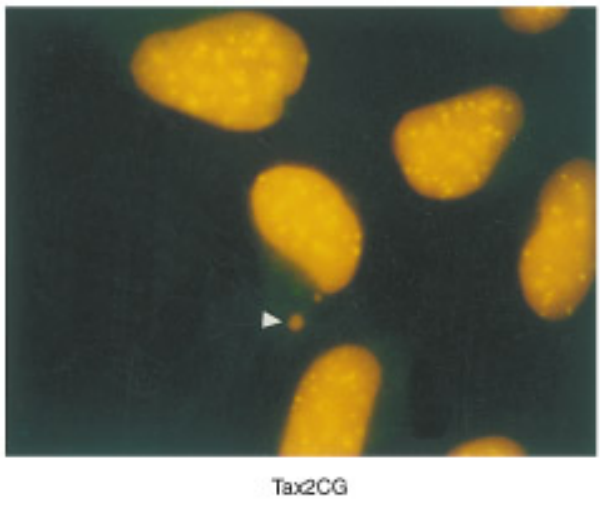
We used these two reporters and an HIV-1 LTR CAT (*44*) plasmid to test the transactivation capacities of Tax1,











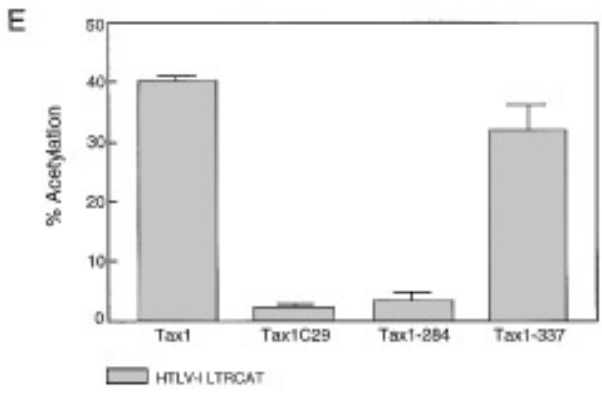


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FIG. 2. Differential induction of micronuclei by Tax1 and Tax2. (A) Schematic representations of the Tax1 and Tax2 constructions used in the micronuclei assays. Tax1 mutant C29S has a cysteine to serine change at amino acid 29 while Tax1-284 and Tax1-337 have stop codons respectively placed at amino acids 284 and 337. Tax2 cDNAs were from two naturally isolated sequences with JD and CG differing at the six indicated amino acids. (B) Micronuclei induction results derived from six Tax expression vectors. Each of the Tax expression vectors has been previously described (*48*) and contains a Tax wild-type/mutant cDNA expressed under the control of the constitutively strong cytomegalovirus immediate–early promoter (*50*). Each assay point was from micronuclei counts from 3000 cells which were repeated in three separate experiments. (C) Micronuclei results from assays performed in the presence of mitomycin C. (D) Example light micrographs of typical micronuclei. An ambient, but low, level of spontaneously occurring micronuclei exists in all cell cultures. (E) *Trans*-activation phenotypes of Tax1 mutants used in the micronuclei induction assays.

