Tax protein of human T-cell leukaemia virus type 1 induces interleukin 17 gene expression in T cells

Madeleine Duc Dodon,1 Zhenlin Li,2 Samir Hamaia1 and Louis Gazzolo1

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INTRODUCTION

Human T-cell leukaemia virus type 1 (HTLV-1), first identified as the aetiological agent of adult T cell leukaemia, was subsequently linked to an inflammatory neurological disease, termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame & Igata, 1989; Poiesz et al., 1980; Yoshida et al., 1982). Furthermore, this human retrovirus has been associated with other inflammatory disorders such as arthritis, uveitis, polymyositis, Sjögren syndrome and alveolitis (Green et al., 1989; Mochizuki et al., 1992; Motokawa et al., 1996; Nishioka et al., 1992). Numerous investigations devoted to the study of the underlying mechanisms of these two different clinical manifestations have underlined the tropism of HTLV-1 for CD4+ and CD8+ T cells in vivo (Grant et al., 2002). In these cells, the provirus is believed to be transcriptionally silent (Bangham, 2000). However, a significant proportion of infected T cells from HAM/TSP patients as well as from asymptomatic HTLV-1 carriers express the Tax protein within a few hours of culture ex vivo (Hanon et al., 2000). Interestingly, the main viral mRNAs detected in these cells were those transcribed from a unique sequence called pX within the proviral genome. One of the open reading frames of this sequence encodes a 40 kDa trans-acting factor known as Tax, which functions in association with the activating transcription factor/CRE-binding protein (ATF/CREB) to enhance virus transcription (Zhao & Giam, 1992). In addition, Tax regulates transcription of many cellular genes through a number of cis-acting DNA elements including the cAMP-responsive element (CRE), the serum-responsive element and NF-kB binding sites, leading to cellular activation and transformation (Fuji et al., 1992; Gatza et al., 2003; Yoshida, 2001). Finally, sustained expression of Tax is thought to cause severe immune dysregulation linked to HAM/TSP. Indeed, several cytokines involved in growth and inflammatory reactions were found to be either induced or upregulated by Tax expression in HTLV-1-infected T cells in vitro (Matusaka et al., 1993; Nishiura et al., 1996; Saib et al., 1993; Villiger et al., 1991).

Human interleukin 17 (IL17), originally identified as cytolytic T lymphocyte-associated antigen 8 (CTLA-8), displays a significant homology with the T-lymphotropic herpesvirus saimiri gene 13 product (HVS13) (Rouvier et al., 1993; Yao et al., 1995a). This cytokine is a 20–30 kDa homodimeric protein, encoded by a 155 aa open reading frame that includes an N-terminal secretion signal sequence of

1Present address: Department of Biochemistry, University of Cambridge, UK.

Tax protein of human T-cell leukaemia virus type 1 (HTLV-1) induces the expression of several cellular genes that are involved in T cell activation and proliferation. In this study, it was observed that Tax upregulated the expression of human interleukin 17 (IL17), a cytokine mainly produced by activated CD4+ memory T cells. Indeed, IL17 mRNA was highly expressed in HTLV-1-infected T cells as well as in Tax-expressing Jurkat T cells, whereas it was not detectable in HTLV-1-negative T cell lines. The clinical relevance of these observations was further demonstrated by quantitative assessment of IL17 expression in lymphocytes isolated from one HTLV-1-infected patient. To define the transcriptional activation of the IL17 gene by Tax, the 5′-flanking region of this gene was cloned and a reporter gene analysis performed. The presence of a Tax-responsive region spanning 614 bp upstream of the initiation start site was identified, in HeLa as well as in Jurkat cells, stimulated with phorbol myristate acetate and Ca2+ ionophore. Finally, Tax mutants were used to show that the transcriptional activation of the IL17 promoter by Tax was dependent on the CREB/ATF pathway. As IL17 upregulates the expression of several pro-inflammatory cytokines, these observations provide new insights into the involvement of the Tax protein in the pathophysiology of HTLV-1-associated inflammatory disorders.
19–23 residues. IL17 expression is induced only in activated T lymphocytes (predominantly of the CD4+ subtype), cultured either with monoclonal antibodies to CD3 and CD28 molecules or with phorbol myristate acetate (PMA) and Ca2+ ionophore (Fossiez et al., 1996; Jovanovic et al., 1998). The human IL17 receptor, identified as a type 1 transmembrane protein, is ubiquitously expressed and has no sequence similarity to any other known cytokine receptors (Moseley et al., 2003; Yao et al., 1997). Such a wide distribution explains the broad set of effects induced by IL17. Thus, this cytokine induces the release of cytokines and prostaglandins from different cell types including fibroblasts, endothelial cells, epithelial cells and macrophages (Fossiez et al., 1996; Jovanovic et al., 1998). Furthermore, both TNF-α and IFN-γ exert a synergistic effect on the IL17-induced secretion of IL6, and the combination of IL17 and TNF-α is effective in promoting granulocyte-macrophage colony-stimulating factor (GM-CSF) release by synovial fibroblasts (Chabaud et al., 2000; Fossiez et al., 1996; Yao et al., 1995a, b). Consequently, IL17 may be considered as another factor in the cytokine network involved in inflammatory reactions. Finally, IL17 has been linked to numerous inflammatory pathologies, such as rheumatoid arthritis (Kotake et al., 1999), asthma (Kawaguchi et al., 2001; Molet et al., 2001) and lupus (Wong et al., 2001). Recently, this pro-inflammatory cytokine has been found to promote angiogenesis and tumour growth (Numasaki et al., 2003), as well as anti-tumour immunity (Benchetrit et al., 2002).

