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Mutational Analysis of Human T-Cell Leukemia Virus Type I Tax: Regions Necessary for Function Determined with 47 Mutant Proteins

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We have made 47 mutations that span the length of the human T-cell leukemia virus type I (HTLV-I) Tax open reading frame. Of the 47 mutations, 38 were substitutions of single amino acids, 5 were missense changes in two or more amino acids, and 4 were deletions. A subset of these mutations includes individual changes of all 26 naturally occurring serines to alanines. By assaying each mutant protein separately on the HTLV-I long terminal repeat (LTR) and the human immunodeficiency virus type 1 (HIV-1) LTR in parallel, we were able to identify regions of Tax selectively necessary for each promoter. A small region in the carboxyl terminus, amino acids 315 to 325, was found to be selectively important for activation of the HTLV-I LTR. Three changes at serine 113, serine 160, and serine 258 were found to specifically affect function on the HIV-1 LTR. Surprisingly, we found that the great preponderance of missense changes (32 of 42) in Tax did not affect function.

Human T-cell leukemia virus type I (HTLV-I) is associated with the development of adult T-cell leukemia (33, 48). It has also been etiologically associated with HTLV-I-associated myelopathy/tropical spastic paraparesis (12, 31). The genomic organization of HTLV-I is similar to that of other retroviruses in containing *gag*, *pol*, and *env* genes, but it has an additional x region in the 3′ portion of the genome. The x region codes for three proteins, Tax, Rex, and p21 (23, 27).

Tax is a 40-kDa protein that regulates viral transcription. Tax activates transcription in *trans* through a 21-bp motif repeated three times in the viral long terminal repeat (LTR) (6, 8, 32, 35, 43). This region of the LTR specifically binds to several cellular factors (1, 17, 26, 30, 44, 47), but there is no evidence that Tax directly interacts with any LTR sequences. One possible scenario that explains the process of *trans* activation is that Tax mediates its action through existing cellular factors (21) that directly bind to the 21-bp motif (3, 49). Consistent with this, Tax has been shown to coprecipitate with cellular proteins of 60 kDa (19, 28) and 95 kDa (28). Although the biological significance of these complexes has not been demonstrated, in one study the amino terminus of a coprecipitated 60-kDa cellular protein is identical to that of hsp60 (28). Recently, two transcription-activating factor (TAF I and TAF II) complexes that bind specifically to the 21-bp repeats of HTLV-I LTR have been described. These two factors were also demonstrated to associate with Tax in vitro (49). The details of how Tax recognizes cellular proteins to form a biologically functional complex are not clear. It is, however, increasingly evident that these protein-protein complexes ultimately dictate functional activity.

In addition to activation of its own viral LTR, Tax has been shown to act upon other unrelated promoters (7, 9, 16, 22, 25, 39, 46). Notable among these heterologous targets is the human immunodeficiency virus type 1 (HIV-1) LTR (4, 39, 50). The HIV-1 LTR contains two consensus NF-κB-binding sites through which Tax exerts its activational effect (4, 39, 50). This activation by Tax can be explained as an indirect effect mediated through interactions of Tax with cellular factors that bind to the NF-κB motifs. Thus, it is suggestive that Tax may form protein-protein complexes with cellular factors other than those that bind to the HTLV-I 21-bp elements.

A step toward understanding function is to elucidate regions within the protein that are important for transcription. Some information is known about the structure-function relationship of Tax. For example, the extreme N terminus of Tax (specifically amino acid 13) has been shown to be important for promoter selectivity between the HTLV-I LTR and the HTLV-II LTR (5). In addition, a second part of the N terminus contains a zinc finger-like domain (36) that may be important for nuclear localization (13, 41) and for overall protein folding (36). Recently, Smith and Greene (40) have used linker-scanning mutagenesis to suggest separate regions in Tax that specify activation of either the HTLV-I LTR or the HIV-1 LTR. These investigators made 52 mutant Tax proteins, of which 49 contained two consecutive amino acid changes and 3 contained single amino acid changes. They found that 27 of these missense mutants could not activate either the HTLV-I or the HIV-1 LTR. Furthermore, many of the resulting proteins also failed to localize to the nucleus. Thus, the inappropriate subcellular location of these proteins is a factor to consider in accounting for these losses in function.

We made a set of Tax mutants designed primarily to change one amino acid at a time. Because Tax has been suggested to be phosphorylated on serine residues (18), we decided to substitute individually an alanine for each of the 26 naturally occurring serines. In addition to these serine changes, we made 21 other mutated forms of Tax that distribute linearly along the open reading frame. In total, 47 mutant proteins were generated and tested for function. Whenever functional deficits were seen, each protein was also characterized for subcellular localization. These results

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allowed us to identify some regions of Tax as being important in protein stability and other regions as being selectively necessary for the activation of either the HTLV-I LTR or the HIV-1 LTR.

