Identification of HTLV-I *tax trans*-activator mutants exhibiting novel transcriptional phenotypes

Matthew R. Smith and Warner C. Greene

Howard Hughes Medical Institute, Department of Medicine and Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710 USA

The type I human T-cell leukemia virus (HTLV-I) encodes a 40-kD nuclear *trans*-regulatory protein termed Tax that transcriptionally activates the HTLV-I long terminal repeat (LTR), as well as selecting cellular and heterologous viral promoters. Tax does not bind DNA specifically but, rather, acts in a more indirect manner. Tax activation of the HTLV-I LTR is mediated through constitutively expressed cellular factors that bind to cAMP response elements (CREs) present within the 21-bp enhancers of the LTR. In contrast, Tax *trans*-activation of the interleukin-2 receptor-α gene (IL-2Rα) and LTR of the type 1 human immunodeficiency virus (HIV-1) involves the induced nuclear expression of NF-κB. We now report the identification of missense mutations within the *tax* gene that functionally segregate these two pathways of *trans*-activation. Additionally, we demonstrate that the carboxyl terminus of the Tax protein, despite its acidic and predicted α-helical structure, is completely dispensable for *trans*-activation through either of these transcription factor pathways. Finally, we demonstrate that mutations within a putative zinc finger domain disrupt the nuclear localization of Tax and abolish *trans*-activation. These results demonstrate that Tax *trans*-activation of viral and cellular promoters involves at least two mechanisms of host transcription factor activation and suggest that this activation is likely mediated through distinct functional domains.

[*Key Words*: cAMP response element; NF-κB; long terminal repeat; zinc finger; nuclear localization]

Received July 17, 1990; revised version accepted August 31, 1990.

Human T-cell leukemia virus (HTLV-I) is the causative agent of the aggressive and often fatal adult T-cell leukemia (Poiesz et al. 1980, 1981; Yoshida et al. 1982). More recently, HTLV-I has been linked with a progressive demyelinating syndrome termed HTLV-I-associated myelopathy or tropical spastic paraparesis (Gessain et al. 1985; Osame et al. 1986). Although the basis for cellular transformation by HTLV-I is not completely understood, recent studies employing transgenic mouse and rat fibroblast models have demonstrated the oncogenic potential of the 40-kD Tax protein encoded within the pX region of the HTLV-I genome (Hinrichs et al. 1987; Pozzati et al. 1990; Tanaka et al. 1990). Tax is a powerful transcriptional *trans*-activator of the HTLV-I long terminal repeat (LTR) (Sodroski et al. 1984; Chen et al. 1985; Felber et al. 1985; Fujisawa et al. 1985). Additionally, Tax induces the expression of select cellular genes involved in T-cell growth, including interleukin-2 (IL-2), the α-subunit of the IL-2 receptor (IL-2Rα), and c-*fos* (Inoue et al. 1986; Cross et al. 1987; Maruyama et al. 1987; Siekevitz et al. 1987a; Fujii et al. 1988; Wano et al. 1988). Tax also activates the human immunodeficiency virus (HIV-1) LTR (Siekevitz et al. 1987b; Böhnlein et al. 1988a), which may contribute to the enhanced HIV-1 gene expression that occurs in T cells dually infected with HTLV-I and HIV-1. Apart from its positive transcriptional effects, Tax negatively regulates the expression of at least one cellular gene, β-polymerase, an enzyme involved in DNA repair (Jeang et al. 1990).

The precise molecular mechanism by which Tax mediates these diverse transcriptional effects remains enigmatic. However, the function of this viral *trans*-activator does not appear to involve its direct binding to regulatory sequences within the various Tax-inducible genes. Rather, Tax functions by inducing or modifying the activity of select host transcription factors. Specifically, Tax induces the nuclear expression of NF-κB (Ballard et al. 1988; Leung and Nabel 1988; Ruben et al. 1988), which activates the related κB enhancer elements present in the IL-2 (Hoyos et al. 1989) and IL-2Rα (Böhnlein et al. 1988b) promoters and the HIV-1 LTR (Nabel and Baltimore 1987) (for review, see Lenardo and Baltimore 1989). In contrast, the Tax-responsive sequence within the HTLV-I LTR corresponds to three tandem 21-bp enhancer elements that are unrelated to the κB enhancer (Paskalis et al. 1986; Shimotohno et al. 1986; Brady et al. 1987). Tax *trans*-activation of the HTLV-I LTR appears to be mediated through constitutively expressed cellular factors that bind to an 8-nucleotide core element, TGACGTCT, present within the 21-bp repeats (Altman et al. 1988; Jeang et al. 1988; Giam et al. 1989; Poteat et al. 1989; Tan et al. 1989a). This octanucleotide

motif has been identified previously as a transcriptional regulatory element present in a wide variety of viral and cellular genes including E1a-activated early genes of adenovirus (Lee et al. 1987) and cAMP-inducible genes (Montminy et al. 1986). Tax *trans*-activation of the HTLV-I LTR likely involves the indirect binding to or modification of a protein(s) that binds to this core element (Jeang et al. 1988; Nyborg et al. 1988). Several candidate proteins have been identified including CRE binding protein (CREB) (Yamamoto et al. 1988), activating transcription factor (ATF) (Hai et al. 1988), and Tax response element-binding protein (TREB) (Tan et al. 1989b). The Tax-responsive sequences in the c-*fos* promoter also contain this octanucleotide motif (Fujii et al. 1988), raising the possibility that Tax activation of both the HTLV-I LTR and the c-*fos* promoter may involve the same host transcription factor(s). However, sequences flanking the CREB/ATF-binding site are required for Tax *trans*-activation of both the HTLV-I LTR (Fujisawa et al. 1989; Montagne et al. 1990) and the c-*fos* promoter (Fujii et al. 1988), suggesting that other interactions may contribute to Tax *trans*-activation of these promoters. Furthermore, more complex interactions may be involved in Tax *trans*-activation of the HTLV-I LTR because Tax appears to interact physically with a protein that binds to the LTR between the two proximal 21-bp enhancers (Marriot et al. 1989).

In this report we describe the identification of HTLV-I *tax* gene mutants that functionally distinguish *trans*-activation through the CREB/ATF and NF-κB host transcription factor pathways. Specifically, five mutants affecting two different regions of the protein were shown to effectively *trans*-activate NF-κB responsive promoters but not CREB/ATF responsive promoters. Conversely, a single Tax mutant was shown to effectively *trans*-activate CREB/ATF responsive promoters but lacked the ability to induce nuclear NF-κB or *trans*-activate NF-κB responsive promoters. Additionally, we demonstrate that the acidic carboxy-terminal domain of Tax is dispensable for *trans*-activation of both CREB/ATF and NF-κB responsive promoters. In contrast, mutations affecting many other regions of the Tax protein were shown to abolish *trans*-activation through each of these transcription factor pathways. Included among this group of transcriptionally inactive *tax* mutants were seven mutations affecting residues within a putative zinc finger domain. Surprisingly, these nonfunctional Tax zinc finger mutants were excluded from the nuclei of expressing cells, suggesting that this structural motif may mediate nuclear localization. Together, these results demonstrate that Tax activation of CREB/ATF and NF-κB responsive promoters proceeds via different intermediate signal transduction pathways and suggest that this activation is mediated by distinct Tax peptide domains.

Results

The *tax* gene of HTLV-I is comprised of two coding exons that, together, specify a protein of 353 amino acids. Initial inspection of the Tax protein sequence revealed two potential structural motifs, a cysteine-rich zinc finger-like domain located between amino acids 23 and 52 and an acidic α-helix involving the carboxy-terminal 31 amino acids. This carboxy-terminal region was particularly interesting because acidic α-helices function as transcriptional activation domains in several well-characterized *trans*-activators, including the GAL4 protein of *Saccharomyces cerevisiae* and the VP16 protein of herpesvirus (for review, see Ptashne 1988).

The mutational analysis of the HTLV-I *tax* gene described here was designed to test the function of these potential structural domains in *trans*-activation by Tax. Of the 52 missense mutations introduced into the *tax* gene, six mutants altered residues that are likely to be involved in the coordination of zinc, and four mutants targeted residues within the acidic carboxyl terminus. To investigate the potential function of additional regions of the Tax protein in *trans*-activation, other mutations were introduced throughout the *tax* gene. The locations of these mutations are represented schematically in Figure 1. Each mutation altered one or two adjacent codons and introduced a unique *Nhe*I or *Bgl*II restriction site into the *tax* gene sequence. The predicted amino acid substitutions introduced by each missense mutation are listed in Table 1. The expression vector, pcTax, which contains a full-length cDNA copy of the HTLV-I *tax* gene under the control of the human cytomegalovirus immediate early promoter, was modified by oligonucleotide-directed in vitro mutagenesis to express the various mutations of Tax.

