Studies of heterologous promoter *trans*-activation by the HTLV-II *tax* protein

Janice L. Williams, Alan J. Cann, Todd Leff, Paolo Sassone-Corsi and Irvin S.Y. Chen

Division of Hematology-Oncology, Departments of Medicine and Microbiology and Immunology,
UCLA School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, CA 90024-1678,
USA

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ABSTRACT

The *tax* protein of HTLV-II increases the level of steady-state mRNA produced from the HTLV-II long terminal repeat (LTR) and also activates heterologous promoters. We have previously shown that the adenovirus E3 promoter, which is *trans*-activated by the adenovirus E1a protein, is also *trans*-activated by the *tax* protein. To investigate the mechanism of *trans*-activation by *tax*, we analyzed E3 promoter deletion mutants to determine nucleotide sequence requirements for activation of this promoter. Our results show that removal of different upstream regions within the promoter does not result in loss of *trans*-activation, indicating that *tax* does not appear to interact with a single DNA binding protein to activate the E3 promoter. In addition, *tax* and E1a together activate the E3 promoter in a greater than additive fashion, suggesting that these proteins function differently. Possible mechanisms of activation by the *tax* protein are discussed.

INTRODUCTION

Human T-cell leukemia virus type II (HTLV-II) is a retrovirus associated with T-cell malignancies in man (1,2). HTLV-II has been isolated from two cases of T-cell variant hairy-cell leukemia (3-5), and *in vitro* transformation of T helper/inducer cells by HTLV-II has been demonstrated (6,7). HTLV-II shares no sequence homology to any known oncogene, and cell lines transformed by HTLV-II show no specific site of provirus integration within the human genome (8). However, HTLV-II, HTLV-I, and the related bovine leukemia virus (BLV) all contain a novel gene, termed *tax* (9-13). The *tax* gene of HTLV-II (previously termed *tat* or *x*) encodes a 37 Kd protein which acts to increase the steady-state level of mRNA produced from the viral LTR (*trans*-activation) (14-17). We previously showed that *tax* activates the adenovirus E3 promoter (18). Activation of heterologous promoters has led to the hypothesis that *tax* may participate in transformation by activating heterologous cellular genes which promote cellular growth (19-21).

The E3 promoter (22-25) is normally, during the course of adenovirus infection, activated in *trans* by the adenovirus E1a protein. Mutational

analysis of the E3 promoter indicated that no specific sequences are required for *trans*-activation by Ela (24). We have investigated *trans*-activation of E3 by the HTLV tax protein in an effort to define the mechanisms of heterologous promoter *trans*-activation by the tax gene. We utilized the same E3 promoter deletions which were previously tested for adenovirus Ela *trans*-activation. Our results indicate that there are no specific sequences in the E3 promoter which are necessary for tax responsiveness, similar to that of Ela, yet the mechanism of *trans*-activation is not entirely similar, since synergistic effects on *trans*-activation are seen with Ela and tax together.

MATERIALS AND METHODS

DNA Constructs

LTR-II-CAT (14), pBC12/CMV/IL-2 (26), the 91023B expression vector (27), and 91023 xII (28) were derived as previously described. E3-CAT is derived from adenovirus 5, and contains sequences which extend 270 nucleotides upstream of the E3 cap site. SV-HTLV (14) contains SV40 promoter and polyadenylation sequences inserted into the 5' and 3' LTRs, respectively, of an infectious HTLV-II clone. SV-HTLV-Cla 5 contains a 2-bp insertion at the Cla site at position 7385 in the HTLV-II tax coding region, resulting in a frameshift mutation. BE5 (18) contains nucleotides 0 to 3323 from the adenovirus 2 genome inserted in pBR322, and expresses Ela gene products from the adenovirus Ela promoter. BE5H/S is derived from BE5, and contains a deletion between nucleotides 1008 and 1571 in the Ela coding region. 91023 13S was derived by ligation of an Ela 13S DNA (29) fragment into the 91023B expression vector.

