Transactivation of the Human Interleukin-6 Gene by Human T-Lymphotropic Virus Type 1 Tax Protein

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Interleukin-6 (IL-6) is a multifunctional cytokine that regulates both humoral and cellular immune responses. Accumulating evidence suggests that the infection of T cells and other cell types with human T-lymphotropic virus type 1 (HTLV-1) results in the constitutive expression of IL-6. However, the underlying molecular mechanisms are little understood. When a reporter plasmid, pIL6-CAT-E3, in which the human IL-6 enhancer/promoter region from -630 to +14 was linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, was transfected, HTLV-1-infected but not uninfected T-cell lines activated the IL-6 promoter. This indicated the presence of a factor transactivating the IL-6 gene in the infected cells. To evaluate the involvement of the HTLV-1-encoded transacting factor (Tax) in this transactivation, we examined the effect of transient cotransfection with the Tax-expression plasmid, pMAX-Neo, on the transcription from the IL-6 promoter by use of COS1 cells. The cotransfected COS1 has about six times greater the CAT activity than that transfected with pIL6-CAT-E3 alone. The analysis of a series of deletions of the IL-6 promoter suggested that the region (-105/-47) containing a NF-xB site was crucial for the Tax responsiveness. We further examined the effect of Tax on endogenous IL-6 gene expression using the Jurkat clone, JXP-9, stably transfected with pMAX-Neo. JXP-9 accumulated steady state transcripts of the endogenous IL-6 gene in response to the induction of Tax expression. Our findings indicate an important role of the Tax protein in the expression of IL-6 in cells infected with HTLV-1.

HUMAN T-LYMPHOTROPIC virus type 1 (HTLV-1) is an etiologic agent of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Furthermore, the association of HTLV-1 with various inflammatory disorders including arthritis, uveitis, polyomylitis, Sjögren syndrome, and alveolitis has recently been suggested. Although the underlying pathogenic mechanisms of these HTLV-1-associated diseases are not fully understood, several cytokines including interleukin (IL)-1, IL-2, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF) are produced by HTLV-1-infected cells, which plays a central role in host defense mechanisms by regulating immune responses, hematopoiesis, and acute phase inflammatory reactions. Although this cytokine is physiologically not produced by human T lymphocytes, recent studies have suggested that infecting T cells with HTLV-1 results in an increase in the expression of IL-6. The constitutive expression of IL-6 is also induced in monocytes, microglia, and synovial cells by HTLV-1 infection. However, our understanding of the molecular mechanisms regulating the IL-6 expression in HTLV-1-infected cells is still incomplete. HTLV-1 encodes a transactivating factor (Tax), which activates not only the transcription of the viral genome but also the expression of various cellular genes. The target cellular genes of Tax so far identified include IL-2, IL-3, IL-4, tumor necrosis factor a (TNFa), transforming growth factor b (TGFb), IL-2 receptor a (IL-2Ra), GM-CSF, and c-fos. This study is the first to show direct evidence for the transactivation of human IL-6 gene by Tax.

**MATERIALS AND METHODS**

**Plasmids.** Reporter plasmids in which various fragments of the enhancer/promoter region of human IL-6 gene were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene were constructed as follows. Three fragments of the IL-6 enhancer/promoter region were amplified from genomic DNA of Jurkat cells by means of the polymerase chain reaction (PCR), using sense primers; S1, 5' GT GGT ACC CCC TAG TTT GTG TCT TGC 3' (−101/−82), S2, 5' G TGG TAC CCT CAC CCT CAAG 3' (−188/−169) and S3, 5' AT GGT ACC TGG AGA CCG GTT GAA 3' (−630/−613) in combination with an antisense primer; AO, 5' CGA GAG GGG AGA TAG AGC TTC 3' (−56/+37). The indicated nucleotide numbers were upstream (−) or downstream (+) of the cap site, and the location and orientation of the primers are shown in Fig. 1. The underlined sequences were added to create Kpn I sites at the 5' ends of PCR products. The products were digested with Kpn I and Xho I, one of which was located 14 bp downstream from the cap site of the IL-6 gene, then ligated to pBluescript KS(+) II. The resulting constructs, pIL-6−E1−, −E2, −E3, and −E4 contained the fragments corresponding to −101/−14, −188/−14, and −630/−14, respectively. Furthermore, pIL-6−E2ΔKB was constructed, in which a HaeIII-Ssp I fragment (−105/−47) containing a NF-xB motif was deleted from the pIL-6−E2. Finally, the BamHI-HindIII fragment of the CAT gene isolated from pSV2-CAT was inserted 51 bp downstream of the IL-6 enhancer/promoter sequences in each of four pIL6 constructs and the resulting reporter plasmids were designated pIL-6−CAT-E1, −E2, −E3, and −E4ΔKB (Fig 1). The pHTLV-1−CAT (pCHL4) contained the CAT gene, the expression of which was regulated by a minimal promoter. Plasmid pRSV-β-gal was used to standardize the transfection efficiency, because the promoter activity of the Rous sarcoma virus (RSV) LTR is not affected by HTLV-1 infection. The Tax expression plasmid pMAX-Neo contained a Tax coding sequence preceded by a murine metallothionein promoter unit, which is induced by heavy-metal ions.