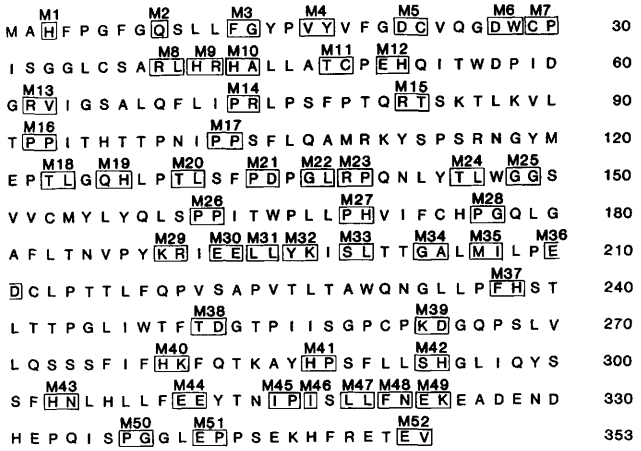

Figure 1. Schematic summary of site-directed mutations introduced into the HTLV-I *tax* gene. The HTLV-I *tax* gene encodes a 353-amino-acid protein with the predicted sequence as shown. Missense mutations affecting either one or two adjacent codons were introduced by oligonucleotide-directed in vitro mutagenesis, as indicated by the boxed residues. These mutations were named according to their location within Tax, with M1 corresponding to the most amino-terminal and M52 the most carboxy-terminal mutation. Each mutation introduced a unique *Nhe*I or *Bgl*II restriction site into the *tax* gene sequence (see Materials and methods). The precise amino acid substitutions introduced by each missense mutation are provided in Table 1.

Table 1. *Phenotypic analysis of the HTLV-I* tax *gene mutants*

CLONE TESTED	MUTATION	% WT ACTIVITY[a] HTLV-I LTR CAT	% WT ACTIVITY[a] HIV-1 LTR CAT	SUBCELLULAR LOCALIZATION[b]
cTax	-	100	100	N
M1	^{3}His → Ser	<5	55	N
M2	^{9}Gln → Ala	>120	>120	N
M3	^{13}PheGly → AlaSer	21	33	N
M4	^{17}ValTyr → AlaSer	<5	11	C>N
M5	^{22}AspCys → AlaSer	<5	<5	C>N
M6	^{27}AspTrp → AlaSer	<5	<5	C>N
M7	^{29}CysPro → AlaSer	<5	<5	C>N
M8	^{39}ArgLeu → AlaSer	<5	7	C>N
M9	^{41}HisArg → AlaSer	<5	<5	C>N
M10	^{43}HisAla → AlaSer	61	49	N
M11	^{48}ThrCys → AlaSer	<5	<5	C>N
M12	^{51}GluHis → AlaSer	<5	<5	C>N
M13	^{62}ArgVal → AlaSer	10	<5	C>N
M14	^{73}ProArg → AlaSer	15	<5	C>N
M15	^{82}ArgThr → AlaSer	<5	12	C>N
M16	^{92}ProPro → AlaSer	50	95	N
M17	^{102}ProPro → AlaSer	<5	<5	C>N
M18	^{123}ThrLeu → AlaSer	<5	<5	C>N
M19	^{126}GlnHis → AlaSer	>120	>120	N
M20	^{130}ThrLeu → AlaSer	<5	<5	C>N
M21	^{134}ProAsp → AlaSer	<5	<5	N
M22	^{137}GlyLeu → AlaSer	56	<5	N
M23	^{139}ArgPro → AlaSer	<5	<5	N
M24	^{145}ThrLeu → AlaSer	54	45	N
M25	^{148}GlyGly → AlaSer	111	115	N
M26	^{161}ProPro → AlaSer	<5	<5	N
M27	^{169}ProHis → AlaSer	<5	<5	N
M28	^{176}ProGly → AlaSer	32	58	N
M29	^{189}LysArg → AlaSer	<5	<5	C=N
M30	^{192}GluGlu → AlaSer	<5	<5	N
M31	^{194}LeuLeu → AlaSer	<5	<5	C=N
M32	^{196}TyrLys → AlaSer	<5	<5	N
M33	^{199}SerLeu → AlaSer	<5	<5	N
M34	^{203}GlyAla → AlaSer	14	29	N
M35	^{206}MetIle → AlaSer	<5	<5	C=N
M36	^{210}GluAsp → AlaSer	6	<5	N
M37	^{237}PheHis → AlaSer	48	62	N
M38	^{251}ThrAsp → AlaSer	65	77	N
M39	^{263}LysAsp → AlaSer	90	49	N
M40	^{279}HisLys → AlaSer	55	36	N
M41	^{287}HisPro → AlaSer	<5	8	N
M42	^{293}SerHis → AlaSer	55	85	N
M43	^{303}HisAsn → AlaSer	60	97	N
M44	^{310}GluGlu → AlaSer	<5	<5	N
M45	^{315}IlePro → ArgSer	22	76	N
M46	^{317}Ile → Arg	16	83	N
M47	^{319}LeuLeu → ArgSer	<5	>120	N
M48	^{321}PheAsn → ArgSer	22	76	N
M49	^{323}GluLys → AlaSer	111	>120	N
M50	^{337}ProGly → AlaSer	103	>120	N
M51	^{341}GluPro → AlaSer	104	>120	N
M52	^{352}GluVal → AlaSer	84	105	N

The position and nature of the amino acid substitutions introduced by each *tax* missense mutation are summarized in the second column.

[a]Functional properties of the Tax mutants were analyzed by cotransfecting Jurkat T cells with the wild-type or a mutant *tax* expression vector and a reporter plasmid containing either the CREB/ATF responsive HTLV-I or NF-κB responsive HIV-1 LTR linked to the CAT reporter gene. CAT activity was measured ~48 hr after transfection and is presented as a percentage of the activity observed in cells transfected with the wild-type pcTax expression vector. The values shown represent the average of two independent transfections. The six boxed mutants (M1, M22, and M45–M48) selectively *trans*-activated one, but not both, retroviral LTR.

[b]The subcellular localization of each mutant Tax protein was analyzed by indirect immunofluorescence. Phase contrast and corresponding immunofluorescence photomicrographs of selected mutants are shown in Fig. 6. Mutants were categorized as displaying either the wild-type nuclear predominant (N), a cytoplasmic, almost nuclear excluded (C > N), or a whole cell (C = N) pattern of expression.

The relative level of expression of each *tax* mutant was determined by Western analysis, using rabbit anti-Tax antisera and lysates prepared from transiently transfected COS cells (Fig. 2). As predicted, the wild-type Tax protein migrated with a relative molecular mass of 40 kD and was readily detected by this analysis (Fig. 2, lane 2). In contrast, COS cell cultures transfected with the parental expression vector containing a cDNA copy of the interleukin-2 gene (pCMV IL-2) yielded no specific signal (Fig. 2, lane 1). Each of the mutant *tax* expression vectors encoded an immunoreactive protein with a mobility comparable to that of the wild-type Tax protein (lanes 3–54). Of note, however, the M27 mutant was expressed at a markedly lower level than the wild-type Tax protein, likely reflecting reduced stability of this mutant polypeptide (Fig. 2, lane 29).