MATERIALS AND METHODS

Construction of mutant Tax-expressing plasmids. All missense mutations were generated by oligonucleotide-directed mutagenesis (29, 45). The wild-type and mutant Tax genes were inserted downstream of a cytomegalovirus immediate-early (IE) promoter (20). IEX denotes the plasmid construct expressing wild-type Tax. All mutants are designated by the amino acid to be changed, followed by the position of the amino acid (e.g., IEXS274). Each mutation (see Fig. 1 and 2) is indicated by the amino acid to be changed, the position of the amino acid, and the single-letter representation of the replacement amino acid (e.g., Q9-G). The deletion mutant IEXΔ2–58 was made by digesting IEX with *Hin*dIII and *Cla*I and repairing with an oligonucleotide that reinserted an ATG while deleting amino acids 2 through 58. Plasmid IEXΔ18–62 was generated by digesting IEX with *Acc*I, repairing the ends with Klenow fragment, digesting with *Mlu*I, blunting with mung bean nuclease, and ligation. IEXΔStu was made by digesting IEX with *Stu*I and ligation. The reporter plasmids pU3RCAT (HTLV-I) (43) and pBennCAT (HIV-1) (11) have been described elsewhere.

DNA transfections and Tax expression assay. CV-1 cells were seeded into six-well tissue culture plates at 10^5 cells per ml in Dulbecco modified Eagle medium containing L-glutamine, 10% fetal calf serum, and penicillin-streptomycin, in a total volume of 5 ml per well. Transfections were done 24 h later by using the calcium phosphate coprecipitation method (15). For each transfection, 0.5 μg of pU3RCAT or pBennCAT was cotransfected with 0.25 to 10 μg of a Tax-expressing plasmid. The transfection medium was removed 16 h later and replaced with fresh medium. The cells were harvested 24 h later by scraping. Total cell extracts were made by the freeze-thaw method, and the supernatants were used for chloramphenicol acetyltransferase (CAT) assays (14).

Immunofluorescence. COS-7 cells or CV-1 cells were seeded at 5×10^4 cells per ml in complete medium onto glass coverslips in 100-mm culture dishes. Cells were transfected with 15 μg of Tax-expressing plasmids. Prior to the immunofluorescence assay, the cells were fixed in 4% paraformaldehyde and permeabilized with 100% methanol. Treated cells were then reacted with a rabbit polyclonal anti-Tax antibody (19) for 1 h at room temperature. After removal of the primary antibody, the cells were exposed to tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat monoclonal anti-rabbit (immunoglobulin G; Cappel) antibody for 30 min at room temperature. Excess secondary antibody was removed with five washes of phosphate-buffered saline containing 0.5% Tween 20, and the coverslips were mounted onto slides. Fluorescence-labeled Tax was visualized with a Zeiss Axiophot photo microscope fitted with a B638 red attenuation filter. All exposure settings were internally standardized.

RESULTS

Generation of mutant Tax-expressing constructs. Our main objective was to make substitutions of individual amino acids, with the intention of making changes that would span the entire length of Tax (Fig. 1). We made 43 missense mutants. A subset of these substitution mutants included those in which all 26 serines of Tax were individually changed. We also made four deletion mutants, IEXΔ41–43, IEXΔ2–58, IEXΔ18–62, and IEXΔStu. Overall, 47 mutant forms of Tax protein were analyzed for their structure-function relationships (Fig. 1).

Ability of mutant Tax to *trans* activate the HTLV-I LTR and the HIV-1 LTR. Tax is known to affect transcription through at least two separate pathways. First, Tax activates the HTLV-I LTR through a cyclic AMP (cAMP)–ATF–AP-1 route (17, 32, 38). Second, Tax can activate certain other promoters that contain NF-κB-binding sites (4, 7, 16, 24, 25, 34, 39). Relevant to the latter observation is the fact that Tax can also *trans* activate the HIV-1 LTR through its two consensus NF-κB elements (4, 39, 50). We used our series of mutant Tax proteins to distinguish the abilities to activate the HTLV-I LTR and the HIV-1 LTR.

In our laboratory, the ability of Tax to *trans* activate the HIV-1 LTR is cell type specific. Tax is an effective transcriptional activator of the HIV-1 LTR in Jurkat and CV-1 cells. However, we did not detect this activity when using either COS-7 or HeLa cells (37). In this study, CV-1 cells were used for the expression assays. Cells were cotransfected with either an HTLV-I LTR (pU3RCAT) or an HIV-1 LTR (pBennCAT) and the appropriate Tax-expressing constructs. Each Tax mutant was subjected to titer determination to measure the activity at concentrations ranging from 0.25 to 10 μg of DNA per dish (Fig. 2A). Slightly higher concentrations of Tax were needed to activate the HIV-1 LTR than the HTLV-I LTR (Fig. 2A). We determined the fold activation as the fold increase in expression based on the percent acetylation of chloramphenicol in the CAT assays (Fig. 2B).

We found that most single-amino-acid changes (32 of 42) in Tax did not significantly perturb biological activity (Fig. 3). By assaying two different LTRs (HTLV-I and HIV-1) in parallel, we were able to identify mutations which were active for one but inactive for the second. Three groups of mutants were identified: those that were inactive for *trans* activation of both HTLV-I LTR and HIV-1 LTR, those that were inactive for HTLV-I LTR only, and those that were inactive for HIV-1 LTR only. Examples of the first group included mutants IEXS10-A, IEXC36-S, IEXS274-A, and the four deletion mutants IEXΔ41–43, IEXΔ2–58, IEXΔ18–62, and IEXΔStu. These were all inactive for both LTRs. The deletion findings are in agreement with those of others (10, 13), who have shown that deletions in Tax were generally inactivating for function. This class of mutations represents changes that either were structurally disruptive or affected a common domain necessary for activating both LTRs. In contrast, a different phenotype was seen for the mutations in the zinc finger region (amino acids 22 through 53). Although some mutations in this region of Tax also resulted in proteins inactive for both HTLV-I and HIV-1 LTRs (36), others exhibited marked selectivity of function. Notably, IEXC23, IEXH41, and IEXH41/H43 were able to *trans* activate the HTLV-I LTR while being defective for the HIV-1 LTR (Fig. 3). Conversely, IEXC29, IEXC49, IEXH52, IEXS273, IEXS274-A, and IEXF276-S were active for the HIV-1 LTR but were inactive for the HTLV-I LTR.