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cells, HTLV-I-infected human T-cell lines, including MT-2 and TL-Su, and uninfected T-cell lines, Jurkat and Molt-4, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). JPX-9 (provided by Dr. Sugamura, Tohoku University) is a Jurkat subclone generated by the stable introduction of the Tax expression plasmid, pMAX-Neo. COS1 was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS.

**Transfection and CAT assay.** Closed circular plasmid DNAs were purified twice by equilibrium centrifugation in cesium chloride-ethidium bromide gradients, then transfected into COS1 by lipofection. Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD) mixed with 5 μg of a reporter plasmid in the presence or absence of 5 μg of pMAX-Neo in 3 mL of serum-free DMEM was added to semiconfluent cultures of COS1. After incubation at 37°C for 8 hours, the culture medium was replaced by DMEM containing 10% FBS. Plasmid DNA was transfected into the T-cell lines by means of diethyl aminoethyl (DEAE) dextran/osmotic shock. Briefly, cells (2.5 × 10⁶) were mixed with 2.5 μg of a reporter plasmid in the presence or absence of 2.5 μg of pMAX-Neo in 0.5 mL of RPMI 1640 medium containing 2 μg of DEAE-dextran. After an incubation at 37°C for 30 minutes, 0.5 mL of a high osmolar solution containing 2.8 mmol/L Tris-HCl, pH 7.3, 40.2 mmol/L sucrose, 2 mmol/L PEG 4000, and 8.4 mmol/L NaCl, was added and the mixtures were incubated at 37°C for 10 minutes. The cells were washed and cultured in RPMI 1640 with 20% FBS. CdCl₂ was added to COS1 and JPX-9 at a concentration of 30 μmol/L 24 hours after transfection to induce the expression of Tax protein. The pRSV-pRSV-β gal (5.0 μg) was cotransfected every time. Cells were harvested 48 hours after transfection and lysed by three cycles of freezing and thawing. Cell extracts (50 μL) containing 10 μg of cellular protein were mixed with 55 μL of 1 mol/L Tris-HCl, pH 7.18, 5 μL of [³²P]-ATP at the 5' termini. Hybridization proceeded in a solution containing 10% formamide at 37°C for 24 hours. After washing of the membrane at 37°C, hybridization signals were quantified by image analysis.

**Activation of the human IL-6 promoter in HTLV-I-infected T-cell lines.** To determine whether or not the mecha-
Fig 2. Both HTLV-1 LTR and the human IL-6 promoter are active in HTLV-1-infected but not in uninfected human T-cell lines. The reporter plasmids were transfected into the indicated cell lines and CAT activity in the cell extract was analyzed. After the difference in transfection frequency among the cell lines was standardized by β-galactosidase activities, the relative CAT activities in Molt-4, MT-2, and TL-Su were determined by a comparison with that in Jurkat. The assays were repeated three times to confirm reproducibility and a representative example is shown.