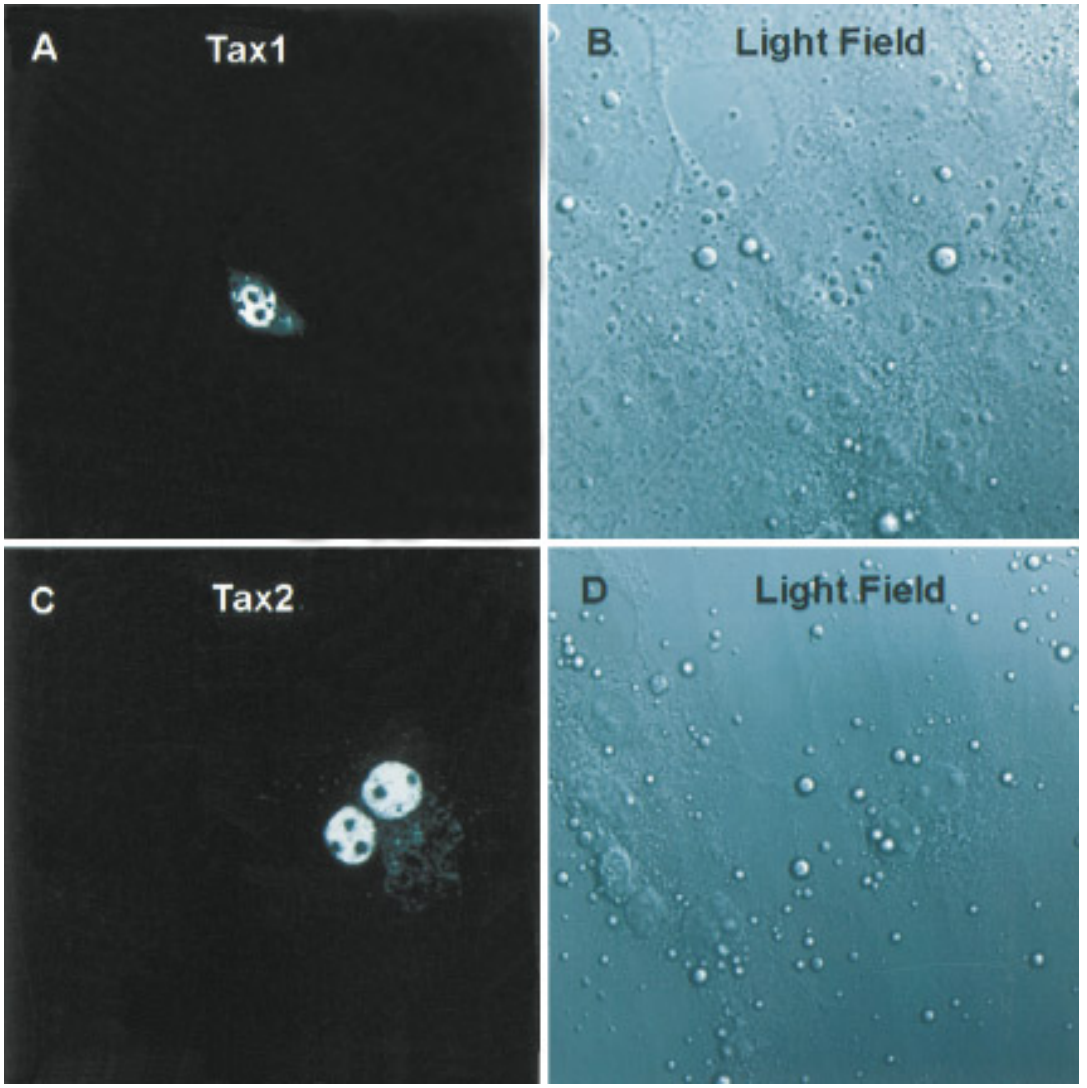


FIG. 6. Localization of Tax1 and Tax2 to the nucleus of Cos cells. Cos cells (1×10^6) were transfected with 1 μg of either Tax1 (A) or Tax2CG (C) expression plasmid. Forty-eight hours later, cells were stained with a rabbit polyclonal antibody that reacts with Tax1 and Tax2. Localization of Tax proteins to nuclei was visualized using confocal microscopy (Zeiss Axiovert 135). Companion light fields (B and D) are shown.

Tax2CG, and Tax2JD (Figs. 3B and 3C). Plasmids were transfected into cells with the indicated transactivator, and CAT activities were assessed 48 hr later. These results revealed that Tax1 and the two Tax2 cDNAs were comparably active in modulating expression from the HIV-1 LTR (Fig. 3C). In agreement with a previous report, we found that Tax2 was also comparably active on both HTLV-I and -II LTRs (Fig. 3B). Reciprocal transactivations with Tax1, however, yielded unexpected findings (Fig. 3B). In these assays, we failed to find any restriction (*41, 42*) in the ability of Tax1 to activate the HTLV-II LTR (Fig. 3B). In addition, we also performed experiments in a T-cell line, Jurkat, and found that both Tax1 (26-fold over control) and Tax2 (21-fold over control) activated the HTLV-II LTR reporter comparably (Semmes, unpublished observations).