We found that a small region in the carboxyl terminus was selectively important for activation of the HTLV-I LTR. Mutations between positions 315 and 325 severely affected HTLV-I LTR activity (Fig. 3). Surprisingly, these changes had little effect on the *trans* activation of the HIV-1 LTR. One interpretation is that this area in Tax may contain a domain important for interaction with effector proteins of the

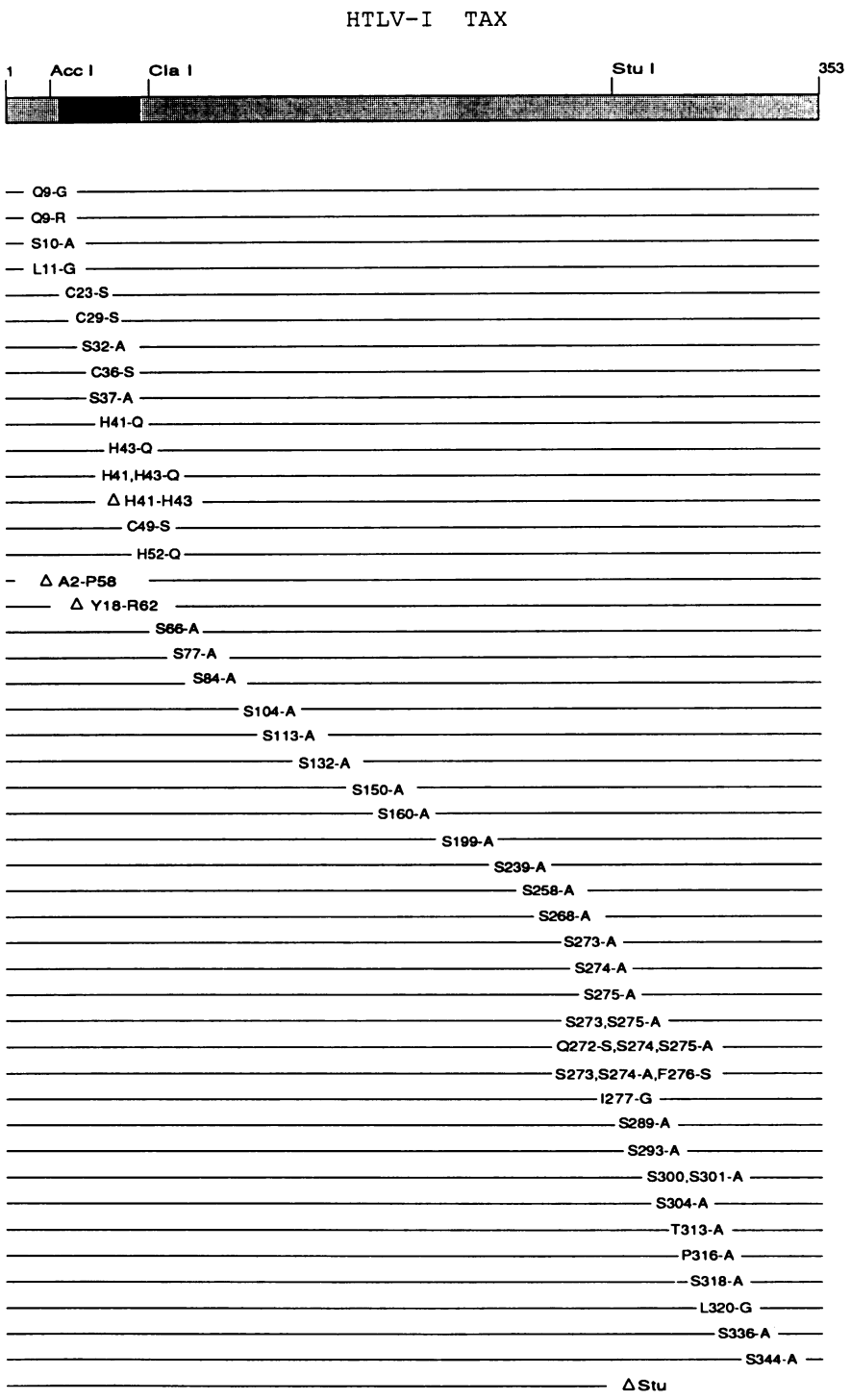


FIG. 1. (Top) Schematic representation of wild-type Tax. The locations of unique restriction sites are indicated. The zinc-finger-like domain (36) is shaded in black. (Bottom) Each of the mutations is represented below with the original amino acid, the position of the amino acid, and the replacement amino acid incorporated into the name. Deletion mutations are indicated by a Δ followed by the inclusive positions of the deleted amino acids.

cAMP–ATF–AP-1 pathway. This contrasts with results for distal mutations in the carboxyl terminus. Mutations here (e.g., S336-A, S344-A) were well tolerated (Fig. 3).