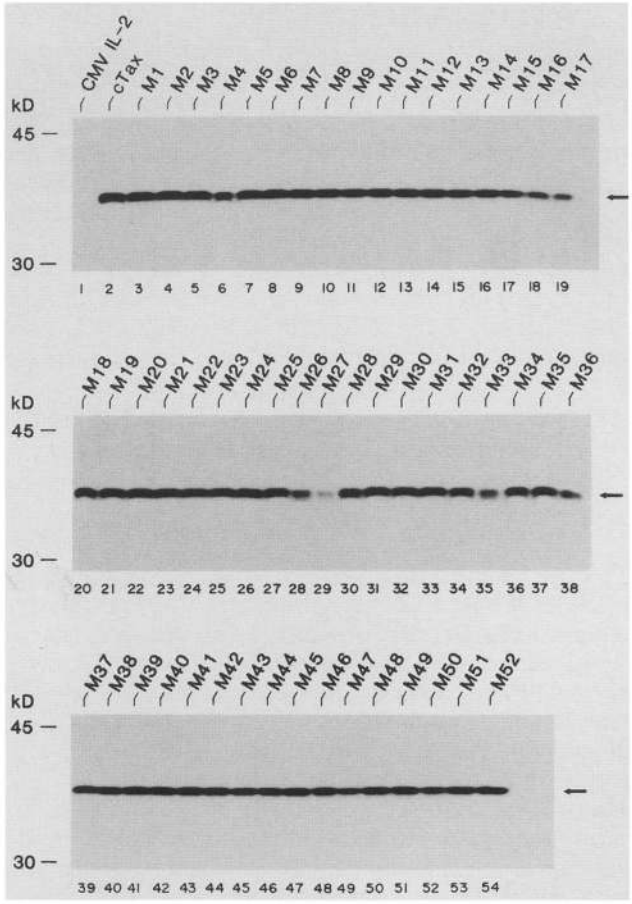

Figure 2. Western blot analysis of the various mutant Tax proteins. Subconfluent COS cell cultures (35 mm) were transfected with 2.5 μg/ml of either the negative control plasmid pCMV IL-2 (lane *1*), the wild-type pcTax expression vector (lane *2*), or each mutant *tax* expression vector (lanes *3–54*). Approximately 48 hr after transfection, cell lysates were prepared and subjected to electrophoresis on 10% discontinuous SDS–polyacrylamide gels. Proteins were then transferred to Immobilon-P membranes and probed sequentially with rabbit anti-peptide antisera directed against amino acid residues 321–353 of the Tax protein (αTax-C), and ^{125}I-labeled protein A. Immunoreactive proteins were visualized by autoradiography (arrows). The migration of known molecular weight standards is indicated at *left*. No other immunoreactive proteins were detected on regions of the Immobilon-P membrane not shown here.

Trans-*activation phenotypes of the HTLV-I Tax mutants*

To assess the functional properties of each mutant Tax protein, Jurkat T cells were cotransfected with each of the mutant *tax* expression vectors and reporter plasmids containing either the CREB/ATF responsive HTLV-I LTR or NF-κB responsive HIV-1 LTR linked to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. As summarized in Table 1, mutants representative of each of the four possible *trans*-activation phenotypes were identified, including (1) mutants that *trans*-activated both the HTLV-I and HIV-1 LTR; (2) mutants that failed to *trans*-activate either the HTLV-I or HIV-1 LTR; (3) mutants that activated the HIV-1 LTR but not the HTLV-I LTR; and (4) a mutant that activated the HTLV-I LTR but not the HIV-1 LTR. Strikingly, most of the 52 missense mutants tested fell within the second category, failing to *trans*-activate either retroviral LTR. These nonfunctional mutants involved residues located throughout most of the linear sequence of the *tax* gene, suggesting that subtle changes in the overall protein structure may have dramatic functional effects on this *trans*-activator. Included within this group of transcriptionally inactive Tax proteins were the clustered series of mutants affecting residues within the putative zinc finger domain of Tax (M5–M12). The only exception to the nonfunctional phenotype of these zinc finger mutants was M10, which exhibited approximately one-half of the biological activity of wild-type Tax on both retroviral LTRs.

The carboxyl terminus of Tax is dispensable for trans-*activation*

None of the four missense mutants affecting residues within the acidic carboxyl terminus of Tax significantly diminished *trans*-activation with either the HTLV-I or HIV-1 LTR (Table 1; M49–M52). Rather, the carboxyl terminus of Tax represented one of the few areas within the protein where mutations appeared well tolerated. Because acidic α-helices function as transcriptional activation domains in several well-characterized *trans*-activators, the apparent dispensability of a structurally similar domain within the Tax protein was unexpected initially. However, sequence alignment of the Tax proteins of HTLV-I and the closely related type II human T-cell leukemia virus (HTLV-II) revealed that although these two proteins are ~74% homologous, the functional 331-amino-acid HTLV-II Tax protein lacks the 22 amino acids corresponding the carboxyl terminus of the HTLV-I Tax protein (Haseltine et al. 1984; Shimotohno et al. 1984). To test formally whether the carboxy-terminal 22 amino acids of the HTLV-I Tax protein were dispensable for *trans*-activation, we introduced a premature stop codon into the wild-type pcTax expression vector. As shown in Figure 3A, this mutant expression vector, designated pcTax^{330}DH → GDStop, encodes a truncated but stable form of Tax recognized by rabbit anti-peptide antisera directed against amino acid residues 106–132 of Tax (αTax-N) but not by antisera specific for the carboxy-terminal 32 amino acids of HTLV-I Tax (αTax-C).

To assess the biological activity of this truncated Tax protein, the pcTax^{330}DH → GDStop expression vector was cotransfected into Jurkat T cells with four different Tax-inducible promoter–CAT constructs. As shown in Figure 3B, this truncated form of Tax *trans*-activated the HTLV-I and HIV-1 LTRs, as well as the c-*fos* and IL-2Rα promoters at levels comparable to that obtained with the wild-type Tax protein. Notably, this phenotype was observed over a 10-fold concentration range of expression vector DNA. These results demonstrate that the carboxy-terminal 22 amino acids of the HTLV-I Tax protein are dispensable for *trans*-activation of CREB/ATF responsive and NF-κB responsive promoters.

Identification of Tax mutants with differential phenotypes of trans-*activation*

The ability of Tax to *trans*-activate viral and cellular promoters through at least two cellular transcription factor pathways raised the possibility that these transcriptional effects may involve distinct mechanisms of activation, perhaps mediated by different peptide domains. The identification of mutants that selectively activated one, but not both, of the retroviral LTR–CAT reporter constructs provides strong support for this hypothesis. Specifically, two Tax mutants, M1 and M47 and, to a lesser extent, the M45, M46, and M48 mutants, displayed nearly wild-type activity on the NF-κB responsive HIV-1 LTR but significantly impaired activity on the CREB/ATF responsive HTLV-I LTR (Table 1). The region of Tax defined by the M45–M48 mutations corresponds to a hydrophobic portion of the protein. Notably, the M1 mutation affects the only charged residue within the otherwise hydrophobic amino terminus of Tax. The inability of the M1 mutant to *trans*-activate the HTLV-I LTR is also consistent with previous functional analyses of HTLV-I and HTLV-II *tax* gene mutants, suggesting an important role for the amino terminus in *trans*-activation of the HTLV LTRs (Wachsman et al. 1987).

A single mutant with the converse phenotype of *trans*-activation was also identified. This mutant, M22, displayed nearly wild-type *trans*-activation of the HTLV-I LTR but failed to significantly activate the HIV-1 LTR (Table 1). The M22 mutant altered the third of four regularly spaced leucine residues in the linear sequence of Tax (see Fig. 1). However, neither of the Tax mutants affecting residues immediately flanking this site (M21, M23) nor mutants affecting the other leucine residues (M18, M20, M24) exhibited the selective phenotype of the M22 mutant.

The functional analysis of the Tax mutants presented in Table 1 involved cotransfection of each of the Tax-inducible retroviral LTR–CAT constructs with a fixed amount of expression vector DNA. To test more rigorously the function of each of the mutants displaying differential phenotypes of *trans*-activation, transfection experiments were performed using four different Tax-in-