DNA Transfections and CAT Assays

COS cells were grown in DMEM media containing 6% calf serum and 3% fetal calf serum. DNA was diluted to a final concentration of 10 μ g/ml, and was added to COS cells after precipitation in calcium phosphate. One and a half hours after addition of DNA, 0.1 mM of chloroquine was added to the cells for 2.5 hours. Cells were then treated with 20% glycerol in phosphate-buffered saline for one minute. Fresh medium was then added. After 36 to 40 hours, cells were harvested and assayed for CAT activity, as described (28). CAT activity was quantitated in the linear range of enzyme activity by measurement of the ratio of acetylated to total chloramphenicol.

S₁ Nuclease Analysis

The oligonucleotide probe used to quantitate E3-specific mRNA contains 88 nucleotides corresponding to positions -20 to +68 in the E3 promoter. The

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                                -220                -200                -180                -160                -140                -120
WT:  TCTTCAAGAATTCTCTCTGAACAGGCGGCTATTACCAACACACCTCGTAATAACCTTAATCCCGTAGTGGCCCGTGCCTGGTGACCAAGGAGTCCCGCTCCCACTGTGGTACTTCCCAAG
233.37:
TCTTCAAGAATT-----
                                -100                -80                -60                -40                -20                +1
WT:  GACGCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCGCAGCTTGCGGGCGGCTTTCGTCACAGGTTGCGGTGCGCCGGGCGAGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
233.37:
-----GGGCAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
111.39:
GACGC-----CCGGGAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
83.37:
GACGCCAGGCCGAAGTTCAGATGACTAACTCA-----GGGCAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
57.39:
GACGCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCGCAGCTTGCGGGCGGCTTTCG-----CCGGGAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
55.39:
GACGCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCGCAGCTTGCGGGCGGCTTTCGTC-----CCGGGAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
48.35:
GACGCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCGCAGCTTGCGGGCGGCTTTCGTCACAGGGT-----GCAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
36.29:
GACGCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCGCAGCTTGCGGGCGGCTTTCGTCACAGGTTGCGGTGCGCCGG-----TATAACTCACCTGACAATCAGAGGGCGAGGTATTCAGCTCAA
55.17:
GACGCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCGCAGCTTGCGGGCGGCTTTCGTC-----GACAATCAGAGGGCGAGGTATTCAGCTCAA

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Figure 1. Sequences of the wild-type and mutant adenovirus E3 promoter constructs. The dotted lines represent deleted nucleotides. "+1" represents the major cap site of E3. +1 to +68 encompasses the fragment which is protected by the DNA probe in S_1 analysis. The name of each deletion mutant is shown above each sequence.

double-stranded probe used to detect cytomegalovirus (CMV) promoter mRNA is 295 bp in length, extending from the Nco I to Hind III sites in pBC12/CMV/IL-2 (26). The probe used to detect Ela 13S mRNA from 91023 13S is 235 bp in length, derived from a construct which produces Ela 13S mRNA from the CMV promoter. The probe used to quantitate the amount of *tax* mRNA produced from the 91023 xII expression vector is 203 bp in length, derived from a construct containing a *tax* cDNA inserted into pUC19. All probes were 5' end-labeled, utilizing $\gamma^{32}\text{P}$ -ATP and T_4 kinase. Fifty μg of whole cell RNA was utilized in overnight hybridizations at 52°C. Two hundred units of S_1 nuclease were then added for one hour at room temperature. S_1 nuclease-resistant fragments were analyzed on 8% acrylamide denaturing gels.

RESULTS

Sequence Requirements for *tax* and Ela *trans*-activation of the E3 Promoter are Similar

The wild-type E3 promoter contains 245 nucleotides upstream of the E3 cap site. Leff et al. (24) generated deletion mutants in the E3 promoter (Figure 1), and tested their activities in the presence and absence of Ela, in order to determine if Ela requires unique nucleotide sequences for *trans*-activation of E3. Their results indicated that Ela *trans*-activation

Figure 2. Schematic diagram of *tax* and *Ela* expression vectors.