In the present study, we examined the expression of IL17 by HTLV-1-infected T cells and evaluated the effect of Tax on the transcription of this cytokine gene. We have shown that expression of IL17 mRNA is induced in HTLV-1-infected T cell lines and in T cells expressing Tax. The 5’-flanking region of the human IL17 gene was isolated and the transcription initiation site was determined. We demonstrated that this region contained a functional promoter by transfecting chimeric reporter constructs into HeLa cells. We showed that signals induced by the addition of PMA and Ca2+ ionophore in combination with Tax expression in Jurkat T cells transiently transfected with these plasmids stimulated the activity of the IL17 promoter. Finally, we identified two regions of the promoter fragment through which Tax was able to transactivate the IL17 gene in these activated T cells.

**METHODS**

**Cells.** The *in vitro* HTLV-1-transformed C91PL, MT2 and HUT-102 cell lines have been described previously (Popovic et al., 1983; Salahuddin et al., 1983). The non-infected Jurkat T cell line was obtained from the American Type Culture Collection (ATCC). IPX-9 cells are derivatives of Jurkat cells, which have a stably integrated *tax* gene under the control of a metallothionein promoter (kindly provided by M. Nakamura, Tohoku University, Sendai, Japan) (Nagata et al., 1989). Cells were incubated at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 μl penicillin ml⁻¹ and 20 μg streptomycin ml⁻¹. To induce Tax expression, the IPX-9 cells were cultured in the presence of 10 μM CdCl3. Peripheral blood lymphocytes (PBLs) were isolated by Ficoll-Paque (Amersham Pharmacia Biotech) density centrifugation of freshly collected heparinized blood from healthy donors. Adherent cells were removed after incubation of the cells for 1 h at 37°C on a plastic Petri dish (Falcon). Non-adherent cells seeded at 2 × 10⁶ cells ml⁻¹ were cultivated in complete RPMI and then stimulated with a mixture of 50 ng PMA ml⁻¹ and 1 μM Ca²⁺ ionophore. HeLa cells, a human epithelial carcinoma line, were grown in minimum essential medium (Invitrogen) supplemented with 10% FCS, 20 μl penicillin ml⁻¹ and 20 μg streptomycin ml⁻¹.

**Plasmids.** The HTLV-1 Tax expression plasmid pTax (kindly provided by W. C. Greene) contained the *tax* sequence under the control of the cytomegalovirus (CMV) promoter. The pCMV plasmid carrying only the CMV promoter was used as the control for all experiments involving the CMV promoter. The M47 and M22 Tax mutant plasmids have been described previously (Smith & Greene, 1990). The LTR–CAT reporter construct contained the chloramphenicol acetyl transferase (CAT) sequence under the transcriptional control of the HTLV-1 LTR. The pRSV-SEAP construct contained the secreted alkaline phosphatase sequence under the control of the rous sarcoma virus (RSV) promoter (a gift from B. C. Cullen). The pBS-IL17 plasmid was constructed by inserting the 450 bp sequence of IL17 into the EcoRI/BamHI sites of the BlueScript plasmid and was used for the preparation of a riboprobe by an *in vitro* transcription assay.