In the center of Tax is a region in which single-amino-acid changes (S113-A, S160-A, and S258-A [Fig. 3]) affected activity on the HIV-1 LTR but had little (S-160) or no (S-113 and S-258) effect on HTLV-I LTR activation. This region, in contrast to the region from positions 315 to 325, might be

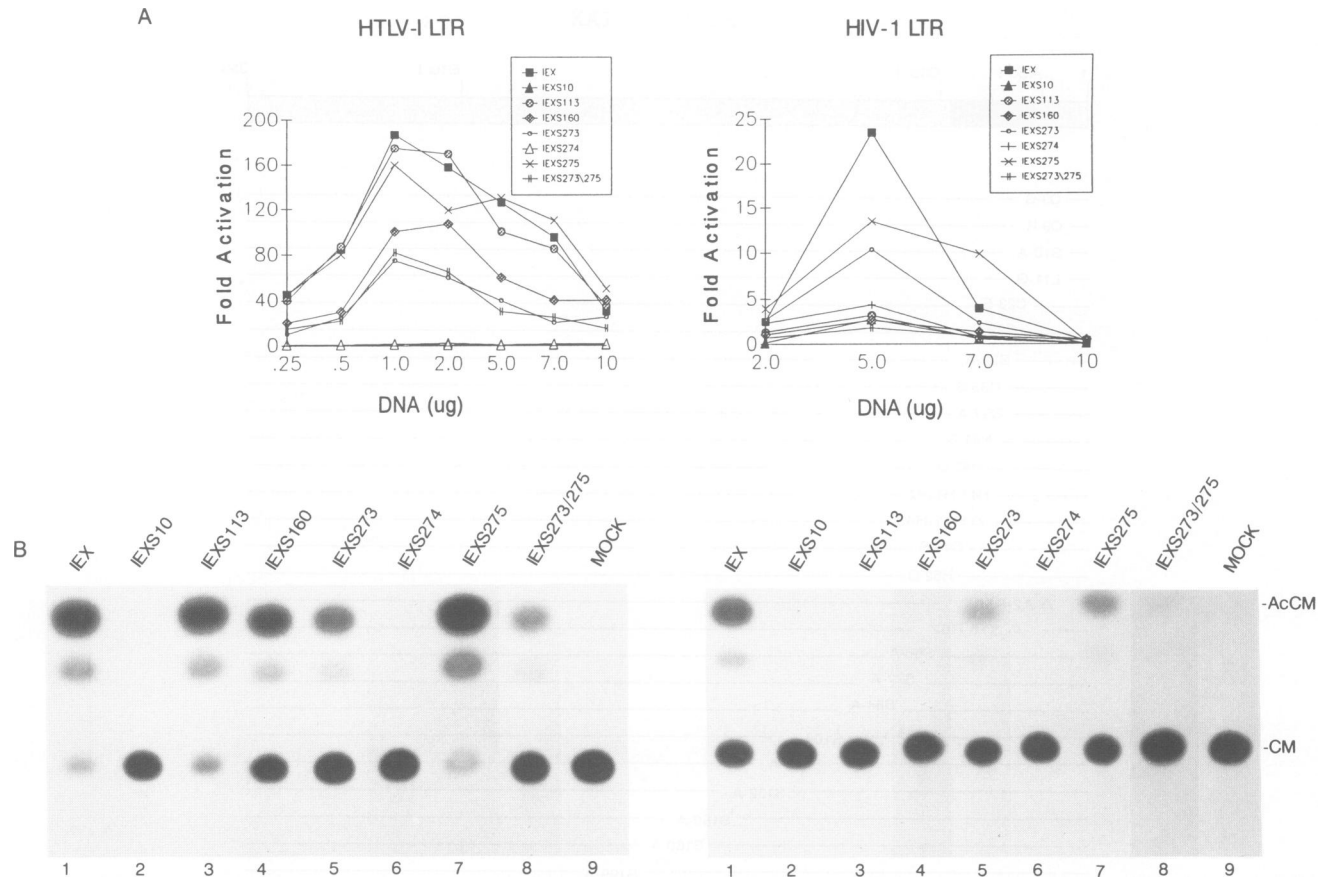


FIG. 2. Some examples of *trans* activation of the HTLV-I and HIV-1 LTRs by wild-type (IEX) and selected mutant Tax constructs. (A) Dose-response curves of the activation by Tax proteins of the HTLV-I LTR (left) and the HIV-1 LTR (right). Fold activation is defined as the fold increase over basal CAT expression. (B) Autoradiogram of a representative CAT assay. The graphic representations in panel A are tabulated from many such CAT assays. Wild-type (IEX) (lane 1) and mutant (lanes 2 to 8) Tax constructs are compared with the basal level (lane 9). Abbreviations: CM, chloramphenicol; AcCM, acetylated chloramphenicol.

important primarily for interaction with factors of the NF-κB pathway.

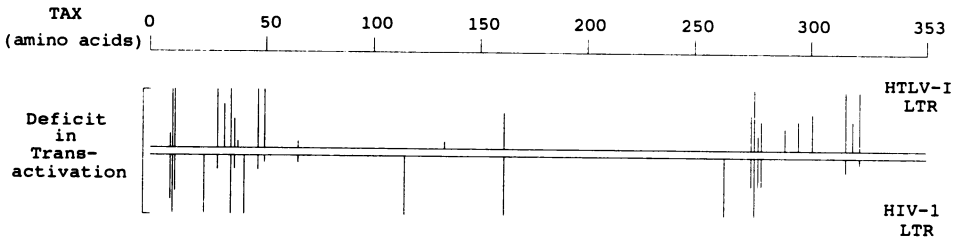
Of the 42 point mutants of Tax, only three proteins (IEXS10, IEXS274, and IEXC36) were simultaneously transcriptionally inactive for both the HTLV-I and HIV-1 LTRs. Surprisingly, all other mutant Tax proteins were competent to *trans* activate either one or both LTRs. This latter finding is particularly important since the retention of activity in the second assay when no function is seen in the first assay implies that proper expression had occurred without global disruption of protein folding and stability.

