Human T-cell Leukemia Virus Type I Tax Protein Transactivates RNA Polymerase III Promoter in Vitro and in Vivo*

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Tax protein of the human T-cell lymphotropic virus type I (HTLV-I) is critical for viral replication and is a potent transcriptional activator of viral and cellular polymerase II (pol II) genes. We report here that Tax is able to transactivate a classical pol III promoter, VA-I. In cotransfection experiments, Tax is shown to increase transcription of the VA-I promoter approximately 25-fold. Moreover, Tax is able to activate VA-I transcription when added exogenously to an in vitro transcription reaction. Using Tax affinity column chromatography, we demonstrate that Tax is able to deplete a HeLa cell extract for components required for transcription of VA-I. The transcriptional activity of the Tax-depleted extract can be restored by the 0.6 M phosphocellulose fraction. Interestingly, a consensus binding site for cAMP-responsive element binding protein (CREB) is located upstream of the VA-I promoter, and deletion of this element results in the loss of Tax responsiveness. When this CREB binding site is replaced by a Gal4 binding site, the VA-I promoter can be transactivated by a Gal4-Tax fusion protein. Taken together, these results suggest that Tax may activate pol III and pol II promoter through a similar mechanism involving the CREB activation pathway. It is also possible that Tax affects pol III transcription by direct interaction with a component of the pol III transcriptional machinery.

HTLV-I long terminal repeat contains overlapping transcription units, a typical pol II promoter, and a unique pol III-like promoter (49). Interestingly, the pol III-like promoter is transactivated by Tax in vitro producing an α-amanitin-resistant transcript (50).

Pol III genes are classified in three subclasses on the basis of their promoter structure and basal factor requirements (for review, see Ref. 51). All pol III genes analyzed so far require TFIIIB and RNA polymerase III for transcription. The tRNA and 5S RNA genes additionally use TFIIIC. TFIIIC binds directly to the internal promoter of tRNA genes facilitating the assembly of TFIIIB and RNA pol III. 5S RNA transcription also requires TFIIIA (51). Both subclasses 1 (encoding 5S RNA genes) and 2 (encoding tRNA, adenovirus VA RNAs, Alu sequences, 7SL, Epstein-Barr-encoded RNA genes) have mostly internal control regions located within the transcribed DNA. However, transcription of some subclass 1 and 2 genes can be influenced either positively or negatively by the 5′-flanking sequences (for review see Ref. 52). The role of upstream sequences is important in the case of the human 7SL RNA and EBER genes. These genes exhibit a hybrid promoter structure since they contain both internal and external control regions, which are essential for accurate transcription of these genes. Interestingly, the upstream regions of these genes contain a TATA box and sequence elements that can be recognized by pol II transcription factors such as ATF and Sp1. Subclass 3 genes (encoding U6 and 7SK genes) have only nontranscribed 5′ control regions containing elements that can be recognized by pol II-like transcription factors. The mechanism by which pol II-like transcription factors activate pol III promoters is largely unknown. It is also unclear if the upstream regions of the VA-I or tRNA genes possess the properties of a pol II-like enhancer.

Several cellular and viral transactivators have been shown to increase the expression of pol III promoters, including HBV (hepatitis B virus) X protein, adenovirus E1A, SV40 small t-antigen, and octamer binding protein (53-58). It appears that each of these transactivators uses a different mechanism to stimulate pol III genes. The hepatitis B virus X protein increases the cellular level of TBP which mediates transactivation of RNA polymerase III genes (59). By using various kinase inhibitors, it was suggested that the HBV X-mediated increases in pol III transcription and TBP levels are dependent upon protein kinase C activation (59). In the case of E1A-mediated transactivation, the target seems to be a component of TFIIIC. A selective increase in the cellular concentration of a 110-kDa subunit of TFIIIC has been shown to be responsible for the increase in pol III activity during infection of cells by adenovirus with concomitant E1A expression (60, 61, 62).

In this report, we present results that demonstrate the ability of HTLV-I Tax to transactivate the VA-I promoter in vivo (cotransfection experiments) and in cell-free extract in vitro. Deletion of sequences upstream of the VA-I initiation site abolished Tax responsiveness without affecting the basal promoter...
activity. Using gel shift assays, we further demonstrated that a CREB binding site is located within these sequences suggesting that the CREB1 recognition element is required for Tax transactivation of VA-I promoter.

