JOURNAL OF VIROLOGY, Mar. 1995, p. 1827–1833 Vol. 69, No. 3
0022-538X/95/$04.00+0

Definition of a Minimal Activation Domain in Human T-Cell Leukemia Virus Type I Tax

OLIVER J. SEMMES AND KUAN-TEH JEANG*

Molecular Virology Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 6 July 1994/Accepted 19 October 1994

Fourteen mutants were used to delineate a minimal activation domain in the Tax protein of human T-cell leukemia virus type I. In an assay using a Gal4-Tax (GalTx) fusion protein and a responsive promoter containing Gal4 consensus binding sites, we found that activation was "squelched" by coexpression of wild-type Tax protein in *trans*. When Tax mutants were tested for squelching, many competed effectively against GalTx. However, those containing changes in amino acids 289 to 322 failed to inhibit activity. In particular, three mutants that were expressed stably, with changes at amino acids 289, 296, and 320 respectively, did not squelch GalTx activity. On the other hand, mutants with individual changes at amino acid 3, 9, 29, 41, 273, and 337 efficiently inhibited GalTx function. Three other mutants failed to be stably expressed. In separate experiments, when fused alone to the DNA-binding domain of Gal4, amino acids 289 to 322 of Tax conferred *trans* activation ability. This fusion protein was able to activate a core promoter. These findings suggest that amino acids 289 to 322 define a region that contacts an essential transcription factor and that this region is a modular activation domain.

Transcriptional *trans* activators generally have two domains (9, 40, 41, 49, 51). One directs recognition (for DNA, RNA, or another protein), while the second modulates transcription. An illustrative example of such an activator is yeast Gal4. Gal4 has a 154-amino-acid N-terminal domain that binds to a cognate DNA sequence and a C-terminal region that directly activates transcription (23). These two domains are separable and can be modularly attached to other proteins, thereby conferring function (50). Another example is herpes simplex virus VP16. VP16 also contains an activation and a recognition domain; the latter recognizes a cellular protein, Oct1 (48, 63), which directs the activator to promoter DNA. Thus, VP16 represents a paradigm for protein-protein interaction used to target an activator to DNA (31, 32, 63, 69).

Distinct from Gal4 and VP16 is the human T-cell leukemia virus type I (HTLV-I) *trans* activator, Tax, which does not bind DNA directly. Tax can associate with cellular factors, but these interactions have been incompletely characterized (2, 6, 17, 26, 44). It is known that one mode of Tax activation is mediated through three 21-bp repeats found in the HTLV-I long terminal repeat (LTR) (16, 28, 37, 54). Each repeat contains a core cyclic AMP (cAMP)-responsive sequence (7, 11, 25, 46, 55, 62). Both AP-1 and CREB have been shown to bind these 21-bp sequences (25, 26, 43, 65, 75), and it has been proposed that Tax is directed to the LTR by protein-protein association with these DNA-binding factors (27, 76).

We have previously characterized 47 mutations in Tax that affect activation (56). These mutants have allowed for an approximate demarcation of regions necessary for function (35, 56, 57). Analyses from these studies suggested that there were minimally two functional domains through which mutations affected the ability of Tax to activate a promoter. Either mutations could affect the ability of Tax to locate properly to the promoter, thus interfering with activation, or they could change directly its activation surface. In the latter instance Tax could still assemble at the promoter, but once there, it lacks the ability to modulate transcription.

In the current work, we have defined a minimal domain in Tax that functionally contacts an essential transcription factor. This definition was based on the ability of different Tax mutants to "squelch" activation by Gal4-Tax (GalTx) of an upstream activating sequence (UAS)-containing reporter plasmid. Using this assay, we identified some mutants which could, and others which could not, titrate for a limiting transcription factor. Amino acids 289 to 322 were found to be a contact domain in Tax for this cellular factor. When amino acids 289 to 322 were fused to the DNA-binding domain of Gal4, the chimeric protein activated a UAS-containing promoter. Thus, these 33 amino acids apparently represent a minimally sufficient activation domain.