Correct subcellular localization of mutant Tax protein. To rule out aberrant subcellular localization as an explanation for loss of function, we examined each mutant Tax protein by indirect immunofluorescence with an anti-Tax antibody. Sixteen examples of the subcellular expression of Tax proteins are shown in Fig. 4 and 5. Overall, expression of protein was assayed in both COS-7 (Fig. 4) and CV-1 (Fig. 5) cells. We have examined all functionally perturbed mutants (37). The typical expression pattern seen for wild-type Tax is shown for comparison (Fig. 4 and 5, IEX). Wild-type Tax is predominantly nuclear, with some apparent nucleolar exclusion. Similarly, all substitution Tax proteins were found to localize to the nucleus (Fig. 4 and 5, IEXH52, IEXP316, etc.). Only deletion mutant IEXΔ2–58 behaved differently (Fig. 4, IEXΔ2–58). The pattern of expression of this mutant was diffusely both cytoplasmic and nuclear. In general, we found that no single-amino-acid change affected nuclear localization. Deletions in the protein, however, often perturbed subcellular distribution. We also found no evidence for any discernible differences in subcellular expression of Tax proteins between different cell types (e.g., COS-7 and CV-1).

Because all of our single-amino-acid substitution mutants migrated into the nucleus, the loss of activity seen with these mutants cannot simply be explained by incorrect subcellular localization. We thus suggest that these single-amino-acid changes may have perturbed the functional domain(s). We conclude that site-directed mutations in Tax can indeed discriminate between its capacity to activate a CREB–ATF–AP-1-dependent versus an NF-κB-dependent promoter.

DISCUSSION

Tax is a potent activator of transcription from the HTLV-I LTR (6, 8, 35, 42, 43) and certain other promoters that contain NF-κB-binding sites (4, 7, 16, 24, 25, 34, 39). In the HTLV-I LTR, Tax acts through three imperfectly repeated 21-bp elements, each containing a core cAMP-responsive motif (17, 32, 38). Tax can also activate NF-κB promoters



Mutation	% Relative Trans-activation HTLV-I LTR	HIV-1 LTR
Q9-G	75	25
Q9-R	75	25
S10-A	<5	<10
L11-G	<5	35
C23-S	100	<10
C29-S	<5	60
S32-A	30	90
C36-S	<5	<10
S37-A	40	65
H41-Q	70	<10
H43-Q	100	<10
H41,H43-Q	75	<10
Δ H41-H43	<5	<10
C49-S	<5	70
H52-Q	<5	80
Δ A2-P58	<5	<10
Δ Y18-R62	<5	<10
S66-A	70	80
S77-A	80	85
S84-A	120	100
S104-A	100	100
S113-A	100	<10
S132-A	75	100
S150-A	110	100
S160-A	35	<10
S199-A	100	85
S239-A	100	100
S258-A	100	<10
S268-A	98	120
S273-A	40	50
S274-A	<5	<10
S275-A	75	50
S273,S275-A	40	<10
Q272-S,S274,S275-A	75	80
S273,S274-A,F276-S	<5	50
I277-G	50	50
S289-A	58	90
S293-A	50	100
S300,S301-A	50	110
S304-A	100	100
T313-A	100	100
P316-A	<5	70
S318-A	50	120
L320-G	<5	75
S336-A	100	100
S344-A	100	120
Δ Stu	<5	<10

FIG. 3. Histogram summarizing the results of mutational analyses. A full-scale deflection on the histogram corresponds to 0% relative activity, whereas no deflection from the baseline corresponds to 100% relative activity. The position of each deflection in relation to the reference scale (top) indicates which mutated amino acid is responsible for the observed activity. Positive scale deflections correspond to *trans* activation of the HTLV-I LTR, and negative scale deflections reflect *trans* activation of the HIV-1 LTR. The data used to generate the histogram are presented in tabular form below. All mutants were directly compared with wild-type Tax in the same experiment, and each assay was repeated three times. Here the single-point value represents the peak activity for a given mutant from a titer determination series (which usually is at 1 μg for the HTLV-I LTR and 5 μg for the HIV-1 LTR).

such as the HIV-1 LTR, although to a more moderate degree (4, 39, 50). One interpretation of this apparently non-sequence-specific activity is that Tax interacts with different effector proteins through protein-protein complexing. These effector proteins, in turn, are sequence specific. They thus transmit the Tax-specific transcription effect onto different promoters, which share no apparent sequence homology. The interaction of Tax with multiple cellular factors may involve different and discrete regions in Tax, which can be viewed as separable activation domains. Using oligonucleotide-directed mutagenesis, we explored the possibility that Tax has one or more separable activation domains. Through 43 missense changes, we defined specific amino acids within Tax that are necessary for activation of either the HTLV-I LTR or the HIV-1 LTR.

There were three classes of activities in our mutants.