**RNA extraction, RNase protection assay and amplification by RT-PCR.** RNA extraction was performed after isotonic lysis of the cells in the presence of 0.5% NP-40 (Chomczynski & Sacchi, 1987). Cytoplasmic RNAs (10 μg) were hybridized for 12 h at 42°C to 100 000 c.p.m. of the labelled RNA probe prepared by transcription of the antisense sequence of pBS-IL17 by T3 RNA polymerase (Promega) according to the manufacturer’s protocol. Hybridization was performed in a 20 μl final volume of hybridization buffer (80% formamide, 0.4 M NaCl, 0.4 M Pipes pH 6.4, 1 mM EDTA). The hybridization reactions were diluted with 200 μl digestion buffer containing 15 U RNase I (Promega) for 60 min at 30°C. Reactions were stopped by the addition of 0.5% SDS and precipitated in ethanol. The protected RNA fragments were analysed on a 5% polyacrylamide gel containing 8 M urea and TBE buffer and visualized by autoradiography of the dried gel using an overnight exposure.

For RT-PCR, RNA (2 μg) was first treated with 10 U RNase-free DNase I (Roche Molecular Biochemicals) for 30 min at 37°C and then for 15 min at 65°C. This RNA sample was then reverse-transcribed at 42°C for 1 h in a total volume of 20 μl reaction buffer (50 mM Tris/ HCl pH 8.3, 30 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol) containing 50 U Expand reverse transcriptase (RT) (Roche Molecular Biochemicals), 100 pmoles oligo(dT)₁₁₋₁₉ (Sigma) and 20 × U RNasin (Promega). A reaction without RT was performed in parallel to serve as a control for the absence of DNA contamination. Five microlitres of the cDNA product was amplified by PCR in a final 50 μl reaction volume containing 20 pmol of gene-specific oligonucleotide primers, 1 mM dNTPs and 2 U mixed thermostable DNA polymerases (Roche Molecular Biochemicals). This mixture was incubated for 30–40 cycles at a 55°C annealing temperature, according to the manufacturer’s instructions. The following primers were used: IL7, 5'-ATGACTCTCCGGGAACACCTCCATGTG-3' (sense) and 5'-TTAGGCAATAGGTGGGACGAC-3' (antisense), generating a 468 bp fragment; IL6, 5'-TCAATGAGGAGACTTGCCTG-3' (sense) and 5'-ATAGTTGGTATCGTCTTGCG-3' (antisense), generating a 260 bp fragment; IL8, 5'-TGGCGAGGTCCTTCTGATT-3' (sense) and 5'-AATCTCTCACAGCCCTCTG-3' (antisense), generating a 248 bp fragment; Tax, 5'-TTGGTGGACACTGTGTAACAGGCG-3' (sense)
and 5′-GTTGTATGATGATTTGCGGTTA-3′ (antisense), generating a 237 bp fragment; and β-actin, 5′-TTGGAAAGAGGG-3′ (sense) and 5′-GGATGAGACAGATGTC-3′ (antisense), generating a 247 bp fragment. Ten microlitres of serial dilutions from each RT-PCR product was analysed by electrophoresis in a non-denaturing 1% agarose gel containing 0.5 µg ethidium bromide ml⁻¹. The gels were then blotted on to a positive nylon membrane (Amersham) for 30 min and hybridized with a 32P-labelled IL17 probe prepared from pBS-IL17 or with an appropriate probe containing the IL6, IL8, tax or β-actin sequence. Labelled DNA products were visualized by autoradiography (Hyperfilm; Amersham).

The intensity of signals was monitored by laser densitometry. For each sample, the amount of mRNA was quantified relative to the respective level of β-actin mRNA. Data were obtained utilizing the following formula: relative mRNA = [optical density (OD) cytokine mRNA transcripts of the sample/OD β-actin transcripts of the sample] [OD cytokine mRNA transcripts of unstimulated cells/OD β-actin transcripts of unstimulated cells].

Real-time quantitative PCRs for IL17 and HTLV-1 Tax/Rex mRNA were performed on a LightCycler System (Roche) using the LightCycler-FastStart DNA Master SYBR Green I kit and the following primers: IL17, 5′-AAACACAGTGACTCTGCGG-3′ (sense) and 5′-GAGACATTTTTGAGATTG-3′ (antisense); RPX3, 5′-ATCCGTTGGGAACACTTCA-3′; and RPX4, 5′-CCTTTGACAGTACGATGTCGATC-3′. The porphobilinogen dehydrogenase (PBGD) gene was amplified as a housekeeping gene to normalize patterns of gene expression using the PBGD primers 5′-GGATGAGACAGATGTCG-3′ (sense) and 5′-CAAGACATTTTTGAGATTG-3′ (antisense). In brief, reactions were performed in a 20 µl final volume and contained 1.5 µl FastStart reaction mix SYBR Green I, 4 mM MgCl₂, 0.5 µM primers and 2 µl cDNA. The reaction conditions were 95 °C for 8 min, followed by 45 cycles of 10 s at 95 °C, 5 s at 61 °C and 10 s at 72 °C. Calibration curves were derived by running two- to threefold serial dilutions of cDNA obtained from a positive cell line. Cellular cDNA samples were run at several dilutions. Controls included RT⁻ RNA samples and water. The threshold cycle values (Ct) were used to plot the calibration curve. Standard curves had a coefficient of variation of at least 0.98. The copy numbers were normalized to the human PBGD values measured in a separate tube. All copy numbers derived were the results of at least two determinations in duplicate.