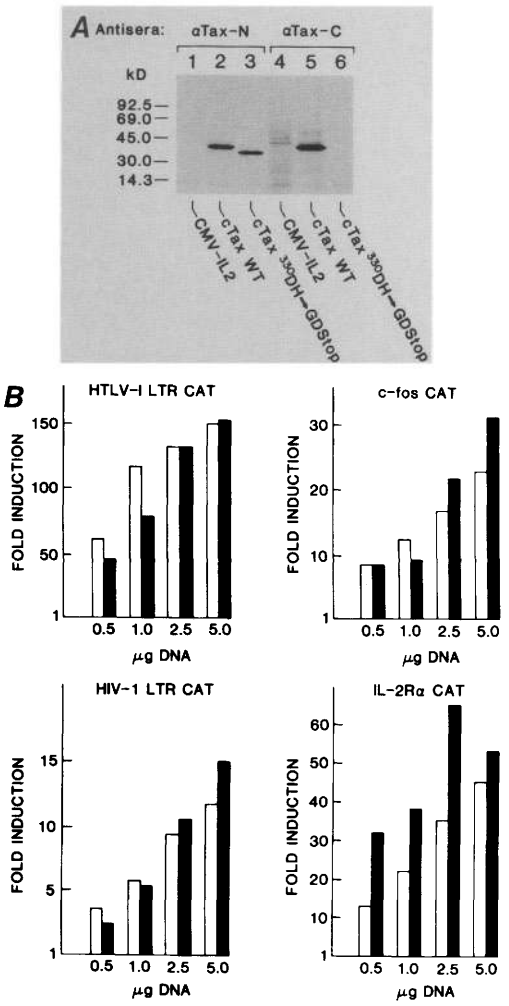

Figure 3. Phenotypic analysis of the carboxy-terminal Tax deletion mutant encoded by the Tax^{330}DH → GDStop expression vector. (*A*) Immunoprecipitation analysis of the Tax^{330}DH → GDStop mutant protein. Subconfluent COS cell cultures were transfected with either pCMV IL-2 (lanes *1* and *4*), the wild-type pcTax (lanes *2* and *5*), or pcTax^{330}DH → GDStop (lanes *3* and *6*). Approximately 48 hr after transfection, cells were metabolically labeled with [^{35}S]cysteine for 4 hr and lysed in RIPA buffer. Cell lysates were immunoprecipitated with either αTax-N (lanes *1–3*) or αTax-C corresponding to rabbit anti-peptide antisera directed against amino acid residues 106–132 or 321–353 of the Tax protein, respectively. Immunoprecipitates were analyzed on 10–20% SDS–polyacrylamide gradient gels. (*B*) Functional analysis of the Tax^{330}DH → GDStop *trans*-activator. Jurkat T cells were cotransfected with either pCMV IL-2, the wild-type pcTax, or pcTax^{330}DH → GDStop and the HTLV-I LTR–CAT, c-*fos*–CAT, HIV-1 LTR–CAT, or IL-2Rα–CAT reporter plasmid. *Trans*-activating function of the truncated Tax protein was analyzed over a 10-fold range of expression vector concentration (0.5–5.0 μg/ml), as indicated, and the total DNA concentration was maintained at 7.5 μg/5 × 10^6 cells for each point by the addition of varying amounts of pCMV IL-2. CAT activity was measured 48 hr after transfection and is represented as the fold induction relative to activity measured in cells transfected with the negative control plasmid pCMV IL-2 and the relevant reporter plasmid. Values shown represent the average of two transfections. (□) pcTax; (■) pcTax ^{330}DH → GDStop.

ducible promoters and variable amounts of each of the mutant *tax* expression vectors. Results of experiments involving the representative mutants M47 and M22 are shown in Figure 4. Over a 10-fold range of expression vector concentration, the M47 Tax mutant displayed <5% of wild-type *trans*-activation of the HTLV-I LTR and c-*fos* promoter while *trans*-activating the NF-κB responsive HIV-1 LTR and IL-2Rα promoter at levels nearly equivalent to the wild-type Tax protein (Fig. 4A). In contrast, the M22 Tax mutant *trans*-activated the HTLV-I LTR and the c-*fos* promoter at levels >50% of wild-type Tax but failed to *trans*-activate significantly either the HIV-1 LTR or the IL-2Rα promoter at all input expression vector concentrations (Fig. 4B). These results confirm the observation that the M22 and M47 Tax mutants functionally segregate *trans*-activation of the CREB/ATF and NF-κB responsive promoters and extend these differential phenotypes of *trans*-activation to two different cellular promoters. Furthermore, these results provide additional indirect evidence that Tax *trans*-activates the c-*fos* promoter and the HTLV-I LTR by a common mechanism.

The M22 Tax mutant fails to induce NF-κB expression in human T cells

To investigate whether the failure of the M22 Tax mutant to activate the NF-κB responsive HIV-1 LTR and IL-2Rα promoter was due to its inability to induce NF-κB, nuclear extracts were prepared from transiently transfected Jurkat T cells and assayed for NF-κB-binding activity. In electrophoretic mobility-shift assays employing the κB enhancer from the IL-2Rα gene, nuclear extracts prepared from cells transiently transfected with the wild-type pcTax expression vector mediated the formation of a single predominant DNA–protein complex (Fig. 5, lane 5). This Tax-inducible complex reflected specific protein binding to the radiolabeled κB probe because the inclusion of a 200-fold molar excess of unlabeled wild-type, but not mutant, IL-2Rα competitor completely blocked its formation (Fig. 5, cf. lanes 5–7). Under identical conditions, nuclear extracts prepared from cells transfected with the negative control plasmid pCMV IL-2 resulted in only trace NF-κB-binding activity (Fig. 5, lanes 2–4). In contrast to the wild-type *tax* expression vector, transfection of Jurkat cells with the M22 mutant *tax* expression vector induced little detectable NF-κB activity (Fig. 5, lanes 8–10). These results suggest that the failure of the M22 Tax mutant to *trans*-activate the HIV-1 LTR and IL-2Rα promoter is likely due to its inability to induce the nuclear expression of NF-κB. As predicted from their functional activities, the M1 and M47 *tax* mutants effectively induced NF-κB activity when transfected into Jurkat T cells (data not shown).

The zinc finger domain of Tax is required for nuclear localization

The Tax protein is localized predominantly in the nuclei of expressing cells (Goh et al. 1985; Kiyokawa et al.

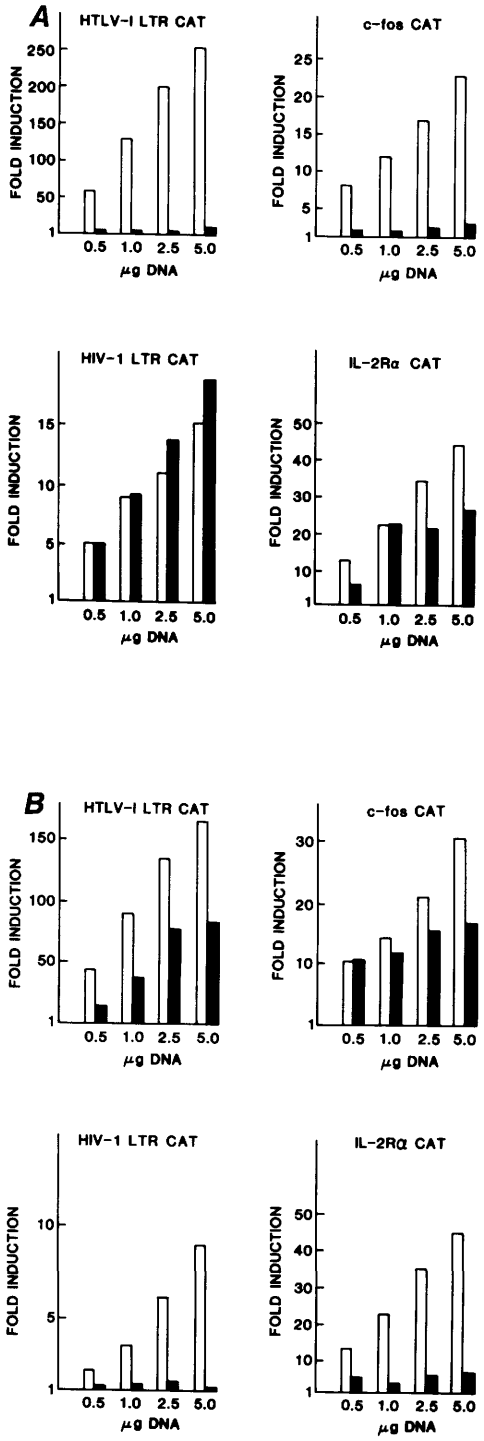

Figure 4. Functional analyses of the *trans*-activation properties of the M47 and M22 Tax mutants. Jurkat T cells were cotransfected with either pCMV IL-2, pcTax, pM47 (*A*) or pM22 (*B*) and the HTLV-I LTR–CAT, c-*fos*–CAT, HIV-1 LTR–CAT, or IL-2Rα–CAT reporter plasmid. Transfections and CAT activity measurements were performed over a 10-fold concentration range of expression vector as described in Fig. 3B. CAT activity is represented as fold induction relative to the activity measured in cells transfected with the indicated reporter plasmid and the control pCMV IL-2 vector. The values shown represent the average of two transfections. (*A*) (□) pcTax; (■) pM47; (*B*) (□) pcTax; (■) pM22.