To check that the results in Fig. 3B were not artifacts emanating from single-point assays, we conducted titration series using both LTR reporters, matched against increasing amounts of either Tax1 or Tax2 (Figs. 4A and 4B). In these series, we also could not discern any difference between the transactivation profiles of Tax1 and Tax2 on either HTLV-I or HTLV-II LTRs (Figs. 4A and 4B). Unlike previous reports (*41, 42*), the activity of Tax1 mirrored that of Tax2 on the HTLV-II LTR (Fig. 4B). We have no clear explanation as to why our findings differ from previous results; however, cell type differences and small nonidentities in reporter constructions could be contributory factors.

It was important that we carefully verify this difference. We reasoned that if Tax1 indeed activated both HTLV-I and HTLV-II LTRs equally then one confirmation

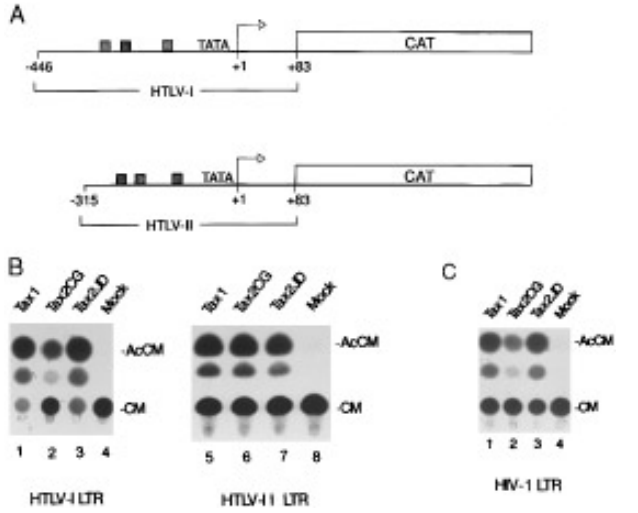
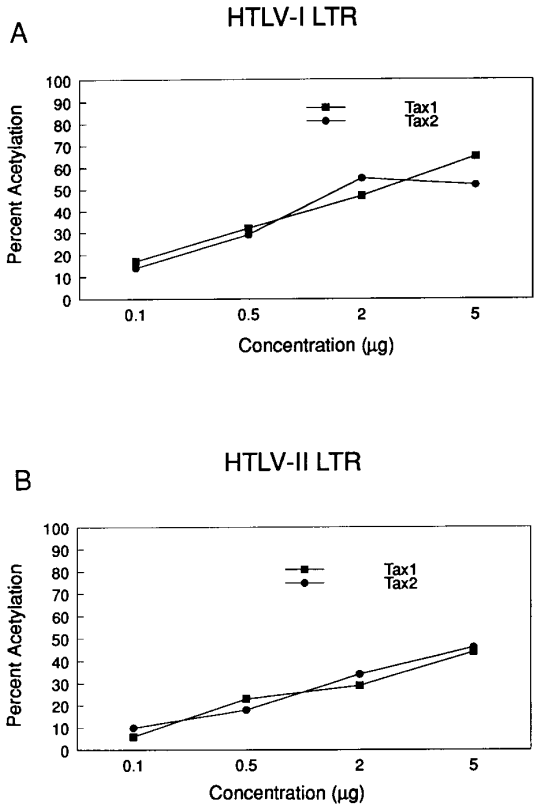


FIG. 3. Transcriptional activities of Tax1 and Tax2 on the reciprocal LTRs and on the HIV-1 LTR. (A) HTLV-I and HTLV-II U3R-CAT reporters that were normalized for the length of the R sequence. (B) Representative CAT assays of the activation of the HTLV-I and HTLV-II reporters by Tax1, Tax2CG, or Tax2JD. (C) Activation of the HIV-1 LTR by Tax1, Tax2CG, or Tax2JD.

of this would be if the activation domain of Tax1 for both could be demonstrated to be the same. We tested this hypothesis using 32 individual Tax1 mutants and wild-type Tax1 (Fig. 4C). In separate transfections (each repeated at least twice), within the resolution afforded by these 33 versions of Tax1, the activation profiles were identical for the two LTRs. For example, mutations that made Tax1 inactive on LTR1 (e.g., L296-G, L320-G, 289tr; Fig. 4C) also inactivated function on LTR2; on the other hand, those Tax1 mutants capable of full activity on LTR1 (e.g., 337tr; Fig. 4C) were fully functional on LTR2. Of interest, those mutants for which activity on LTR1 was partially impaired (e.g., S32-A, S37-A, S160-A, S273-A, S289-A; Fig. 4C) were similarly impaired for LTR2. Thus to the extent that function(s) could be resolved in a 353-amino-acid protein by 32 discrete mutations, we concluded that the same domain (and very likely the same mechanism) is used by Tax1 to activate both LTRs. Mechanistically, we believe that Tax1 also activates LTR2 through CREB/ATF motifs, since two copies of the HTLV-II 21-bp CREB-containing motifs when fused to a minimal promoter–CAT reporter sufficiently conferred responsiveness to HTLV-I Tax (data not shown).