MATERIALS AND METHODS

Plasmid constructions. Nomenclatures for mutants and for wild-type Tax were as previously described (56). The deletion mutants TxΔ3, TxΔ3-4, TxΔ3-6, and TxΔ3-9 were made by digesting IEX (56) with *Nco*I and *Acc*I and repairing with appropriate synthetic oligonucleotides. Termination mutants were constructed by digesting IEX with *Stu*I (Tx289trm) or *Sma*I (Tx337trm) and ligating to an oligonucleotide pair containing a stop codon. Tax mutants designated M21, M23, M26, and M27 were gifts from W. C. Greene (59). GalTx fusion protein expression vectors were made by positioning wild-type and mutant Tax cDNAs in frame with the Gal4-binding domain (amino acids 1 to 147). Tax/VP16 fusion proteins were expressed by ligating the appropriate Tax open reading frame to the VP16 activation domain (amino acids 413 to 490). Gal4/VP16 (GalVP) was a gift from Peter O'Hare (10). The reporter plasmids uasHT and uasHV were generated by inserting four copies of the Gal4-binding sequence (GGAAGACTCTCCTC CGA) into a unique *Xba*I site.

Calcium phosphate transfection. HeLa cells were seeded at 5×10^5/25-mm^2 dish in Dulbecco's modified Eagle medium containing 2 mM L-glutamine, 10% fetal calf serum, and 100 U of penicillin-streptomycin per ml. Calcium phosphate-mediated transfections were performed 24 h later as previously described (20). Cells were washed twice and then fed with 5 ml of medium 16 h after introduction of calcium phosphate precipitates. The cells were harvested 48 h after transfection by scraping. Extracts were made by freeze-thawing and were assayed for chloramphenicol acetyltransferase (CAT) activity (19).

Electroporation of F9 cells. F9 cells were cultured to 80% confluence in multiple dishes. The cells were detached by trypsinization, washed once in phosphate-buffered saline (PBS), and then resuspended into PBS at 2.5×10^7/ml. DNA was diluted to the appropriate concentration in 50 μl of distilled water and placed into sterile Bio-Rad Gene Pulser electroporation cuvettes (electrode gap,

* Corresponding author. Phone: (301) 496-6680. Fax: (301) 402-0226.

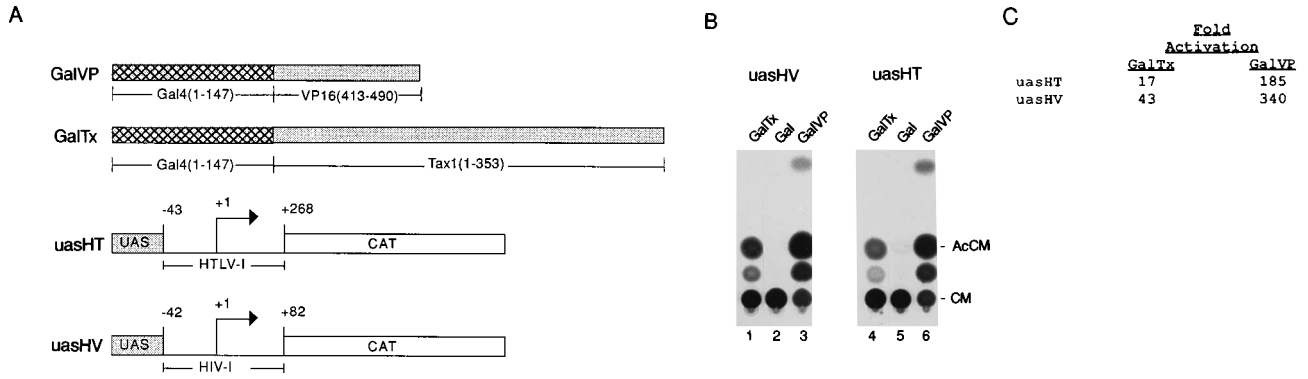

FIG. 1. *trans* activation by GalTx of enhancerless promoters. (A) Schematic representations of the constructions used in the analyses. The Gal4 DNA-binding domain, amino acids 1 to 147, is crosshatched. The VP16 activation domain, amino acids 423 to 490 (GalVP), and the complete Tax coding sequences, amino acids 1 to 353 (GalTx), are shaded. The reporter plasmids uasHT and uasHV are diagrammed below. Four copies of the upstream activating sequence (UAS), a cognate DNA-binding site for Gal4, are indicated. −43 to +268 and −42 to +82, sequences that surround the HTLV-I and the human immunodeficiency virus TATAA elements, respectively. +1 is defined as the initial nucleotide of transcription. (B) *trans* activation of uasHT and uasHV by GalTx, Gal4 (Gal), and GalVP expression plasmids. An autoradiogram of a representative CAT assay is shown. Acetylated chloramphenicol (AcCM) and unacetylated chloramphenicol (CM) are indicated. (C) Quantitation of the activities of GalTx and GalVP on uasHT and uasHV. Fold activation is defined as the fold increase over Gal activation measured as percentage of acetylation.