- A. The top line is a representation of the SV-HTLV vector with the HTLV-II *gag*, *pol*, *env* genes, and the *X* region (which contains the *tax* gene) indicated. Dark regions represent SV40 sequences. The shaded region represents coding sequences for the neomycin gene. Below the genome the spliced mRNA which encodes the *tax* protein is shown. The *Cla* I site used to construct the SVH-Cla vector is shown. The exons used to construct the non-spliced *tax* gene inserted into the 91023B expression vector (91023 *xII*) are shown below the spliced mRNA.
- B. The top line depicts the BE5 vector containing the *Ela* coding region of the adenovirus 2 genome. Below are the potential spliced mRNA products encoding the *Ela* proteins. The *Sma* I and *Hpa* I sites used to construct BE5H/S are shown. Below the spliced mRNAs the exons used to construct the non-spliced 13S *Ela* mRNA inserted into the 91023B vector are diagrammed.

does not require a unique sequence within the E3 promoter.

HTLV *tax* *trans*-activation of the E3 promoter mutants was tested by transient co-transfections in COS cells. Activation of the mutants by co-transfections with an *Ela* expression vector were also performed for comparison. The promoter activities were measured by quantitative S_1 nuclease analysis. The expression vectors used in these experiments are shown in Figure 2. The SV-HTLV vector utilizes the SV40 promoter to produce the *tax* protein, while SV-HTLV-Cla contains a frameshift in the coding region of *tax*, and was used as a negative control in the co-transfections. The BE5 expression vector produces *Ela* gene products, while BE5H/S, which contains a deletion in the coding region of *Ela*, was used as a negative control for *Ela trans*-activation.

Figure 3. S_1 nuclease analysis of representative E3 promoter mutants co-transfected in COS cells with either tax or E1a. The E3 promoter mutants are described in Figure 1. The protected band specific for E3 is 68 nucleotides in length, while the CMV-specific band is 77 nucleotides in length.

Panel A, wt + SVH, wt + SVHCla5, wt + BE5, and wt + BE5H/S.

Panel B, 111.39 + SVH, 111.39 + SVHCla5, 111.39 + BE5, and 111.39 + BE5H/S.

Panel C, 55.17 + SVH and 55.17 + SVHCla5.

Panel D, 57.39 + SVH, 57.39 + SVHCla5, 57.39 + BE5, and 57.39 + BE5H/S.

This result of this transfection and others are summarized in Table I.

Representative E3 promoter mutants, which were examined using S_1 nuclease analysis, are shown in Figure 3. The pattern seen at the position indicated as "E3" (68 nt) on the gel represents the sites of initiation of transcription. This is the same pattern as that seen in adenovirus-infected cells (data not shown). The 77-nucleotide fragment seen above the E3 band corresponds to the mRNA produced from the cytomegalovirus immediate early promoter (BC12), which was added to each transfection as an internal control. Lane A shows the wild-type E3 promoter co-transfected with the SV-HTLV and SV-HTLV-Cla vectors. Basal activity of the E3 promoter in Lane 2 compared to

TABLE I
***Trans*-activation of E3 Promoter Mutants by tax and Ela**

E3 mutants	+ tax/- tax	+ Ela/-Ela
<u>Wild-type</u>	100%	100%
233.37	---	---
111.39	49%	58%
83.37	58%	68%
57.39	98%	64%
55.39	115%	60%
48.35	79%	81%
36.29	107%	85%
55.17	64%	---

Transcriptional activation of E3 promoter deletion mutants determined by densitometry. Results were obtained from three separate experiments. Activation of the wild-type promoter by tax in Column I was determined by dividing the densitometric value obtained with SV-HTLV by the value obtained with SV-HTLV-Cla. *Trans*-activation of wild-type E3 by Ela in Column II was obtained by dividing the densitometric value obtained with BE5 over that obtained with BE5H/S. All densitometry readings of E3 promoter activities were normalized to the CMV promoter used as an internal transfection control. Wild-type E3 promoter activation by tax was four-fold, and given a value of 100% in Column I. Activation of wild-type E3 by Ela was six-fold, and given a value of 100% in Column II. Activation of the mutant promoters by the tax and Ela proteins was expressed as a percentage of wild-type activation by either tax or Ela in Row 1.