MATERIALS AND METHODS

Plasmids and Oligonucleotides—pVA-dl182 and pVA-dl27 were constructed by cloning, respectively, a 219-bp XbaI-NheI fragment of pVA-I (containing the VA-I gene from −27 to +192) into the XbaI-restricted pGEM-3Z and a 374-bp EcoRI-NheI fragment of pVA-I (containing the VA-I gene from −182 to +192) into Smal-XbaI-restricted pGEM-3Z (Promega). pVA-Gal was constructed by inserting a 17-mer-containing pVA-I template (65) as indicated under “Results.” Restrictions were incubated for 60 min at 30°C before being stopped by addition of 400 μl of 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, 0.2% SDS. RNA was extracted with phenol/chloroform, ethanol precipitation, and separated by denaturing gel electrophoresis (6% polyacrylamide gel containing 7 M urea). Gels were exposed to phosphorus screens (Kodak) and bands corresponding to full-length transcripts quantitated by PhosphorImager (Molecular Dynamics, Inc).

The HeLa WCE was fractionated onto a phosphocellulose column (PC), as described previously (66), to generate the 0.3 and 0.6 M KCl fractions. Recombinant Tax was purified as described previously (67). For experiments testing the effect of antibodies, Tax was preincubated for 30 min at 30°C with Tax monoclonal or control normal IgG antibodies (amounts of antibodies are as indicated in the figure legend) before the DNA template and the nucleotides were added. RNasin, an RNase inhibitor (Promega), was added to all the reactions containing antibodies. The amount of VA-I DNA used in these assays was titrated over a wide range. In order to measure the level of transactivation, we used about 5 ng of VA-I vector. All experiments were repeated at least three times.

RNase Protection Assay—Total cellular RNA was isolated by using the guanidinium thiocyanate/phenol/chloroform extraction method using the RNAzol B reagent and following the manufacturer’s protocol. The VA-I RNA probe was generated by cloning of a XbaI-BamHI fragment from pVA-I (containing the VA-I gene from −27 to +63) into pGEM-3Z vector (Promega) and transcribing the antisense VA-I RNA with T7 polymerase in the presence of α-32PUTP. A β-actin probe generated from pTRI-B-actin human plasmid DNA by using T3 polymerase was used to detect the internal β-actin gene.

Cell Culture and Transfection—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum, 2 mM l-glutamine, and 100 units of penicillin/streptomycin per ml. Transfections were performed by using the lipofectamine reagent (Life Technologies, Inc.). Cells were seeded 24 h prior to transfection at 6 × 10^5 cells per 60-mm dish. When the cells were 50–80% confluent, they were washed twice with Opti-MEM Reduced Serum medium (Life Technologies, Inc.) and then transfected with a mixture of 25 μl of lipofectamine reagent and the indicated amount of reporter and expression vectors. The amount of DNA used in each transfection was held constant at 6 μg. 5 h after adding the lipofectamine reagent, the transfection media was removed and cells were incubated for another 24 h in fresh Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum. Total RNA was then isolated from the transfected cells.

EMSA—An oligonucleotide containing the upstream region of the VA-I gene from −44 to −18 was synthesized and end-labeled with γ-32P-ATP (Amersham Corp.) using T4 polynucleotide kinase. The labeled oligonucleotide was purified twice through Micro Select G-25 spin columns (SPErime-3Prime, Inc.). In a final volume of 8 μl, 0.2 μl of purified human CREB-1 (254–327), Santa Cruz Biotechnology, Inc.) was incubated with 0.1 μg of the VA-I DNA probe for 10 min at room temperature in a buffer containing 15 mM KCl, 0.5 mM dithiothreitol, 4 mM Hepes-KOH (pH 7.7), 15 mM MgCl2, 0.01% Nonidet-40, 10% glycerol, and 3% of CHAPS. For competition, oligonucleotides containing a CREB binding site, CREB consensus (5’AGAGATGGGCTACGTCA- GAGGCTAG3’), and HTLV-I TRE-1 (14), or control oligonucleotides, Ets consensus (14), and 5’CAACTTCCGGAGAACACAGC3’ corresponding to the sequence between nucleotide −58 and nucleotide −79 in the P2 promoter of the parathyroid hormone-related peptide (PTH-P) (14) were used. The reaction mix was loaded onto a 4% polyacrylamide gel and electrophoresed in 0.25 × TBE for 30 min at 100 V in a mini-Protean II cell (Bio-Rad).

Affinity Column—Affinity columns were performed as described previously (68). 6 mg of HeLa WCE proteins were loaded on the Tax, bovine serum albumin, carbonic anhydrase, and buffer columns at 4°C. The flowthrough was collected and tested for transcriptional activity in the in vitro transcription assay.