0.4 cm). To each cuvette was added 200 μl of resuspended F9 cells with DNA. The electrical pulse was 300 V at a capacitance of 960 μF, generated with a Bio-Rad Gene Pulser. After pulsing, cells were resuspended into 10 ml of Dulbecco's modified Eagle medium containing 10% fetal calf serum and plated onto 100-mm-diameter culture dishes. Forty-eight hours following electroporation, cells were harvested, washed twice in PBS, resuspended into 100 μl of 0.25 M Tris-HCl, pH 8.0, subjected to three cycles of freeze-thawing, and assayed for CAT (19).

Western (immunoblot) analysis. HeLa cells were grown to 60% confluence in 100-mm tissue culture flasks. Tax plasmids were transfected with calcium phosphate. Forty-eight hours later, cells were washed once in 10 ml of PBS, scraped into 1 ml of PBS, and pelleted by centrifugation. Total protein was solubilized by addition of 250 μl of 1× Laemmli buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1% bromophenol blue) with heating at 90°C for 10 min followed by clarification in a Beckman Microfuge (15,000 rpm for 5 min).

Proteins were resolved in an 12% SDS–polyacrylamide gel and transferred to Immobilon P membranes by using a Millipore semidry blotting apparatus per manufacturer's protocol. The membranes were then incubated with rabbit anti-Tax polyclonal antibody (55a) at a 1:1,000 dilution in BLOTTO (2% milk in PBS) at 4°C overnight. Tax protein was visualized by chemiluminescence (Tropix Western-Light) using an anti-rabbit alkaline phosphatase-conjugated second antibody.

RESULTS

A GalTx protein activates enhancerless promoters. To define an activation domain, one could start with an assumption that Tax has modular components. Thus, separate regions that determine activation and/or promoter targeting would exist in Tax. Theoretically, these domains can be defined through complementation assays using heterologous counterparts. Hence, one line of reasoning dictates that mutations affecting the ability of Tax to target to the promoter would be complemented when the mutant is fused to the Gal4 DNA-binding domain and then assayed on a promoter containing Gal4-binding sites. These GalTx chimeras should be active so long as the activation domain in Tax is intact.

To validate the hypothesis that a fusion protein reflects Tax activity, we constructed a GalTx in which the Gal4 DNA-binding domain (amino acids 1 to 147) was linked, in frame, to the complete Tax open reading frame (Fig. 1A). GalTx was assayed by using reporter CAT plasmids containing the minimal promoter from either HTLV-I (uasHT) or human immunodeficiency virus type 1 (uasHV) attached to four copies of Gal4 consensus-binding sites (UAS) (Fig. 1A). Since others (13, 72) have proposed that cAMP-responsive elements, commonly found upstream of many TATA elements, are required for Tax activation, it was of interest to ask whether GalTx could transactivate uasHT or uasHV, because neither contained cAMP-responsive elements. We found that GalTx activated uasHT and uasHV 17- and 43-fold, respectively (Fig. 1B [compare lanes 1 and 2 and lanes 4 and 5] and C). This activation is comparable to that normally observed for Tax activation of the complete HTLV-I LTR.

As a control we tested both uasHT and uasHV for responsiveness to a GalVP fusion protein (Fig. 1B, lanes 3 and 6). Like GalTx, GalVP also activated uasHV better than uasHT. This suggested that these small differences arose from variations in basal activities between the two promoters. Nonetheless, on the same promoters GalTx shares some of the qualities of GalVP (Fig. 1B and C).

We also tested GalTx in F9 cells. F9 cells are deficient in expression of AP-1 (4, 8) and CREB (55a). Furthermore, the activity of the HTLV-I promoter is low in F9 cells (26). Thus, the functional requirements for either CREB or AP-1 in Tax-mediated activation, as suggested by others (13, 72), would not be met. We cotransfected GalTx into F9 cells with either uasHT or uasHV. Resulting CAT activities showed that GalTx effectively activated expression (Fig. 2, lanes 1 and 3). Similar to the findings with HeLa cells, the levels of *trans* activation were 40-fold for uasHT and 95-fold for uasHT (Fig. 2). Therefore, although AP-1/CREB-like factors may contribute to HTLV-I promoter function, in the setting of GalTx activation of a minimal promoter they are not required. In this context, activation might be mediated through different factors (15) or by direct contact with TBP (6).