the activity of E3 in the presence of tax shows the overall level of *trans*-activation of this promoter by the tax protein. Similarly, comparison of the wild-type E3 promoter co-transfected with BE5 and BE5H/S shows the level of *trans*-activation of the E3 promoter by the Ela protein. Panel B shows the 111.39 E3 promoter mutant, which lacks nucleotides -40 to -110 upstream of the E3 cap site; Panel C shows the 55.17 mutant, which lacks nucleotides -54 to -18; and Panel D shows the 57.39 mutant, which lacks sequences -40 to -56. The levels of *trans*-activation are low when compared to those of the HTLV LTR; however, they are reproducible. These results show that although each of these mutants have different basal activities relative to the wild-type promoter, they are still *trans*-activated by tax.

Table I shows the levels of *trans*-activation by tax and Ela of each promoter mutant, as measured by densitometry of the E3 promoter-specific band normalized to the densitometry values obtained from S₁ nuclease analysis of the BC12 control present in each transfection. Column I shows, relative to wild-type E3, the levels of *trans*-activation by tax of each promoter.

Figure 4

- A.** Activation of the E3 promoter by the tax and Ela proteins. Five µg of E3-CAT were transfected in the presence of 5-20 µg 91023 xII alone (open circles), 5-20 µg 91023 13S alone (closed circles), and 5-20 µg each of 91023 xII and 91023 13S (triangles). Each transfection contained a total of 45 µg DNA, using the 91023B vector as carrier DNA. The graph represents an average of three separate transfections.
- B.** Additive effect of the tax and Ela activation of E3 promoter in COS cells. Each lane represents a transfection with 3.33 µg of E3-CAT.
 Lane 1: 6.66 µg 91023B
 Lane 2: 3.33 µg 91023 xII, 3.33 µg 91023B
 Lane 3: 3.33 µg 91023 13S, 3.33 µg 91023B
 Lane 4: 3.33 µg 91023 xII, 3.33 µg 91023 13S
 CAT activity values for this transfection are shown in Table II.
- C.** Levels of steady-state mRNA produced from the 91023 xII and 91023B Ela expression vectors in COS cells utilizing S₁ nuclease analysis. The protected band specific for tax RNA of 91023 xII is 176 in length (xII), and the protected band specific for Ela RNA is 110 in length (Ela). Each lane represents analysis of 25 µg of whole cell RNA. The amounts of DNA used in each transfection are listed as follows:
 Lane 1: 5 µg of 91023 13S, 10 µg of 91023 xII; Lane 2, 5 µg of 91023 13S, 10 µg of 91023B vector; Lane 3, 10 µg of 91023 xII, 5 µg of 91023 vector; Lane 4, 15 µg of 91023B vector. Lanes 1-4 each contain 5 µg of E3-CAT.

TABLE II
Effect of *tax* Plus *Ela* *Trans*-activation of the E3 Promoter

<u><i>tax</i></u>	<u><i>Ela</i></u>	<u>% Conversion</u>
---	---	0.97
91023 xII	---	7.9
---	BE5	4.7
91023 xII	BE5	50
SVH	---	1.3
---	91023 13S	4.2
SVH	91023 13S	8.9
---	---	2.3
91023 xII	---	27
---	91023 13S	9.3
91023 xII	91023 13S	80

tax and *Ela trans*-activation of the E3 promoter. Separate experiments were performed using 3.33 μ g of E3-CAT was co-transfected with 3.33 μ g of each of the expression vectors listed above. Each transfection contained a total of 10 μ g, with either SVHCla5, BE5H/S or 91023B used as carrier DNA, and as negative controls. CAT activity was quantitated by the percent conversion of total chloramphenicol to acetylated forms.