RESULTS

In Vivo Stimulation of Pol III Transcription by Tax—The effect of Tax on pol III transcription was first analyzed in vivo by measuring the level of expression of a classical pol III promoter, VA-I, using transient transfection assay. HeLa cells were cotransfected with a plasmid containing the VA-I promoter (pVA-I) and increasing amounts of Tax expression vector (pcTax). Twenty-four hours after transfection, total RNA was isolated and analyzed by RNase protection assay using either a VA-I-specific probe (upper panel) or an actin-specific probe (lower panel). β, quantitation of the in vivo transactivation of VA-I promoter by Tax.

FIG. 1. HTLV-I Tax up-regulates pol III VA-I promoter in vivo in HeLa cells. A, HeLa cells were transiently transfected as described under “Materials and Methods” with 30 ng of pVA-I DNA (lanes 1–7) and indicated amounts of pcTax. Total RNA concentration in each transfection was kept constant by adjusting it to 6 μg with pGEM DNA. After 24 h of transfection total RNA was isolated and analyzed by RNase protection assay using either a VA-I-specific probe (upper panel) or an actin-specific probe (lower panel). B, quantitation of the in vitro transactivation of VA-I promoter by Tax.
The HTLV-I Tax Protein Stimulates Transcription of an Exogenous Pol III Promoter in Vitro—The effect of Tax on pol III transcription was then analyzed in vitro. Therecombinant Tax purified from Escherichia coli extracts used in the in vitro transcription reactions was about 80–90% pure as shown by silver stain in Fig. 2A. A dose-dependent activation of VA-I was observed when increasing amounts of Tax were added to the transcription reaction (Fig. 2B). The highest level of activation, about 25-fold, was obtained when 100 ng of recombinant Tax was used (Fig. 2, lanes 2 and 6). To address the specificity of Tax-mediated activation of the VA-I promoter, purification Tax was preincubated with Tax monoclonal antibodies (lanes 4 and 5). In vitro transcription reactions were performed in HeLa whole cell extract with VA-I promoter (5 ng) and 0 ng (lane 1), 20 ng (lanes 2 and 4), and 200 ng (lanes 3 and 5) of purified Tax. Tax was preincubated with Tax monoclonal antibodies (lanes 4 and 5). In vitro transcription reactions were performed in HeLa whole cell extract with VA-I promoter (5 ng) and 200 ng of Tax (lanes 2 and 4), mock Tax E. coli HB101 extract (lane 3). In lane 4, Tax was preincubated for 30 min at 4 °C with an unrelated antibody prior to performing the transcription reactions. In lane 5, the anti-Tax antibody was tested on basal VA-I.

The HTLV-I Tax Protein Stimulates Transcription of an Exogenous Pol III Promoter in Vitro—The effect of Tax on pol III transcription was then analyzed in vitro. The recombinant Tax purified from Escherichia coli extracts used in the in vitro transcription reactions was about 80–90% pure as shown by silver stain in Fig. 2A. A dose-dependent activation of VA-I was observed when increasing amounts of Tax were added to the transcription reaction (Fig. 2B). The highest level of activation, about 25-fold, was obtained when 100 ng of recombinant Tax was used (Fig. 2, B and C). To address the specificity of Tax-mediated activation of the VA-I promoter, purified Tax was preincubated with anti-Tax antibodies prior to addition in the transcription mixtures. As shown in Fig. 2D, lanes 4 and 5, anti-Tax monoclonal antibodies inhibited the stimulation of VA-I promoter activity by Tax but did not affect the basal transcriptional activity (Fig. 2E, lanes 1 and 5). In contrast, control antibodies did not affect Tax-mediated activation of the VA-I promoter (Fig. 2E, lanes 2 and 4). The control mock extract from E. coli HB101 had no stimulatory activity on VA-I expression (Fig. 2E, lanes 1 and 3). Furthermore, when Tax was added to in vitro reactions containing the pol II ADML promoter, no increase in transcription was observed (data not shown). From these experiments, we concluded that Tax is able to specifically transactivate the VA-I pol III promoter in vitro.