Because the micronuclei induction assays were performed in Cos cells, we wanted to verify that both Tax1 and Tax2 were expressed in comparable quantities and were appropriately localized to the nucleus of Cos cells. Three sets of experiments were performed. First, we assayed directly for the expression levels of Tax1 and Tax2 by immunoblotting (Fig. 5A). Cos cells were transfected with either Tax1 (Fig. 5A, lane 5) or Tax2CG (Fig. 5A, lane 6) and were compared to proteins from mock-transfected Cos cells (Fig. 5A, lane 7) and Tax-nonexpressing Jurkat cells (Fig. 5A, lane 2). A cadmium chloride inducible cell line (JPX-9; lane 1; ref. *51*) that expresses Tax upon induction (Fig. 5A, lanes 3 and 4) was analyzed in parallel as positive control. Proteins were resolved by SDS–PAGE, transferred to PVDF membrane, reacted with anti-Tax serum that recognizes both Tax1 and Tax2, and visualized by chemiluminescence (Tropix; Bedford, MA). In these settings, we found essentially identical levels of expression of Tax1 and Tax2. Second, we measured levels of functional expression using dose titrations (Fig. 5B). When 1 μg of HTLV-I LTR CAT reporter was titrated in Cos cells against increasing amounts of cotransfected Tax1- or Tax2CG-expressing plasmids, the results tracked in an indistinguishable manner. This suggested that at the functional level the introduced plasmids expressed comparably either Tax1 or Tax2 protein. Third, Tax1 and Tax2 proteins in Cos cells expressed after transfection were visualized using confocal immunomicroscopy (Fig. 6). We found appropriately that the localization of Tax1 (Fig. 6A) and Tax2 (Fig. 6C) was strictly to the nucleus.

We have compared the HTLV-I and -II Tax proteins in two selected aspects, micronuclei induction and transcriptional activation of LTRs. While Tax1 and Tax2 are >77% identical in amino acid sequences, the two comparisons do reveal instructive differences. For instance, our results (Figs. 2 and 4) indicate that the region of greatest divergence between Tax1 and Tax2 (i.e., the carboxyl amino acids in Tax1 that are missing from Tax2) explains, in part, differences in micronuclei induction. [Interestingly, one subtype of HTLV-II has an extended 356-amino-acid Tax protein (*49*); however, even here, these carboxyl amino acids (331 to 356) are essentially unrelated to their linear counterparts in Tax1.] On the other hand, as measured by transactivation of the two HTLV LTRs and the HIV-1 LTR, this C-terminal divergence in the two Tax proteins does not determine transcriptional selectivity for viral LTRs. Currently, while our evidence indicates a contribution, we do not believe that the carboxyl fragment of Tax1 is independently sufficient for micronuclei induction. Most likely, in Tax1, it cooperates with another portion(s) of protein to fulfill function. A more detailed mapping of this function, which has been complicated by the fact that some point mutations selectively destabilize the entire Tax protein (*40*), is in progress. The divergence between Tax1 and Tax2 in micronuclei induction might be one reflection of the complex differences between HTLV-I and HTLV-II in lymphoproliferative disorders. We suggest that this molecular observation correlates well with biological findings of DNA damage in ATL cells (*4, 5*) and encourages further investigation in this area.