Similarly, Column II shows the levels of *trans*-activation of each promoter mutant by *Ela*. The lower levels of *trans*-activation measured with the mutant promoters relative to the wild-type E3 promoter may be due to overall lower absolute levels of activity of the mutants. The 233.37 mutant, which has only the TATA box region and lacks nucleotides -232 to -36, shows a complete loss of measurable activity, in agreement with previously published results from Leff (24). The 55.17 mutant is activated by *tax*, but we were unable to demonstrate significant activation by *Ela*. However, Leff (24) has shown that this mutant is activated by *Ela*. This lack of agreement may be due to our inability to quantitate the low levels of mRNA produced from this construct.

It is noteworthy that the 111.39, 83.37, and 57.39 mutants lack a 5-nucleotide sequence (TGACG) located on the non-coding strand at -54 and -58 upstream of the E3 cap site. The TGACG sequence is also found in the three 21-nucleotide repeats in the HTLV-II LTR enhancer. This sequence and the TATA element represent the only significant nucleotide sequence homologies found between the E3 promoter and the LTR. It has been shown that the 21-nucleotide repeats of the LTR are necessary for *trans*-activation of the HTLV LTR by *tax* (30-32). Removal of the TGACG sequence in the E3 promoter does not result in loss of *trans*-activation by *tax* and *Ela*, indicating that this sequence is not necessary to confer *tax* inducibility on the E3 promoter.

The E3 promoter mutants used in these studies cover an extensive region

within E3, ranging from -110 to -15 upstream of the E3 cap site. All mutants which retain transcriptional activity reproducibly display higher amounts of activity in the presence of *tax*. In addition, we have also tested an E3 promoter deletion mutant lacking upstream sequences from -236 to -130, which are present in our other deletion mutants, and found that this mutant is *trans*-activated by both *tax* and *Ela* (data not shown). The results from these experiments indicate that the nucleotide sequence requirements for *tax* and *Ela trans*-activation of the E3 promoter are similar, suggesting that *tax*, like *Ela*, does not require specific upstream promoter elements to *trans*-activate the E3 promoter. Mutations downstream of the E3 cap site were not tested for *trans*-activation.

The *tax* Protein May Activate the E3 Promoter by a Different Mechanism than the *Ela* Protein

The results obtained from analysis of the E3 promoter mutants suggested that at least in regard to sequences necessary for *trans*-activation, *Ela* and *tax* act similarly. We further investigated whether *tax* and *Ela* function similarly by determining whether *tax* and *Ela*, acting together on the E3 promoter, provide greater *trans*-activation than either protein alone. Initially we determined the concentrations of *tax* and *Ela*, individually, at which *trans*-activation of the E3 promoter does not increase further. The expression vectors used in these experiments were 91023 xII and 91023 13S, which utilize the adenovirus major late promoter and the SV40 enhancer to express high levels of non-spliced *tax* mRNA from HTLV-II, and 13S mRNA from adenovirus, respectively (see Figure 2). As seen in Figure 4A, addition of 5-10 μ g of 91023 xII results in maximum levels of E3-CAT activity. Addition of greater amounts (15-20 μ g) of 91023 xII results in no further increase in E3-CAT activity. Similarly, maximum levels of E3-CAT activity occur in the presence of 5-10 μ g of 91023 13S. At these same concentrations of *tax* and *Ela* added to the transfections, when both are present together, a synergistic effect is observed. CAT activity is twice the amount of the maximum level of activity observed with *tax* alone, and twenty times the maximum level of *Ela* activation. Figure 4B shows a representative CAT assay demonstrating this effect, using the 91023 xII and *Ela* 13S expression vectors. S_1 nuclease analysis of E3-CAT showed that the increase in CAT activity seen in Figure 4 correlates with an increase in steady-state levels of mRNA produced from E3-CAT (data not shown).