Tax Binds Specifically to a Component of the Pol III Transcriptional Machinery—To test for a possible interaction between Tax and the pol III transcription factors, we used an affinity column assay that has been previously described (68). Tax or control proteins were immobilized on an Affi-Gel 10 matrix (see “Materials and Methods”). HeLa WCE was chromatographed over the Tax or control columns, and the flowthroughs were tested for transcriptional activity on pol III VA-I or pol II ADML templates (Fig. 3A). The cell extract chromatographed through the Tax affinity column was significantly reduced in its ability to transcribe the VA-I promoter, whereas the same extract was able to transcribe the ADML pol II promoter (Fig. 3A, lane 2). The extracts chromatographed through the control columns did not show any significant reduction in transcriptional activity of either the pol II or pol III promoters. Cellular factors required for pol III transcription can be functionally fractionated by chromatography on a phosphocellulose column and separated into four fractions 0.1, 0.3,
the 0.6 M phosphocellulose fraction but not with the 0.3 M fraction (Fig. 3). Tax-depleted extract. The Pol III activity could be restored with their ability to restore pol III transcriptional activity in the fractions (49). We tested these phosphocellulose fractions for 0.6, and 0.8 M PC fractions (66). Pol III transcription on the lane 5 m with phosphocellulose 0.3 and 0.6 M fractions. The Tax-depleted extract and methods. "Depleted extract were then tested for pol II (Adml) or pol III (VA-I) transcriptional activity. In vitro transcription reactions were performed as described under "Materials and Methods." B, reconstitution of transcription on the VA-I promoter with phosphocellulose 0.3 and 0.6 M fractions. The Tax-depleted extract (lanes 2–5) was supplemented with 5 μl of PC 0.3 M fraction (lane 3), 5 μl of PC 0.6 M fraction (lane 4), 5 μl of PC 0.3 M and 5 μl of PC 0.6 M fractions (lane 5).

Fig. 3. Analysis of Tax affinity column chromatography by in vitro transcription. A, extracts were depleted on Tax, bovine serum albumin (BSA), carbonic anhydrase protein (CA), or buffer columns as described under "Materials and Methods." Depleted extracts were then tested for pol II (Adml) or pol III (VA-I) transcriptional activity. In vitro transcription reactions were performed as described under "Materials and Methods." B, reconstitution of transcription on the VA-I promoter with phosphocellulose 0.3 and 0.6 M fractions. The Tax-depleted extract (lanes 2–5) was supplemented with 5 μl of PC 0.3 M fraction (lane 3), 5 μl of PC 0.6 M fraction (lane 4), 5 μl of PC 0.3 M and 5 μl of PC 0.6 M fractions (lane 5).

0.6, and 0.8 M PC fractions (66). Pol III transcription on the VA-I promoter can be reconstituted with the 0.3 and the 0.6 M fractions (49). We tested these phosphocellulose fractions for their ability to restore pol III transcriptional activity in the Tax-depleted extract. The Pol III activity could be restored with the 0.6 M phosphocellulose fraction but not with the 0.3 (Fig. 3B), 0.1, or 0.8 M fractions (data not shown). The reconstitution of pol III transcription with the 0.6 M fraction confirmed that the affinity column chromatography did not alter the transcriptional properties of the extract but rather specifically removed an activity required for transcription of the VA-I promoter. We concluded from this experiment that Tax binds a factor specifically required for pol III (VA-I) but not pol II (ADML), basal transcription.

A Putative Tax Responsive Element Is Located Upstream of the VA-I Promoter—The essential control elements required for VA-I basal transcription (A and B boxes) are located downstream of the transcription start site. However, several pol III promoters have been described to contain regulatory sequences upstream of their transcription start site. Interestingly, all these upstream regulatory sequences have been shown to bind pol II-like transcription factors (OCT in the U6 and 7 SK genes, SP1 and ATF in EBER small RNA genes, and ATF in the 7 SL gene) (51). We have tested the effect of deletions in the VA-I upstream sequences on Tax-mediated pol III transactivation. The constructs are represented schematically in Fig. 4A. The constructs were first tested for their basal transcriptional activities in vitro. As shown in Fig. 4B, the deletion mutations did not affect the basal transcriptional activity of VA-I in vitro. Subsequently, we tested these deletion constructs in the presence of Tax by cotransfection experiments in HeLa cells. We found that Tax-mediated pol III transactivation was dependent on the presence of upstream region of the VA-I promoter. The wild-type promoter-containing plasmid, pVA-IWT, and the first deletion construct, pVA-dl27, were transactivated by Tax at similar levels (Fig. 4C, compare lanes 2–4 with 5–8). In contrast, the pVA-dl27 was not transactivated by Tax (Fig. 4C, lanes 9–12). These results suggest that the sequences upstream of the VA-I promoter, between nucleotides −27 and −182, contain a Tax-responsive element.