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Tax1	% Relative Trans-activation HTLV-I LTR	HTLV-II LTR
WT	100	100
Q9-G	85	80
S10-A	<5	<5
F13-Y	105	100
C23-S	95	75
C29-S	<5	<5
S32-A	35	50
C36-S	<5	<5
S37-A	40	40
H41-Q	70	85
H43-Q	100	70
C49-S	<5	<5
H52-Q	<5	<5
S66-A	75	60
S77-A	75	65
S84-A	110	100
S104-A	95	100
S113-A	100	100
S132-A	80	85
S150-A	95	100
S160-A	30	25
S199-A	100	70
S258-A	100	100
S273-A	45	60
S274-A	<5	<5
S289-A	55	50
L296-G	<5	<5
S304-A	100	100
S318-A	75	85
L320-G	<5	<5
S344-A	100	100
337trm	75	100
284trm	<5	<5

FIG. 4. Comparative activation of the HTLV-I and HTLV-II LTRs by Tax1 or Tax2. Dose–response analysis of the activation of the HTLV-I (A) or the HTLV-II (B) LTRs by Tax1 or Tax2. HeLa cells were transfected with 1 μg of reporter plasmid and increasing amounts of the indicated activator DNA. Each datum point is presented as the percentage conversion of chloramphenicol to acetylated chloramphenicol. Basal activities for the HTLV-I or the HTLV-II reporter plasmids without activators were equivalent (0.5% conversion) and are not shown. (C) Parallel comparisons of the ability of 32 Tax1 mutants and Tax1 wild type in the activation of the HTLV-I and the HTLV-II LTRs. Shown are the relative activities of Tax1 mutants compared to wild-type Tax1 protein. For comparative purposes, the activity of wild-type Tax1 on HTLV-I or HTLV-II LTR was set at 100%, and the activities of the mutants were quantitated as a percentage of this value. The Tax1 mutants have been described elsewhere (*40, 48*). The nomenclature of the point mutants follows the convention of the original amino acid, the position number of the amino acid, and the resulting substituted amino acid. For 337trm and 284trm, trm indicates inserted termination codons.

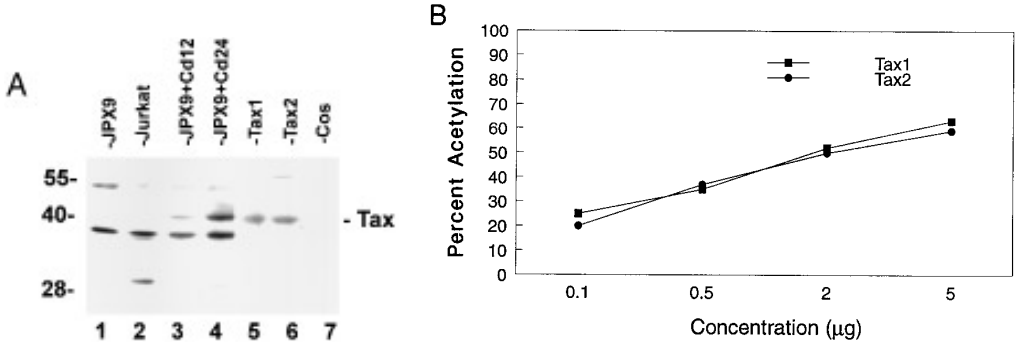

FIG. 5. Expression and *trans*-activation of Tax1 and Tax2 in Cos cells. (A) Immunoblot analysis of 1×10^7 Cos cells transfected with 10 μg of either Tax1 (lane 5) or Tax2CG (lane 6) expression plasmid. Total transfected cell proteins were harvested 48 hr after introduction of DNA and resolved by SDS–PAGE followed by transfer to PVDF membrane. The membrane was probed with a polyclonal anti-Tax1 rabbit serum (*23*) that cross-reacts with Tax2. The migration position of Tax at approximately 40 kDa is indicated. The band in lane 6 representing Tax2 demonstrates a faster relative migration upon longer electrophoresis. Lane 7 contains mock-transfected Cos cells. Lanes 1 through 4 contain control cell samples from the engineered JPX-9 cell line (*51;* lane 1) which produces Tax protein upon induction with $CdCl_2$ (lanes 3, induced for 12 hr with 30 μM $CdCl_2$; lane 4, induced for 24 hr). Jurkat cells (lane 2) which do not synthesize Tax serve as a negative control for JPX-9. (B) Dose titration of Tax1 and Tax2 activities in Cos cells. One microgram of HTLV-1 LTR-CAT reporter was transfected into Cos cells and titrated against increasing amounts of Tax1 or Tax2CG expressing plasmid. CAT activities were assayed 48 hr later. The curves represent averaged values from three assays.

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