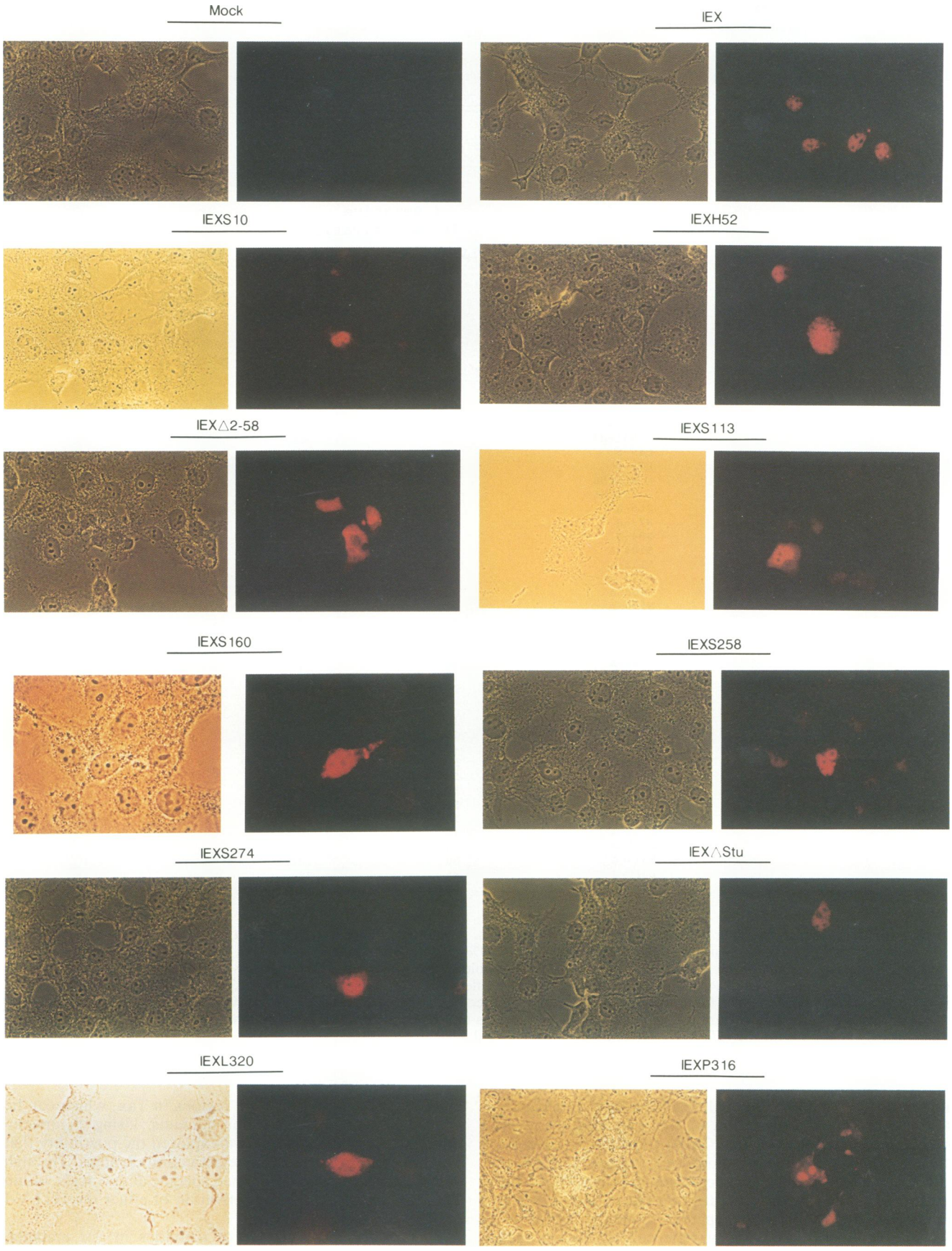

Mock
IEX
IEXS10
IEXH52
IEX△2-58
IEXS113
IEXS160
IEXS258
IEXS274
IEX△Stu
IEXL320
IEXP316

FIG. 4. Bright-field and fluorescent images of the subcellular localization of wild-type Tax (IEX) and 10 biologically affected mutant proteins in COS-7 cells. Cells were transfected in 100-mm dishes with 15 μg of Tax plasmid DNA, fixed and permeabilized 48 h after transfection, and subjected to indirect immunofluorescence. The primary antibody was a rabbit polyclonal anti-Tax serum (19), and the secondary antibody was TRITC-conjugated goat anti-rabbit immunoglobulin G. The transfected Tax construct is indicated above each pair of photographs. "Mock" represents control cells transfected with the Tax expression plasmid containing a reverse-oriented Tax gene. All mutant Tax proteins, except IEXΔ2-58, localized to the nucleus.

These were mutations that abolished activity in both LTR assays, mutations that were defective for the HTLV-I LTR alone, and mutations that inactivated *trans* activation of the HIV-1 LTR. The HTLV-I LTR and the HIV-1 LTR have unrelated upstream elements that respond to Tax. Therefore, it was reasonable to find mutations that would distinguish the ability of Tax to activate through the enhancer region of the HTLV-I LTR from the ability to activate through that of the HIV-1 LTR. A simple interpretation, which does not exclude others, is that these mutations define regions in Tax specific for different activation routes. This contrasts with mutations that decrease activities on both LTRs. Although these mutations could have affected regions of the protein that are crucial to both activation functions, they could be equally well explained as changes that caused distortions in protein folding and stability.