In order to exclude the possibility that greater levels of *Ela* or *tax*

are expressed when transfected together than a comparable amount transfected alone, we examined the levels of *Ela* or *tax* expression by S_1 nuclease analysis. Each lane in Figure 4C represents an S_1 nuclease analysis using two probes: one specific for 91023 13S, and the other specific for 91023 xII. Lane 1 shows the amount of mRNA produced from 91023 xII and 91023 13S when the two expression vectors are transfected together; Lane 2 shows the steady-state message produced from 91023 13S transfected alone; and Lane 3 shows the amount of *tax* steady-state mRNA produced from 91023 xII transfected alone. The results show that *Ela* and *tax* mRNA are the same, whether transfected alone or together, and thus, increases in levels of *trans*-activator proteins cannot account for the effects observed.

The synergistic effect of *tax* and *Ela* activation was also evident using different combinations of expression vectors (Table 2). Our results show that *tax* and *Ela* act in a greater than additive manner to activate the E3 promoter. One explanation for this result is that *tax* and *Ela* may interact with different cellular factors to *trans*-activate the E3 promoter.

DISCUSSION

There is no direct evidence to explain the mechanism of *trans*-activation by the HTLV-II *tax* protein. Data generated from *in vitro* footprinting experiments show that *tax* does not appear to be a DNA binding protein (33). The results from our experiments indicate that *tax* may activate the E3 promoter by interacting with multiple DNA binding proteins, each recognizing different promoter sequence motifs.

DNase I footprinting experiments have demonstrated four major protected regions within the E3 promoter. The protected region found at position -183 to -154 upstream of the E3 cap site contains the consensus sequence for NF-1. A protected region is found at position -96 to -87, and contains the AP-1 consensus sequence. The TGACG sequence is located on the non-coding strand at -58 to -54 of the E3 promoter. Factors which bind this protected sequence have been referred to as ATF (34), CREB (35), E4F1 (36), E2A-EF (37), E3F2 (38), and may be identical or related proteins. The protected region found at position -33 to -7 contains the TATA box. The two regions of limited homology between the E3 promoter and the HTLV-II LTR are the TGACG core sequence and the TATA element. The results from these experiments showed that removal of either the TATA region or the TGACG region alone does not result in loss of *trans*-activation by the *tax* protein, indicating that *tax* does not interact solely with either the TATA-binding protein or the TGACG-binding protein alone

to activate E3 transcription. However, since we did not test whether an E3 promoter mutant which lacks both the TATA box and the TGACG regions is *trans*-activated by *tax*, it is possible that *tax* activates the E3 promoter by activating both the TATA box and the TGACG-binding proteins, but that loss of either one of these regions alone does not result in loss of *trans*-activation by *tax*. Clearly, for activation of the E3 promoter, *tax*, like *Ela*, does not appear to interact with a single DNA binding protein to promote E3 transcription, since the consensus sequences for NF-1, AP-1, ATF, and TATA can be eliminated, and *trans*-activation still occurs.

Although a similar lack of sequence requirements is apparent for both *tax* and *Ela*, the results from the experiments utilizing both proteins in transfections indicate that *tax* and *Ela* activation differ, since E3 promoter activity is increased in a greater than additive fashion. One possibility is that different DNA binding proteins can recognize the same sequences in the E3 promoter, but are differentially responsive to the effects of *Ela* or *tax*. Another explanation for the synergistic effect is that *tax* and *Ela* interact with different non-DNA binding proteins to activate the E3 promoter.

Heterologous activation of cellular promoters by the *tax* protein may contribute to cellular transformation by HTLV. It is interesting to note that our results suggest that a single promoter can be activated by different mechanisms. Cellular promoters may also be modulated by multiple *trans*-acting proteins. It may be possible that multiple mechanisms of activation of a cellular gene can be selectively utilized to express varying amounts of mRNA from a given cellular promoter. Activation of the appropriate cellular genes in T cells by *tax* may lead to cell transformation.

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