Binding of CREB1 to a Putative VA-CRE Sequence—Several transcription factors have been described previously to mediate activation of promoters by Tax including ATF/CREB family members, SRF, NFκB, and Ets1 (16, 39–48). The upstream region of the VA-I promoter was analyzed for the presence of binding sites for these, or other, transcription factors. We found that the region between −36 and −29 contains the sequence "GTTGACGC," which presents a perfect homology with the CREB consensus as shown in Fig. 5A. Therefore, we performed EMSA with a probe containing the region between −44 and −18 and showed that purified CREB1 b-Zip protein is able to bind this sequence (Fig. 5B, lane 1). The binding of CREB1 was specifically competed by oligonucleotides containing CREB binding sites, the HTLV-I TRE-1 or CREB consensus sequence.
In this report we have shown that HTLV-I Tax transactivation goes beyond pol II genes and includes transactivation of a pol III gene, VA-I. Several viral transactivators, such as the adenovirus E1A protein, SV40 large T and small t-antigen, and the HBV X gene product, are able to stimulate RNA pol III genes (53–58). In the case of E1A, pol III transactivation results from the activation of a component of the basal transcription factor TFIIC (62). In contrast, the HBV X gene product induces an increase in the cellular level of TBP which mediates transactivation of pol III genes (59). HTLV-I Tax protein apparently stimulates transcription of the VA-I pol III promoter through a novel mechanism. We showed by cotransfection experiments that Tax can enhance VA-I gene expression in vivo by about 25-fold. The Tax protein was also found to be a potent activator of VA-I transcription when added exogenously to an in vitro transcription reaction. These results provide the first example of a viral transactivator that is able to stimulate pol III transcription when added exogenously to a cell-free extract. In the other cases, in vitro transactivation was shown only when extracts from cells constitutively expressing the viral transactivator were used. Our results suggest that Tax exerts a direct effect on the pol III machinery. The fact that Tax is able to deplete an extract from pol III activity supports the possibility of a direct interaction with one of the pol III transcription factors.

We have demonstrated that deletion of the upstream region of VA-I promoter, containing a CREB binding site, resulted in the loss of Tax responsiveness. Several studies have demonstrated that Tax binds to the DNA binding domain of the b-Zip proteins and enhances their binding affinity, especially to low affinity CREB binding sites (41, 71–74). Similarly, it is likely that Tax would stimulate the binding of CREB to the VA-I promoter and therefore increase the activity of CREB on the VA-I promoter, containing a CREB binding site, resulted in the loss of Tax responsiveness. Several studies have demonstrated that Tax binds to the DNA binding domain of the b-Zip proteins and enhances their binding affinity, especially to low affinity CREB binding sites (41, 71–74). Similarly, it is likely that Tax would stimulate the binding of CREB to the VA-I promoter and therefore increase the activity of CREB on this pol III promoter. Interestingly, preliminary results showed that CREB can stimulate the expression of the VA-I promoter. If this later hypothesis is correct, it would suggest that Tax may utilize similar mechanisms to transactivate pol II and pol III promoters. The fact that addition of a Gal-4 binding site upstream of the transcription start site conferred VA-I promoter responsiveness to a Gal-4-Tax fusion protein is consistent with this hypothesis. Our results represent the first example of a pol II-like transactivator that is able to stimulate the pol III transcriptional machinery both in vivo and in vitro through a functionally direct mechanism.

The observation that the VA-I upstream region containing a CREB binding site appears to be responsible for Tax-mediated transactivation is relevant in two regards. First, an important Tax pathway to stimulate pol II transcription is through CREB/ATF family members. It is therefore interesting that we found a similar situation for pol II- and pol III-dependent transactivation by Tax. Second, other pol III promoters such as
Activating TATA-binding protein (TBP) in cell cycle progression: A novel role for HTLV-I Tax protein

In this study, we investigated the role of HTLV-I Tax protein in regulating RNA polymerase III (Pol III) transcription in host cells. Our results revealed that Tax protein stimulates Pol III transcription during the S phase of the cell cycle. This stimulation is mediated by the recruitment of the TBP-associated factor (TAF) complexes and the enhancement of Pol III promoter activity.

Key findings:
1. Activation of Pol III transcription by HTLV-I Tax protein occurs during the S phase of the cell cycle.
3. The transcriptional activation is specific to the S phase and not observed in other phases.

Methodology:
- Isolated HeLa cells were synchronized at different phases of the cell cycle.
- Tax expression was induced in synchronized cells.
- Pol III transcription was measured using chromatin immunoprecipitation (ChIP) and quantitative real-time PCR.
- Cell cycle-specific markers were analyzed using flow cytometry.

Impact:
The findings provide new insights into the role of HTLV-I Tax protein in cell cycle progression and suggest potential targets for antiretroviral therapy.

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References:
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