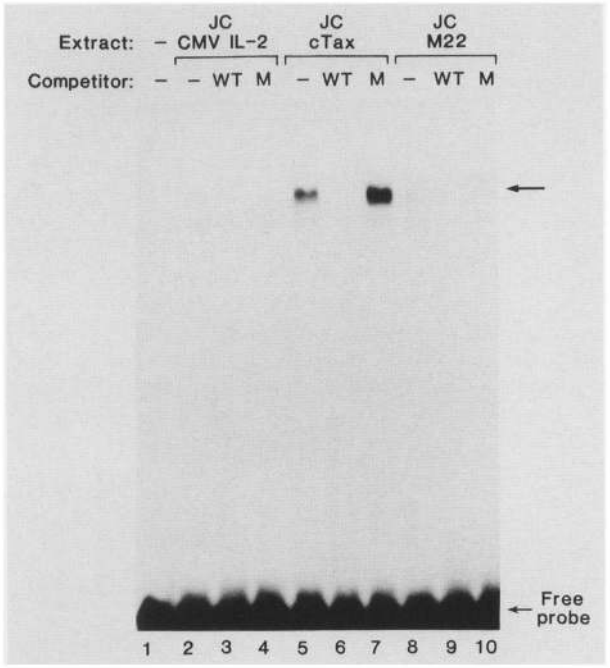

Figure 5. The M22 Tax mutant fails to induce nuclear expression of NF-κB. Nuclear extracts were prepared from Jurkat T cells transiently transfected with either pCMV IL-2 (lanes *2–4*), pcTax (lanes *5–7*), or pM22 (lanes *8–10*), as described in Materials and methods. These extracts were incubated with a 27-bp ^{32}P-labeled oligonucleotide probe spanning the IL-2Rα κB site. The resultant DNA–protein complexes were resolved on native 6% polyacrylamide gels. To confirm the sequence specificity of DNA–protein interactions, extracts were preincubated with either no specific competitor (lanes *2*, *5*, and *8*) or a 200-fold molar excess of unlabeled oligonucleotide competitor corresponding to either wild-type κB (WT, lanes *3*, *6*, and *9*) or mutant κB (M, lanes *4*, *7*, and *10*) IL-2Rα sequence. This mutant competitor contained a 4-nucleotide substitution (GGGA → ATCT) within the IL-2Rα κB sequence shown previously to disrupt the binding of NF-κB (Leung and Nabel 1988). No extract or specific competitor was present in the binding reaction shown in lane *1*. Position of the prominent Tax inducible κB-specific complex is indicated with an arrow.

1985). This subcellular localization of wild-type Tax was confirmed by indirect immunofluorescence (Fig. 6A,B). Similarly, the subcellular localization of each missense mutant was determined and categorized (Table 1). The majority of Tax mutants, including M1, M22, and M45–M48, displayed nuclear predominant patterns of expression (N) indistinguishable from that of the wild-type protein. Phase-contrast and corresponding immunofluorescence photomicrographs of the M22 and M47 Tax mutants are presented in Figure 6, C–F. These results indicate that the contrasting functional phenotypes of these mutants are not explained by an altered pattern of subcellular expression. Strikingly, each of the nonfunctional zinc finger mutants displayed a cytoplasmic predominant, almost nuclear-excluded, (C > N) pattern of expression (Table 1; M5–M12). The subcellular localization of two representative zinc finger mu-

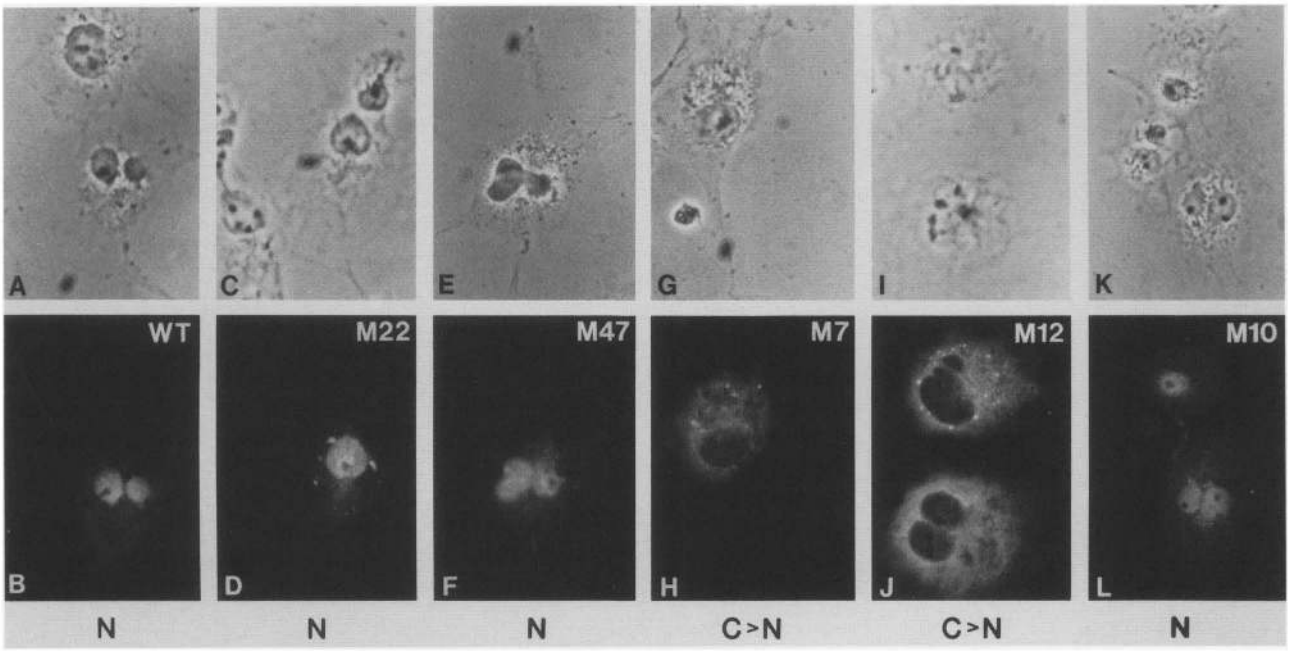

Figure 6. Subcellular localization of Tax mutants by indirect immunofluorescence. Phase-contrast (*top*) and corresponding immunofluoresence (*bottom*) photomicrographs of fixed transfected COS cells are presented. Cells were treated with rabbit αTax-C antisera followed by goat anti-rabbit IgG conjugated to rhodamine isothiocyanate. Three patterns of subcellular localization were observed: (N) nuclear predominant, with some cytoplasmic expression; (C > N) cytoplasmic predominant with little detectable nuclear expression; and (C = N) random distribution throughout the entire cell. Representative examples of nuclear predominant and cytoplasmic predominant patterns of subcellular localization are shown. The subcellular pattern of expression for each missense *tax* mutant is summarized in Table 1.

tants, M7 and M12, is shown in Figure 6, G–J. In contrast, M10, the single biologically active zinc finger mutant, exhibited a wild-type nuclear predominant pattern of expression (Fig. 6K and L). These results suggest that the zinc finger domain of Tax is required for its nuclear expression. Additionally, several mutants affecting residues outside the proposed zinc finger were also excluded from the nucleus (Table 1; M4, M13–M15, M17, M18, M20), indicating that additional regions of Tax may also contribute to its nuclear targeting. Notably, each of the Tax mutants excluded from the nucleus uniformly failed to *trans*-activate either the HTLV-I LTR or the HIV-1 LTR. These findings argue further that nuclear expression of Tax may be required for its transcriptional activities.

Discussion

We used transient gene expression analysis to assess the phenotype of a series of missense mutants of the HTLV-I *tax* gene. Using this approach, we identified mutants affecting residues within three distinct regions of the Tax protein that differentially *trans*-activate CREB/ATF and NF-κB responsive promoters. These findings demonstrate that Tax activation of these two cellular transcription factor pathways proceeds via distinct mechanisms and suggest that activation is mediated by different Tax domains. Specifically, the M1 and M47 *tax* mutants displayed markedly attenuated abilities to *trans*-activate the CREB/ATF-dependent HTLV-I LTR while retaining essentially wild-type activation of the NF-κB-dependent HIV-1 LTR and IL-2Rα promoter. Interestingly, these mutations are located within two widely separated hydrophobic domains of the Tax protein. However, it remains unknown how these hydrophobic domains participate in the activation of CREB/ATF host transcription factors.