Of the 26 serine substitution mutations generated in this study, only mutations at S-10 and S-274 inactivated the Tax effect on the HTLV-I LTR. These two point mutants were also inactive for *trans* activation of the HIV-1 LTR. Thus, these mutations could be in an area of shared function or could be structurally disruptive and destabilizing. We noted, however, that the IEXS274-A protein, when expressed in cells pretreated with cycloheximide, had a shorter half-life than that of wild-type Tax (37); this is compatible with the latter possibility. Under the same conditions, IEXS10 was comparable to wild-type Tax in stability (37). Thus, S-10 may represent a necessary amino acid for the activation of both HTLV-I and HIV-1 LTRs. Conversely, S-274 could be a site essential for protein stability.

Mutations in the zinc finger-like domain revealed this region to be important for both activities of Tax. Each mutation in this region was designed to evaluate the importance of the cysteine and histidine residues. Amino acid changes which abolished *trans* activation of the HTLV-I LTR all affected the protein motif necessary for folding into a zinc finger structure (36). However, the amino acid changes that affected the activation of the HIV-1 LTR (C23-S, C36-S, H41-Q, and H41/43-Q) did not affect the zinc finger structure. Thus, although the zinc-finger amino acids

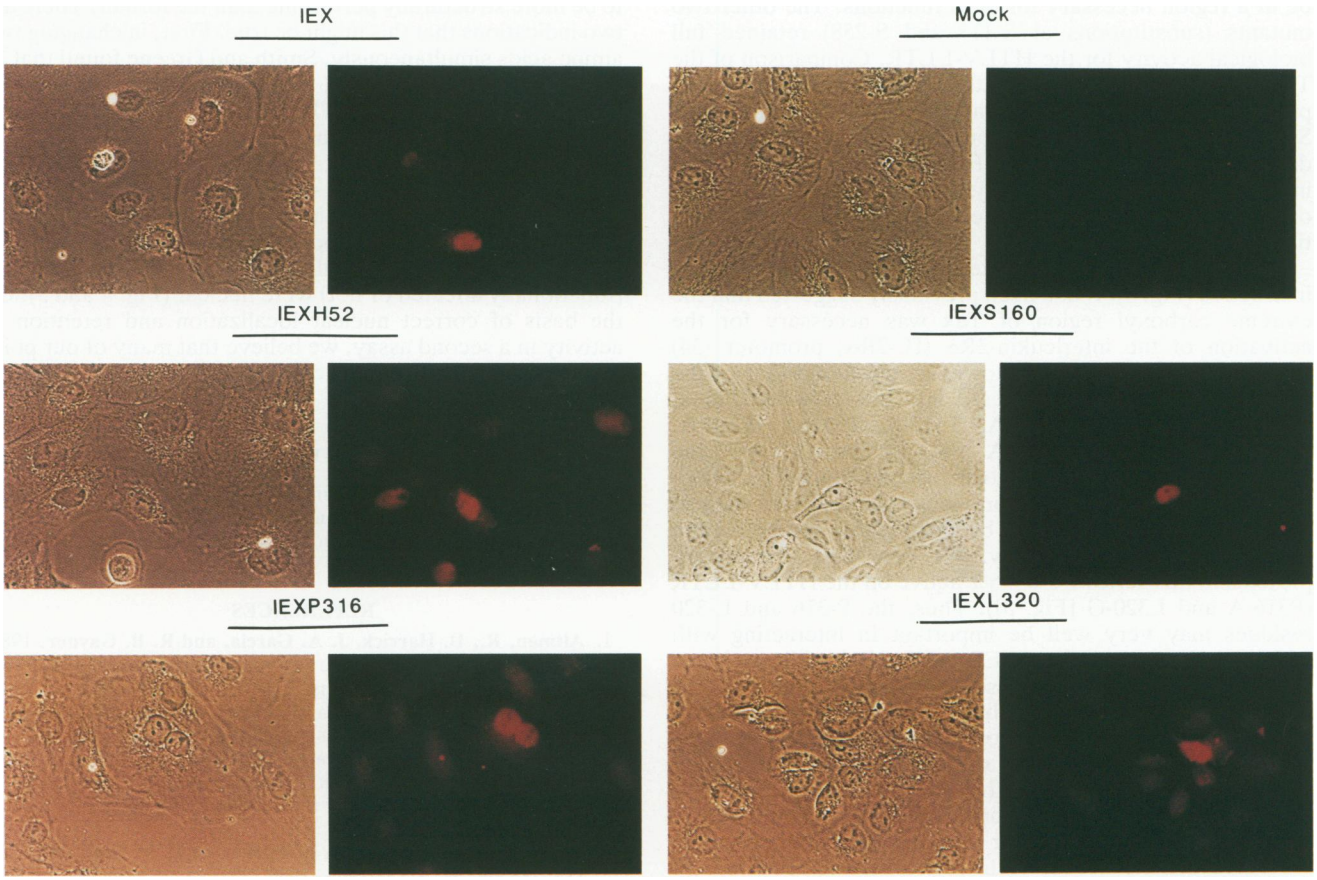


FIG. 5. Subcellular localization of Tax proteins in CV-1 cells. Immunofluorescence was performed as described in the legend to Fig. 4. All mutant Tax proteins were defective in functional assays but localized to the nucleus.