Isolation of a genomic fragment containing the IL17 5′-flanking region and construction of human IL17 reporter plasmids. The isolation of the genomic fragment containing the IL17 5′-flanking region was performed by PCR screening of a human P1 library (Genome Systems). The primers used were 5′-CACAGTCCCTCCTGCAGCCG-3′ (sense) and 5′-ACCTCCTGTGTGGAAAGAGGG-3′ (antisense). One clone was obtained and subsequently subcloned into the Bluescript vector. Finally, a clone containing a 6.6 kb EcoRI insert with 4.6 kb 5′-flanking sequence and 2 kb of coding and intronic sequences of the human IL17 gene was isolated and sequenced. The 5′-deletion mutants were prepared by insertion of a series of 5′-deletion mutants (from −1043 to −85 fragment) upstream of the bacterial CAT reporter gene in the pBLCAT plasmid. Sequencing of these mutants was carried out by the dyeoxy chain termination method (Sanger et al., 1977) using a Sequenase sequencing kit.

Analysis of the IL17 transcription initiation site by 5′ rapid amplification of cDNA ends (RACE). Mapping of the IL17 transcription initiation site by RACE was performed using a commercial kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Total mRNAs isolated from human PBLs and from HUT-102 cells by standard techniques were submitted to reverse transcription using a reverse primer (24-mer) located at the end of exon 3 (5′-TTAGCCACATGTTGGCACAATCGG-3′). The 3′-tailed cDNA was submitted to 30 rounds of PCR amplification using an oligo(dT) sense primer and a reverse primer (5′-TGGATGGGGCAGAGTTCATGTC-3′) followed by a nested PCR using a reverse primer located on exon 2 (5′-AGATTCCAGGTGAGTGGATCGG-3′). PCR included a denaturation step at 96 °C for 30 s, an annealing step at 55 °C for 30 s and an elongation step at 72 °C for 1 min, after an initial denaturation at 94 °C for 5 min, and was followed by a 10 min elongation. The PCR products were separated by agarose gel electrophoresis, purified and sequenced by the dyeoxy chain termination method.

Transfection, CAT and SEAP assays. HeLa cells seeded at 6 × 10⁴ cells per well of a 12-well plate were transfected 20 h later, using the Fugene transfection reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. The respective concentrations of plasmid DNA used in each transfection assay (which was performed in triplicate) are given in the relevant figure legends, pRSV-SEAP (40 ng) always being included as an internal control. Two days after transfection, cells were harvested and cell pellets were resuspended in 60 µl lysis buffer (100 mM Tris/HCl pH 7.8, 0.7 % NP-40) and lysed by five repeated freeze–thaw cycles and analysed for CAT activity. The obtained values were normalized for transfection efficiency related to values of SEAP activity, determined using a chemiluminescent reporter kit (Aurora-NEN).

Transfection of the Jurkat T cells was performed by a DEAE–dextran procedure with chloroquine treatment (Grosschedl & Baltimore, 1985). Briefly, the CAT reporter constructs (1.5 µg) were co-transfected into Jurkat cells (2 × 10⁶ cell) with pcTax (200 ng) or empty vector (pCMV) as control. The transfected cells were divided into aliquots and incubated for 24 h at 37 °C. One aliquot was further treated with 50 ng PMA ml⁻¹ (Sigma) and 1 µM Ca²⁺ ionophore (A23187; Calbiochem) for 6 h. Cells were then harvested and centrifuged. Cell pellets were lysed in 60 µl lysis buffer by five repeated freeze–thaw cycles. Cell extracts were normalized for protein content using the Bradford technique before assays for CAT activity (Neumann et al., 1987), CAT results are the mean value of three experiments performed in triplicate ± SD.