Conversely, the M22 *tax* mutant fails to activate the NF-κB-dependent HIV-1 LTR and IL-2Rα promoter while displaying nearly wild-type *trans*-activation of the HTLV-I LTR and c-*fos* promoter. Interestingly, the M22 mutation alters the third of four regularly spaced leucine residues in the linear sequence of Tax. This region of the protein, however, contains four proline residues and does not likely form a leucine zipper because these structural motifs are distinguished by their α-helical properties (Landschulz et al. 1988; O'Shea et al. 1989). Furthermore, mutants altering the other regularly spaced leucine residues did not recapitulate the functional phenotype of the M22 mutant. The M22 Tax mutant was also shown to lack the ability to induce nuclear expression of NF-κB, thus explaining its inability to *trans*-activate either the HIV-1 LTR or IL-2Rα promoter. Recent studies have shown that the induction of NF-κB in many cells involves its dissociation from a cytoplasmic inhibitor, termed IκB (Baeuerle and Baltimore 1988), initiated by protein kinase-mediated phosphorylation of IκB (Ghosh and Baltimore 1990). The mecha-

nism by which the wild-type nuclear Tax protein induces NF-κB dissociation from IκB in the cytoplasm remains unknown. Preliminary experiments using either in vitro-translated Tax or recombinant Tax partially purified from *Escherichia coli* would appear to exclude a simple direct interaction of Tax and IκB (M.R. Smith and W.C. Greene, unpubl.).

Our mutation analysis of Tax also demonstrates that the carboxyl terminus, although acidic in nature and predicted to fold as an α-helix, is not required for Tax activation through either the CREB/ATF or the NF-κB pathways. Furthermore, this subregion of Tax does not function as a transcriptional activation domain when fused to the DNA-binding domain of GAL4 (M.R. Smith and W.C. Greene, unpubl.). The apparent dispensability of the carboxyl terminus is consistent with the absence of 22 of these amino acids in the homologous HTLV-II Tax protein, which is a potent *trans*-activator of both the HTLV-I and HTLV-II LTRs (Pavlakis et al. 1988; Cann et al. 1989), as well as the HIV-1 LTR and the IL-2Rα promoter (M.R. Smith and W.C. Greene, unpubl.). In addition, several laboratories have reported that hybrid proteins consisting of the truncated HTLV-II carboxyl terminus fused to the HTLV-I amino terminus are functional *trans*-activators (Pavlakis et al. 1988; Cann et al. 1989). Our results, however, differ with a previous study suggesting that the carboxyl terminus of the HTLV-I Tax protein may be required for *trans*-activation of the L-2Rα promoter (Ruben et al. 1989).

These studies have revealed that mutations within a putative zinc finger domain of Tax abolish *trans*-activation of both CREB/ATF and NF-κB responsive promoters. Currently, formal proof of metal binding by this potential structural domain is lacking. Recombinant Tax partially purified from *E. coli* has been reported to bind zinc chelating columns (Marriot et al. 1990). However, recombinant Tax immobilized on nitrocellulose does not bind zinc specifically under conditions where known metal-binding proteins bind divalent metals (M.R. Smith and W.C. Greene, unpubl.). Furthermore, the precise functional role of this structural domain in *trans*-activation is difficult to assess, because each of the transcriptionally inactive zinc finger mutants is excluded from the nuclei of expressing cells.

Nuclear proteins typically contain short highly basic peptide domains responsible for their nuclear targeting (for review, see Dingwall and Laskey 1986). In contrast, the Tax protein does not contain a canonical basic nuclear localization signal. Our mutational analyses, however, revealed that the zinc finger domain is required for nuclear expression of this viral *trans*-activator. These results raise the possibility that this structural motif may represent a new class of nuclear localization signal. Consistent with this interpretation, we identified a minimal sequence encompassing this structural motif (residues 17–48) that is sufficient to retarget β-galactosidase, a large cytoplasmic protein, to the nuclei of expressing cells (M.R. Smith and W.C. Greene, unpubl.). Notably, several biologically inactive Tax mutants affecting residues outside this nuclear localization signal also failed to localize to the nucleus (Table 1). We suspect that anomalous folding of these mutant proteins may preclude their entry into the nucleus, either by disrupting the conformation of the nuclear localization signal or by a more indirect mechanism. Notably, each of the nuclear-excluded Tax mutants lacked detectable *trans*-activator function, suggesting that nuclear localization of Tax may be required for its biological function.

In summary, these studies highlight several structural and functional properties of the HTLV-I Tax protein that underlie its diverse transcriptional activities. The identification of Tax mutants with differential transcriptional phenotypes demonstrates that Tax activation of the CREB/ATF and NF-κB transcription factors involves different biochemical pathways and suggests further that these processes may be mediated through distinct Tax peptide domains. These mutants should prove valuable in future studies of the molecular mechanisms of Tax *trans*-activation and cellular transformation.

Materials and methods

Construction of expression vectors

An M13 bacteriophage system (Amersham) for oligonucleotide-directed in vitro mutagenesis (Nakamaye and Eckstein 1987) was used to introduce targeted nucleotide substitutions into a cDNA copy of the *tax* gene encoded by the pcTax expression vector (Böhnlein et al. 1988a). The majority of mutations involved the introduction of a unique *Nhe*I site into the *tax* gene, resulting in the substitution of the dipeptide alanine–serine for the original amino acid residues. Mutants M45–M48 targeted specifically a hydrophobic region of the protein. To create mutations more likely to disrupt hydrophobic interactions within this region, mutants M45–M48 contain arginine–serine dipeptide substitutions that created a unique *Bgl*II site within the *tax* gene. The pcTax^{330}DH → GDStop mutant expression vector, encoding a truncated form of Tax, was constructed by mutating the nucleotide sequence of codons 330–332 to insert the dipeptide glycine–aspartic acid followed by a translational termination codon. A unique *Bgl*II site was also introduced downstream of this stop codon to facilitate the identification of this mutant. The presence of each mutation within the *tax* gene was determined by diagnostic restriction mapping. The DNA sequence of the majority of introduced mutations, including M1, M10, M22, M47, pcTax^{330}DH → GDStop, was confirmed by dideoxynucleotide sequencing. The Tax-inducible HTLV-I LTR–CAT and HIV-1 LTR–CAT (pU3R-I and pU3R-III; Sodroski et al. 1985), c-*fos* CAT (−356 *fos*–CAT; Gilman et al. 1986), and IL-2Rα–CAT (−317 WT; Ballard et al. 1988) reporter plasmids have been described previously.

Cell transfections and CAT assays

The Jurkat human T-cell line was cultured in RPMI 1640 media supplemented with 7.5% fetal calf serum, 50 μg/ml gentamicin, and 250 ng/ml amphotericin B. Jurkat cells were transfected with 2.5 μg of reporter plasmid DNA and 5.0 μg of expression vector DNA per 5×10^6 cells using DEAE dextran and chloroquine as described previously (Holbrook et al. 1987). Cell extracts were assayed for CAT activity (Neumann et al. 1987) ~48 hr after transfection. COS cells were cultured in Iscove's media supplemented as described above and transfected with

5.0 μg of plasmid DNA/ml using DEAE dextran and chloroquine (Cullen 1987). In transfections performed for subsequent immunofluorescence analysis, the amount of plasmid DNA was reduced to 2.0 μg/ml.

Analysis of mutant proteins

Western blot analysis of lysates prepared from COS cells transiently transfected with the wild-type and mutant *tax* expression vectors was performed as described (Winston et al. 1989). Briefly, these lysates were subjected to electrophoresis on discontinuous 10% polyacrylamide gels, followed by transfer of proteins to Immobilon-P (Millipore Corp., Bedford, MA) polyvinylidene difluoride membranes. Membranes were then probed using rabbit anti-peptide antisera directed against amino acid residues 321–353 (αTax-C; 1 : 2000 dilution) and ^{125}I-labeled protein A (10^4 cpm/cm^2 Immobilon-P membrane). The carboxy-terminal deletion mutant Tax ^{330}DH → GDStop was analyzed by immunoprecipitation. Transfected COS cell cultures (~10^6 cells) were metabolically labeled for 4 hr with [^{35}S]cysteine (200 μCi/ml) and lysed in RIPA buffer (Malim et al. 1989). Metabolically labeled proteins were then immunoprecipitated with either αTax-C or rabbit anti-peptide antisera directed against amino acid residues 106–132 (αTax-N) (1 : 200 dilution). Immunoprecipitates were analyzed on 10–20% SDS–polyacrylamide gradient gels under reducing conditions.

Indirect immunofluorescence

Indirect immunofluorescent staining (Cullen 1987; Cullen et al. 1988) was used to assess the subcellular patterns of expression of the various mutant Tax proteins within transfected COS-7 cells. After paraformaldehyde fixation, cells were treated with rabbit αTax-C antisera (1 : 200 dilution) and stained with goat anti-rabbit IgG antibody conjugated to rhodamine isothiocyanate (Boehringer Mannheim) (1 : 100 dilution).