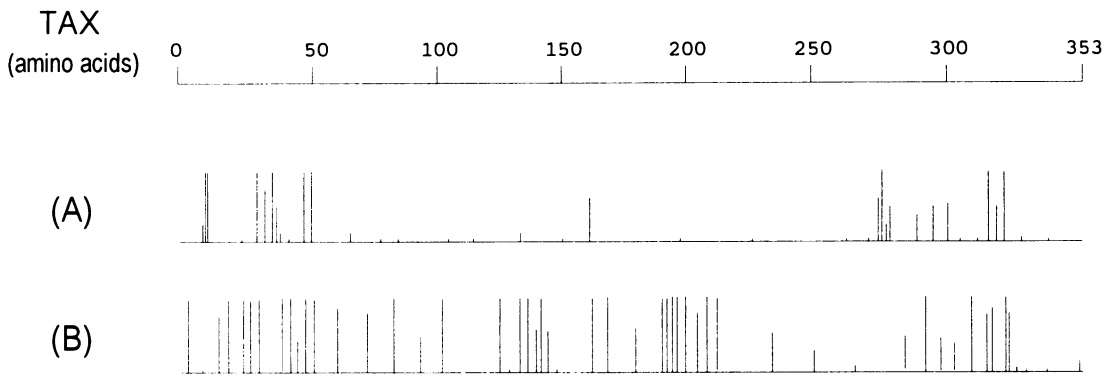


FIG. 6. Comparison of mutations that affect Tax activation of the HTLV-I LTR between this study (A) and that of Smith and Greene (40) (B). A linear scale representing the 353-amino-acid Tax protein is shown at the top. Full-scale deflections correspond to 100% loss of activity. Fully active mutant proteins are represented by the smallest vertical hatch marks. In all, 33 mutants in panel A are compared against 52 mutants in panel B. Some mutants used in the present study contained multiple changes at the same site or at multiple sites and are not depicted in the comparison. Striking differences between the two studies are seen for changes engineered into the amino acids between positions 50 and 225. There is good agreement between the two studies for amino acid changes at the N and C termini.

are important for HTLV-I activation, other neighboring amino acids outside the structure were found to be necessary for HIV-1 LTR activation. Disruption of the zinc finger does not directly correlate with HIV-1 LTR activation by Tax.

Three serine-to-alanine substitutions (at S-113, S-160, and S-258) completely ablated Tax *trans* activation of the HIV-1 LTR. The S160-A mutant also reduced activity (35% of wild type) on the HTLV-I LTR. Thus, the S160-A mutation may be in a region necessary for both functions. The other two mutants (substitutions at S-113 and S-258) retained full biological activity for the HTLV-I LTR. Comparison of the Tax amino acid sequence with a compiled data base of protein motifs (2) revealed that the sequence surrounding S-113 (RKYS) is a perfect consensus motif for a cAMP-dependent kinase substrate. However, we have not yet investigated whether phosphorylation of Tax occurs at S-113 or whether a phosphorylation event is required for interaction of Tax with NF-κB.

Mutations in the carboxyl terminus of Tax were highly instructive (Fig. 3). It had been previously suggested that the extreme carboxyl region of Tax was necessary for the activation of the interleukin-2Rα (IL-2Rα) promoter (34) through its NF-κB motifs. However, we found that of the nine point mutations engineered into this region (S289-A, S293-A, S300/301-A, S304-A, T313-A, P316-A, S318-A, L320-G, S336-A, and S344-A [Fig. 3]), none affected the activation of the HIV-1 LTR to any significant degree. These results agree with one other previous study (40) and suggest that this region is likely to be dispensable for activation through the NF-κB pathway. We note that two mutant proteins in this series were <5% active on the HTLV-I LTR (P316-A and L320-G [Fig. 3]). Thus, the P-316 and L-320 residues may very well be important in interacting with factors that influence the CREB–ATF–AP-1 pathway.

We have compared our missense mutations (Fig. 6A) with a series of changes published by Smith and Greene (40) (Fig. 6B). A qualitative analysis of the two sets of mutations highlights areas of agreement and disagreement (Fig. 6). For example, focusing on the HTLV-I LTR, both studies agree on the importance of the regions between positions 0 and 50 and positions 275 and 325 for function. However, whereas Smith and Greene (40) found the region between positions 50 and 250 to be extremely sensitive to change, we found that 11 of our 12 point mutants with changes in this region retained function (Fig. 6). The one exception (S160-A) still retained 35% of wild-type activity. An explanation of this difference may lie in the two different mutagenesis approaches. In our series, used for comparison in Fig. 6, we made only single-amino-acid changes. In contrast, each of the mutants used by Smith and Greene contained a simultaneous change of two consecutive amino acids as a unit, usually to an Ala-Ser pair (40). The latter approach is likely to be more structurally perturbing than the former. There are two indications that this might be true. First, in changing two amino acids simultaneously, Smith and Greene found that 27 of 49 mutants (55%) had 15% or less (compared with the wild type) activity on both the HTLV-I and HIV-1 LTRs. In our study, although many mutants lost activity on either the HTLV-I or the HIV-1 LTR, only 3 of 38 single-amino-acid changes (7%) lost activity simultaneously on both LTRs. Second, 17 of the mutants used by Smith and Greene that lost function also failed to localize properly into the nucleus. However, we found that all our single missense changes (functionally affected or not) were nuclear (Fig. 4 and 5). On the basis of correct nuclear localization and retention of activity in a second assay, we believe that many of our point mutations delineate amino acids that are directly involved in function.

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