 Activation of the human IL-6 promoter by transient cotransfection with a Tax expression plasmid in COS1 cells. To determine whether Tax transactivates the IL-6 promoter, we examined the effect of transient cotransfection with the Tax-expression plasmid, pMAX-Neo, on transcription from the IL-6 promoter. The COS1 cell line was used because it can be efficiently transfected. COS1 cotransfected with pMAX-Neo and pIL6-CAT-E3 was cultured in the presence of CdCl2. As shown in Fig 3, cotransfected COS1 has about six-times greater CAT activity than that transfected with pIL6-CAT-E3 alone. The generation of functional Tax in the pMAX-Neo–transfected COS1 was confirmed by the evidence that transcription from HTLV-1 LTR was potently stimulated by transient co-transfection with pMAX-Neo. To roughly identify the Tax-responsive region, we generated two 5′ deletion constructs, pIL-6-CAT-E1 and -E2, in which the regions –101/+14 and –188/+14 were, respectively, linked to the CAT gene (Fig 1). Plasmids pIL-6-CAT-E1, -E2, and -E3 were transfected with or without pMAX-Neo into COS1 and the levels of Tax-mediated transactivation were estimated from the ratio between CAT activities in the presence and in the absence of Tax. As shown in Fig 1, all
the reporter plasmids, including the shortest pIL-6-CAT-E1 (−101/+14), appeared responsive to Tax. The levels of transactivation were almost equivalent and the relative values of the Tax-mediated CAT activity in pIL-6-CAT-E1, -E2, and -E3-transfected cells were 4.9, 3.9, and 5.7, respectively. This indicated that the region between −101 and +14 including a NF κB motif (−69/−57) contained a Tax-responsive element. To evaluate the contribution of this region, the sequence between −105 and −47 was deleted from the pIL-6-CAT-E2 and the Tax-responsiveness of the resulting construct, pIL-6-CAT-E2ΔKB (Fig 1), was tested. As shown in Fig 1, the Tax-mediated transactivation decreased to a level of 1.3.

Activation of the endogenous IL-6 gene in the stable Tax transfectant of Jurkat cell line, JPX-9. Because the results of the transient co-transfection experiments were not always applicable to endogenous genes, we further examined the effect of Tax on the expression of endogenous IL-6 gene using the Jurkat clone, JPX-9, stably transfected with pMAX-Neo. The expression of Tax protein in JPX-9 was induced by CdCl₂. Transcription from both the HTLV-I LTR and IL-6 promoter of the respective reporter plasmids, pHTLV-I-CAT and pIL6-CAT-E3, was activated in the CdCl₂-treated JPX-9 but not in Jurkat (Fig 4). However, IL-6 mRNA was undetectable in the culture fluid of JPX-9 by Northern blotting and the concentration of soluble IL-6 in the culture medium was below the sensitivity of the assay (<20 pg/ml), indicating that the IL-6 expression level was much lower than those in MT-2 and TL-Su. The expression of IL-6 mRNA in JPX-9 in the presence of CdCl₂ was therefore analyzed by more sensitive RT-PCR and Southern blotting. The GAPDH mRNA was concurrently amplified and the relative levels of the IL-6 mRNA were estimated by the ratio between the intensities of the hybridization signals of IL-6 and GAPDH (Fig 5). IL-6 mRNA was barely detectable in untreated JPX-9 cells, but CdCl₂ induced the expression of IL-6 mRNA. IL-6 mRNA was readily induced 3 hours after CdCl₂ was added. IL-6 mRNA accumulated thereafter and reached the maximal level, a 7.2-fold increase, within 24 hours. This induction did not occur in the parent Jurkat cells.

DISCUSSION

Production of IL-6 in HTLV-I-infected cells was first shown in an infected human T-cell line, TCL-Nal, from which IL-6 was purified and its gene was isolated. Since this discovery, evidence for the involvement of HTLV-I in the IL-6 gene expression has accumulated. Villiger et al showed the constitutive production of IL-6 by HTLV-I-infected T cells and cell lines. The elevated expression of the IL-6 mRNA was also noted in HTLV-I-infected T-cell lines. Furthermore, the IL-6 production by other cell types, such as monocytes, microglial and synovial cells, is enhanced by HTLV-I infection. By analogy with other cellular genes, the involvement of Tax in the expression of IL-6 in cells infected with HTLV-I has been speculated. Here, we presented that transient cotransfection with the Tax-expression plasmid activated the transcription from the human IL-6 promoter in COS1 cells. This finding directly indicates the potential of Tax for transactivating the human IL-6 gene. Moreover, the effect of Tax on the endogenous IL-6 gene was confirmed by the evidence that steady state IL-6 mRNA accumulated in the Jurkat clone, JPX-9, that was stably transfected with pMAX-Neo in response to induced Tax expression. These together indicate that Tax is involved in the constitutive expression of IL-6 in cells infected with HTLV-I.