Tax squelches the activity of GalTx. The above results verified that GalTx capably activated a minimal promoter. This finding could be extended to define domains in Tax on the basis of squelching. Specifically, one reasons that wild-type Tax and/or some mutants of Tax, when coexpressed, would squelch GalTx-mediated activation (Fig. 3A). Depending on which mutants squelch (or fail to squelch), one can map the region(s) in Tax necessary for contacting the essential transcription factor(s). This region is likely to be the activation domain for Tax.

Current thinking suggests that Tax targets to the HTLV-I LTR via interactions with cellular factors docked at the 21-bp repeats (Fig. 3A, upper panel) (2, 17, 75, 76). Tax then contacts

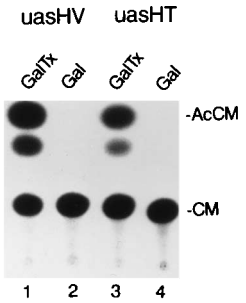

FIG. 2. *trans* activation of uasHV and uasHT by GalTx in F9 cells. Plasmids were introduced into F9 cells by electroporation. The activities of GalTx on uasHV and uasHT are compared with the activities of Gal4 (Gal) on the same reporters. Acetylated chloramphenicol (AcCM) and unacetylated chloramphenicol (CM) are indicated.

a factor(s) at the promoter. In GalTx activation, the activator binds DNA directly and then contacts a limiting transcription factor (Fig. 3A, middle panel). In squelching, one envisions that a domain in Tax would compete with the same domain in GalTx for contact with a critical factor. Tax mutants perturbed in this domain would not compete. Thus, in using a panel of Tax mutants and measuring their respective abilities to compete with GalTx, one could deduce this contact domain.

We first squelched GalTx using wild-type Tax (Fig. 3A, lower panel). Coexpression of increasing amounts of Tax indeed reduced GalTx activity (Fig. 3B, lanes 3 and 4). In parallel comparisons, neither increasing amounts of cytomegalovirus vector DNA (Fig. 3B, lanes 1 and 2) nor increasing amounts of pUC19 carrier plasmid (Fig. 3B, lanes 5 and 6) affected GalTx activity. These results validated the hypothesis that Tax does titrate, in *trans*, for a limiting transcription factor.

Tax mutants map a contact domain. We and others had previously constructed many Tax mutants (56, 59). A subset of these mutants was transcriptionally inactive when assayed on the HTLV-I LTR. There are two reasons for this inactivation. First, loss of activity could result from an inability of the mutant to target to the promoter. Second, the mutant, despite targeting properly to the promoter, lacks activity because its activation domain has been perturbed. In terms of squelching, these mutants are expected to fall into two groups, one that retains, and the other that does not retain, the ability to inhibit GalTx.

In total, 14 inactive Tax mutants were tested (Fig. 4). Mutants TxΔ3, TxG9, TxΔ41-43, M21, M23, M26, M27, TxA273S274, and Tx337tm efficiently squelched GalTx (Fig. 4). However, TxA10, TxS29, TxA274, Tx284tm, TxG296, and TxG320 failed to affect GalTx activity. Thus, mutations either in the N terminus or in the C terminus of Tax apparently abolish the ability to squelch GalTx (Fig. 4).

To clarify these findings, we sought to distinguish between mutations that affected protein-protein contact from those that rendered the overall protein unstable. In the latter instance, the mutants do not achieve effective concentrations necessary to titrate a limiting factor, although in squelching assays they would resemble phenotypically mutants affected in a contact domain. To check this, proteins unable to compete with GalTx were analyzed by immunoblotting to determine steady-state stability (Fig. 5). We found that, with the exception of TxA10 and TxA274, mutants that did not squelch were expressed at levels comparable to the level of wild-type Tax. TxA10 and TxA274 were drastically reduced in amounts of protein, and TxS29 was expressed at a lower level than the wild type, which correlated with its lower efficiency of squelching. Hence, we