Electrophoretic mobility-shift assays

Jurkat T cells (10^8) were transfected with 100 μg of plasmid DNA using DEAE dextran and chloroquine and nuclear extracts were prepared (Dynan 1987) ~48 hr later. Electrophoretic mobility-shift assays (EMSAs) were performed as described previously (Ballard et al. 1989) by use of 5 μg of extracted proteins and a radiolabeled 27-bp probe containing the IL-2Rα NF-κB-binding site. This probe was prepared at high specific radioactivity (~5×10^7 cpm/pmole) by primer extension. To determine the κB-specific nature of binding, a 200-fold molar excess of oligonucleotide competitor, containing either the wild-type or mutant (GGGA→ATCT; Leung and Nabel 1988) IL-2Rα κB element, was added to some reactions. Binding reactions were analyzed on native 6% polyacrylamide gels.

Acknowledgments

We thank Drs. Steve Gilman, William Haseltine, and Mark Ptashne for providing plasmid DNAs, Dr. Richard Randall and Mary R. Jones for oligonucleotide synthesis, Dr. Dean Ballard for assistance with EMSAs, Dr. Sarah Hanly for assistance in performing the immunofluorescence analyses, and Dr. Michael Malim for many useful scientific discussions. M.R.S. is supported by the Medical Scientist Training Program.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Altman, R., D. Harrich, J.A. Garcia, and R.B. Gaynor. 1988. Human T-cell leukemia virus types I and II exhibit different DNase I protection patterns. *J. Virol.* **62:** 1339–1346.

Ballard, D.W., D.W. Böhnlein, J.W. Lowenthal, Y. Wano, R.B. Franza, and W.C. Greene. 1988. HTLV-I Tax induces cellular proteins that activate the κB element in the IL-2 receptor-α gene. *Science* **241:** 1652–1655.

Ballard, D.W., E. Böhnlein, J.A. Hoffman, H.P. Bogerd, E.P. Dixon, B.R. Franza, and W.C. Greene. 1989. Activation of the interleukin-2 receptor α gene: Regulatory role for DNA-protein interactions flanking the κB enhancer. *New Biologist* **1:** 83–92.

Baeuerle, P. and D. Baltimore. 1988. IκB: A specific inhibitor of the NF-κB transcription factor. *Science* **242:** 540–546.

Böhnlein, E., M. Siekevitz, D.W. Ballard, J.W. Lowenthal, L. Rimsky, H. Bogerd, J. Hoffman, Y. Wano, B.R. Franza, and W.C. Greene. 1988a. Stimulation of the HIV-1 enhancer by the HTLV-I *tax* gene product involves the action of inducible cellular proteins. *J. Virol.* **63:** 1578–1586.

Böhnlein, E., J. Lowenthal, M. Siekevitz, D.W. Ballard, B.R. Franza, and W.C. Greene. 1988b. The same inducible nuclear proteins regulate mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. *Cell* **53:** 827–836.

Brady, J., K.-T. Jeang, J. Duvall, and G. Khoury. 1987. Identification of p40^x-responsive regulatory sequences within the HTLV-I LTR. *J. Virol.* **61:** 2175–2181.

Cann, A.J., J.D. Rosenblatt, W. Wachsman, and I.S.Y. Chen. 1989. In vitro mutagenesis of the human T cell leukemia virus types I and II *tax* genes. *J. Virol.* **63:** 1474–1479.

Chen, I.S.Y., D.J. Slamon, D.J. Rosenblatt, N.P. Shah, S.G. Queen, and W. Wachsman. 1985. The *x* gene is essential for HTLV replication. *Science* **229:** 54–58.

Cross, S.L., M.B. Feinberg, J.B. Wolf, N.J. Holbrook, F. Wong-Staal, and W.J. Leonard. 1987. Regulation of the human interleukin-2 receptor α chain promoter: Activation of a nonfunctional promoter by the transactivator gene of HTLV-I. *Cell* **49:** 47–56.

Cullen, B.R. 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* **71:** 684–704.

Cullen, B.R., J. Hauber, K. Campbell, J.G. Sodrowski, W.A. Haseltine, and C.A. Rosen. 1988. Subcellular localization of the human immunodeficiency virus trans-acting *art* gene product. *J. Virol.* **62:** 2498–2501.

Dingwall, C. and R.A. Laskey. 1986. Protein import into the cell nucleus. *Annu. Rev. Cell Biol.* **2:** 367–390.

Dynan, W.S. 1987. DNase footprinting as an assay for mammalian gene regulatory proteins. In *Genetic engineering: Principles and methods* (ed. J.K. Setlow), vol. 9, pp.75–87. Plenum Press, New York.

Felber, B.K., H. Paskalis, C. Kleinmann-Ewing, F. Wong-Staal, and G.N. Pavlakis. 1985. The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. *Science* **229:** 675–680.

Fujii, M., P. Sassone-Corsi, and I.M. Verma. 1988. *c-fos* promoter trans-activation by the tax$_1$ protein of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci.* **85:** 8526–8530.

Fujisawa, J., M. Seiki, T. Kiyokawa, and M. Yoshida. 1985. Functional activation of the human T-cell leukemia virus type I by trans-acting factor. *Proc. Natl. Acad. Sci.* **82:** 2277–2281.

Fujisawa, J., M. Toita, and M. Yoshida. 1989. A unique enhancer element for the trans-activator of human T-cell leu-

kemia virus type I that is distinct from cyclic AMP and 12-*O*-tetradecanoyl phorbol-13-acetate-responsive elements. *J. Virol.* **63:** 3234–3239.

Gessain, A., F. Barin, J.C. Vernant, O. Gout, L. Maurs, A. Calender, and G. de The. 1985. Antibodies to the human T lymphotropic virus type-1 in patients with tropical spastic paraparesis. *Lancet* **2:** 407–410.

Ghosh, S. and D. Baltimore. 1990. Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. *Nature* **344:** 678–682.

Giam, C.-Z. and Y.-L. Xu. 1989. HTLV-I *tax* gene product activates transcription via pre-existing cellular factors and cAMP response element. *J. Biol. Chem.* **264:** 15236–15241.

Gilman, M.Z., R.N. Wilson, and R.A. Weinberg. 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. *Mol. Cell. Biol.* **6:** 4305–4316.

Goh, W.C., J.G. Sodroski, C.A. Rosen, M. Essex, and W.A. Haseltine. 1985. Subcellular localization of the product of the long open reading frame of human T-cell leukemia virus type I. *Science* **227:** 1227–1228.

Hai, T., F. Liu, A. Allegretto, M. Karin, and M.R. Green. 1988. A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. *Genes Dev.* **2:** 1216–1226.

Haseltine, W.A., J. Sodroski, R. Patarca, D. Briggs, D. Perkins, and F. Wong-Staal. 1984. Structure of the 3′ terminal region of type II human T lymphotropic virus: Evidence for a new coding region. *Science* **225:** 419–421.

Hinrichs, S.H., M. Nerenberg, R.K. Reynolds, G. Khoury, and G. Jay. 1987. A transgenic mouse model for human neurofibromatosis. *Science* **237:** 1340–1343.

Holbrook, N.J., A. Gulino, and F.W. Ruscetti. 1987. Cis-acting transcriptional regulatory sequences in the gibbon ape leukemia virus (GALV) long terminal repeat. *Virology* **157:** 211–219.

Hoyos, B., D.W. Ballard, E. Böhnlein, M. Siekevitz, and W.C. Greene. 1989. Kappa B-specific DNA binding proteins: Role in regulation of human interleukin-2 gene expression. *Science* **244:** 457–460.

Inoue, J., M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida. 1986. Induction of interleukin 2 receptor gene expression by p40^{x} encoded by human T-cell leukemia virus type I. *EMBO J.* **5:** 2883–2888.

Jeang, K.-T., I. Boros, J. Brady, M. Radonovich, and G. Khoury. 1988. Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40^{x}-responsive 21-base-pair sequence. *J. Virol.* **62:** 4499–4509.

Jeang, K.-T., S.G. Widen, O.J. Semmes, and S.H. Wilson. 1990. HTLV-I trans-activator protein, Tax, is a trans-repressor of the human β-polymerase gene. *Science* **247:** 1082–1084.

Kiyokawa, T., M. Seiki, S. Iwashita, K. Imagawa, K. Shimuzu, and M. Yoshida. 1985. Association of the pX gene product of human T-cell leukemia virus type-I with nucleus. *Virology* **147:** 462–465.

Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1988. The leucine zipper; a hypothetical structure common to a new class of DNA binding proteins. *Science* **240:** 1759–1764.

Lee, K.A.W., T.-Y. Hai, L. SivaRaman, B. Thimmappaya, H.C. Hurst, N.C. Jones, and M.R. Green. 1987. A cellular protein, activating transcription factor, activates transcription of multiple E1a-inducible adenovirus early promoters. *Proc. Natl. Acad. Sci.* **84:** 8355–8459.