Although the accumulation of IL-6 mRNA was obviously induced by Tax in JPX-9, the level appeared to be much lower than those in the cell lines productively infected with HTLV-I, namely MT-2 and TL-Su, and soluble IL-6 was undetectable in the culture fluid of JPX-9. The difference may simply reflect the levels of Tax expression in the cells. However, the levels of IL-6 promoter transactivation seem to be roughly equivalent among the cell lines (Fig 2 and 4). Moreover, TL-Su produces nearly 10 times more soluble IL-6 than MT-2, despite their similar transactivation levels of both the HTLV-I LTR and the IL-6 promoter. These findings
indicate that factors of either viral or cellular origin other than Tax are also involved in the constitutive expression of IL-6 in HTLV-1-infected cells, probably at the posttranscriptional level. Many of the genes encoding cytokines including IL-6, contain AU-rich sequences in the 3′ untranslated region of their mRNAs, which decreases the half-life of the mRNAs and causes transient expression of these genes under physiologic conditions. HTLV-1 infection may alter the physiologic metabolism of IL-6 mRNA in cells. Such alteration may result in the constitutive IL-6 expression in coordination with transcriptional activation by Tax.

Tax is located predominantly in the nucleus, but is not capable of binding DNA directly. It is considered to function by inducing or modifying the activity of certain host transcription factors. In the IL-6 promoter region, a number of cis-acting elements, including NF κB, CREB, NF-IL6, and AP-1 binding sites, have been identified (Fig 1). One or a combination of these elements could be involved in the Tax-mediated transactivation. Our deletion analysis indicates that a Tax-responsive element is localized between 105 and 47 bp upstream of the transcription initiation site. A role of the NF κB motif present in this region is strongly suggested. The finding that synthetic oligonucleotides, GGGATTTT-CCC, spanning the NF κB motif of the IL-6 gene is responsive to Tax supports this argument. NF κB is implicated in the Tax-mediated transactivation of various other genes, including those encoding IL-2 and IL-2Rα.

The major form of NF κB consists of 50- and 65-kDa subunits (p50 and p65), both of which belong to the Rel family. NF κB is present in the cytoplasm in the inactive form complexed with inhibitory proteins, I κB. Various stimuli, such as phorbol ester, bacterial lipopolysaccharide, IL-1, and TNFα, result in the dissociation of NF κB/I κB complex and the translocation of NF κB to the nucleus. Tax physically associates with the 105-kD NF κB precursor, p105, which acts as an I κB. Interaction with Tax could activate NF κB, which subsequently binds to the NF κB motif of the IL-6 gene. However, IL-6 is not physiologically produced by T lymphocytes, which are the major target cells of HTLV-1 infection. Moreover, the NF κB motif of the IL-6 gene is unresponsive to TNFα in Jurkat cells, although both Tax and TNFα are equally effective in the NF κB-mediated activation of the IL-2Rα gene in these cells. The expression of IL-6 may be negatively regulated through the NF κB motif in T-lymphoid cells. The mechanisms through which Tax overcomes such regulation, remain to be elucidated.

HTLV-1 causes a wide spectrum of diseases including ATL, HAM/TSP, and inflammatory disorders of various organs. Tax is considered to play a central role in the pathogenesis of these HTLV-1-related diseases. Transgenic mice overexpressing Tax protein develop tumors of various tissues and diseases quite similar to Sjögren syndrome and rheumatoid arthritis, the pleiotropic IL-6 aberrantly produced through Tax-mediated transactivation is likely to play an important pathogenic role in HTLV-1 infection.

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REFERENCES

24. Gorman CM, Merlino GT, Willington MC, Pastan I, Howard BH: The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells. Proc Natl Acad Sci USA 79:6777, 1982
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