Lenardo, M.J. and D. Baltimore. 1989. NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58:** 227–229.

Leung, K. and G.J. Nabel. 1988. HTLV-I transactivator induces interleukin-2 receptor expression through an NF-κB like factor. *Nature* **333:** 776–778.

Malim, M., S. Böhnlein, J. Hauber, and B.R. Cullen. 1989. Functional dissection of the HIV-1 Rev trans-activator-derivation of a trans-dominant repressor of Rev function. *Cell* **58:** 205–214.

Marriot, S.J., I. Boros, J.F. Duvall, and J.N. Brady. 1989. Indirect binding of the human T-cell leukemia virus type I tax_1 to a responsive element in the viral long terminal repeat. *Mol. Cell. Biol.* **9:** 4152–4160.

Marriot, S.J., P.F. Lindholm, S.D. Gitlin, K.M. Brown, M.F. Radonovich, J.F. Duvall, and J.N. Brady. 1990. Molecular analysis of trans-activation in the HTLV-I LTR. In *Human retroviruses* (ed. J.E. Groopman, I.S.Y. Chen, M. Essex, and R.A. Weiss), pp. 13–22. Wiley/Liss, New York.

Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J. Inoue, M. Yoshida, and T. Taniguchi. 1987. Evidence for abberant activation of the interleukin-2 autocrine loop by HTLV-I encoded p40x and T3/Ti complex triggering. *Cell* **48:** 343–350.

Montagne, J., C. Beraud, I. Crenon, G. Lombard-Platet, L. Gazzolo, A. Sergeant, and P. Jalinot. 1990. Tax1 induction of the HTLV-I 21 bp enhancer requires cooperation between two cellular DNA-binding proteins. *EMBO J.* **9:** 957–964.

Montminy, M.R., K.A. Sevarino, J.A. Wagner, G. Mandel, and R.H. Goodman. 1986. Identification of a cyclic-AMP responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci.* **83:** 6682–6686.

Nabel, G. and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326:** 711–713.

Nakamaye, K. and F. Eckstein. 1987. Inhibition of restriction endonuclease Nci I by phosphorthioate groups and its application to oligonucleotide-directed mutagenesis. *Nucleic Acids Res.* **14:** 9679–9698.

Neumann, J.R., C.A. Morency, and K.O. Russian. 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* **5:** 444–447.

Nyborg, J.K., W.S. Dynan, I.S.Y. Chen, and W. Wachsman. 1988. Binding of host factors to DNA sequences in the long terminal of human T cell leukemia virus type I: Implications for viral gene expression. *Proc. Natl. Acad. Sci.* **85:** 1457–1461.

Osame, M., K. Usuku, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, and H. Tara. 1986. HTLV-I associated myelopathy, a new clinical entity. *Lancet* **1:** 1031–1032.

O'Shea, E.K., R. Rutkowski, and P. Kim. 1989. Evidence that the leucine zipper is a coiled coil. *Science* **243:** 538–542.

Paskalis, H., B.K. Felber, and G.N. Pavlakis. 1986. Cis-acting sequences responsible for the transcriptional activation of human T-cell leukemia virus type I constitute a conditional enhancer. *Proc. Natl. Acad. Sci.* **83:** 6558–6562.

Pavlakis, G.N., B.K. Felber, G. Kaplin, H. Paskalis, N. Grammatikakis, and M. Rosenberg. 1988. Regulation of expression of the HTLV family of retroviruses. In *The control of human retrovirus gene expression* (ed. B.R. Franza, B.R. Cullen, and F. Wong-Staal), pp. 281-289. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Poiesz, B.F., F.W. Ruscetti, A.F. Gazdar, P.A. Bunn, J.D. Minna, and R.C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci.* **77:** 7415–7419.

Poiesz, B.F., F.W. Ruscetti, M.S. Reitz, V.S. Kalyanaraman, and R.C. Gallo. 1981. Isolation of a new type C retrovirus

(HTLV) in primary uncultured cells from a patient with Sezary T-cell leukemia. *Nature* **294:** 268–271.

Poteat, H.T., P. Kadison, K. McGuire, L. Park, R.E. Park, J.G. Sodrowski, and W.A. Haseltine. 1989. Response of the human T-cell leukemia virus type 1 long terminal repeat to cyclic AMP. *J. Virol.* **63:** 1604–1611.

Pozzatti, R., J. Vogel, and G. Jay. 1990. The human T-lymphotropic virus type I *tax* gene can cooperate with the *ras* oncogene to induce neoplastic transformation of cells. *Mol. Cell. Biol.* **10:** 413–417.

Ptashne, M. 1988. How eukaryotic transcriptional activators work. *Nature* **335:** 683–689.

Ruben, S.M., H. Poteat, T.-H. Tan, K. Kawakami, R. Roeder, W. Haseltine, and C.A. Rosen. 1988. Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-I *tax* gene product. *Science* **241:** 89–91.

Ruben, S.M., A. Perkins, and C.A. Rosen. 1989. Activation of NF-κB by the HTLV-I trans-activator protein Tax requires an additional factor present in lymphoid cells. *New Biologist* **1:** 275–284.

Shimotohno, K., D.W. Golde, M. Miwa, T. Sugimura, and I.S.Y. Chen. 1984. Nucleotide sequence analysis of the long terminal repeat of human T-cell leukemia virus type II. *Proc. Natl. Acad. Sci.* **81:** 1079–1083.

Shimotohno, K., M. Takano, T. Terunchi, and M. Miwa. 1986. Requirements of multiple copies of a 21-nucleotide sequence in the U3 region of human T-cell leukemia virus type I and type II long terminal repeats for trans-acting activation of transcription. *Proc. Natl. Acad. Sci.* **83:** 8112–8116.

Siekevitz, M., M.B. Feinberg, N. Holbrook, F. Wong-Staal, and W.C. Greene. 1987a. Activation of interleukin-2 and interleukin-2 receptor (Tac) promoter expression by the trans-activator (*tat*) gene product of human T-cell leukemia virus, type I. *Proc. Natl. Acad. Sci.* **84:** 5389–5393.

Siekevitz, M., S.F. Josephs, M. Dukovitch, N. Peffer, F. Wong-Staal, and W.C. Greene. 1987b. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. *Science* **238:** 1575–1578.

Sodroski, J.G., C.A. Rosen, and W.A. Haseltine. 1984. Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. *Science* **225:** 381–385.

Sodroski, J., C. Rosen, W.C. Goh, and W. Haseltine. 1985. A transcriptional activator protein encoded by the x-lor region of the human T cell leukemia virus. *Science* **228:** 1430–1434.

Tan, T.-H., R. Jia, and R.G. Roeder. 1989a. Utilization of signal transduction pathway by the human T-cell leukemia virus type I transcriptional activator Tax. *J. Virol.* **63:** 3761–3768.

Tan, T.-H., M. Horikoshi, and R.G. Roeder. 1989b. Purification and characterization of multiple nuclear factors that bind to the Tax-inducible enhancer within the human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **9:** 1733–1745.

Tanaka, A., C. Takahashi, S. Yamaoka, T. Nosaka, M. Maki, and M. Hatanaka. 1990. Oncogenic transformation by the *tax* gene of human T-cell leukemia virus type I in vitro. *Proc. Natl. Acad. Sci.* **87:** 1071–1075.

Wachsman, W., A.J. Cann, J.L. Williams, D.J. Slamon, L. Souza, N.P. Shah, and I.S.Y. Chen. 1987. HTLV *x* gene mutants exhibit novel transcriptional regulatory phenotypes. *Science* **235:** 674–677.

Wano, Y., M. Feinberg, J.B. Hosking, H. Bogerd, and W.C. Greene. 1988. Stable expression of the *tax* gene of the type I human T cell leukemia virus in human T cells activates specific cellular genes involved in growth. *Proc. Natl. Acad. Sci.* **85:** 9733–9737.

Winston, S.E., S.A. Fuller, and J.G.R. Hurrell. 1989. Western blotting. In *Current protocols in molecular biology* (ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl), vol 2, sect. 10.8. John Wiley/Greene, New York.

Yamamoto, K.K., G.A. Gonzalez, W.H. Biggs, and M.R. Montminy. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334:** 494–498.

Yoshida, M., I. Miyoshi, and Y. Hinuma. 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci.* **79:** 2031–2035.

Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes.

M R Smith and W C Greene

Genes Dev. 1990, **4:**
Access the most recent version at doi:10.1101/gad.4.11.1875