RESULTS

Detection of IL17 mRNA in HTLV-1-infected and uninfected T cells

To determine whether HTLV-1 infection induces the expression of IL17, we first performed ribonuclease protection analysis of total RNAs isolated from untreated PBLs, PBLs activated with PMA and Ca²⁺ ionophore, or HTLV-1-infected T lymphocytes (HUT-102). Whereas IL17 mRNAs were not detected in unstimulated PBLs (Fig. 1A), these transcripts were observed as early as 2 h after addition of PMA and Ca²⁺ ionophore and increased in cells stimulated for 4 and 6 h. IL17 mRNAs were found to be abundant in HUT-102 cells. Next, to delineate whether IL17 expression correlated with Tax expression, cytoplasmic RNAs isolated from HUT-102 cells, from two other HTLV-1-infected T cell lines (MT2, C91PL) and from HTLV-1-negative Jurkat T cells were analysed in a semi-quantitative RT-PCR assay for the expression of IL17, Tax and actin. IL17 transcripts were not found in Jurkat cells (Fig. 1B). Conversely, they were exclusively detected in the three HTLV-1-infected T cell lines that also expressed Tax mRNAs. These observations, which
provide evidence for the constitutive expression of IL17 mRNA by HTLV-1-infected T cells, indicate that Tax might be involved in this IL17 expression.

**HTLV-1 Tax induces IL17 mRNA expression**

To investigate the effect of Tax on the induced expression of IL17, we examined IL17 mRNA expression in JPX-9 cells, a subclone of Jurkat cells, in which the tax gene is under the control of an inducible metallothionein promoter responsive to heavy-metal ions. In addition, we looked for the expression of two other pro-inflammatory cytokines, IL6 and IL8, known to be upregulated by Tax. Expression of these cytokines was assayed by RT-PCR performed on cytoplasmic mRNAs obtained from cells cultivated for 24 h either in medium alone or in medium containing 20 µM CdCl₂. Under these conditions, Tax mRNA was not detected in untreated cells (Fig. 2A). These cells, which were found to express IL6 and IL8, did not express IL17. In contrast, Tax mRNA was detected after addition of CdCl₂. In these Tax-expressing cells, levels of IL6 and IL8 expression increased as shown by a respective 1.8-fold and 2.9-fold increase when compared with untreated cells (Fig. 2A and B). Likewise, Tax and IL17 mRNAs accumulated during the 24 h treatment and reached a level that was 11-fold and eightfold that of actin mRNAs (Fig. 2B). Consequently, Tax expression appears to be associated with the transcriptional induction of the IL17 gene.

**Structure of the 5’-flanking region of the human IL17 gene**

To analyse the regulatory mechanism of IL17 gene transcription, we cloned the promoter region of the IL17 gene.
One clone, which contained 4 kb sequences upstream of the ATG initiation codon for IL17, was identified and sequenced. This 4 kb 5′-flanking region, as well as four 5′ serial deletions of this region (−1663, −1283, −1043 and −814 bp), were isolated and ligated to the CAT reporter gene. CAT activity was then examined with or without a Tax expression vector in HeLa cells. In the absence of Tax, a weak promoter activity was detected with each reporter plasmid (Fig. 3A). Tax expression (as assessed by immunoblotting; data not shown) was found to induce promoter activity of these constructs. Furthermore, these results showed that most of the promoter activity was associated with the −1043 reporter plasmid.

To show that the transcription initiation site was contained in this fragment, we then performed a 5′ RACE analysis of total mRNAs isolated either from human T lymphocytes activated with PMA and Ca²⁺ ionophore or from HUT-102 cells. A major transcription initiation site was located 57 nt upstream of the translation initiation codon, as indicated by the 5′-untranslated sequence obtained from both PBL and HUT-102 cells (Fig. 3B). Furthermore, sequences related to a typical TATA box were found approximately 30 bp upstream of this site. Finally, the nucleotide sequence of the 1043 bp fragment upstream of the major site was determined (Fig. 3C). Comparison of this IL17 5′-flanking nucleotide sequence with the non-redundant nucleotide databases and the eukaryotic promoter database using BLAST revealed no homology to any gene or promoter sequences described. This 5′-flanking sequence was mapped to human chromosome 6. Furthermore, potential transcription factor binding sites were identified by the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH) based on the TRANSFAC databases (Heinemeyer et al., 1998) with a threshold of over 80.

Transactivation of the IL17 promoter by Tax

To examine the promoter activity of each subregion in the regulatory region, a series of eight 5′-deletion mutants (−814, −614, −461, −351, −264, −191, −135 and −85 fused to CAT) was generated from the −1043 bp fragment. Each of these reporter constructs was first co-transfected into HeLa cells in combination, with or without a Tax expression plasmid. In the absence of Tax, the low basal activity observed for each deletion construct was not significantly different from that observed with the pBL-CAT construct (Fig. 4, white boxes). In presence of Tax, the IL17/CAT reporter plasmids spanning from −1043 to −614 and from −351 up to the transcription initiation site were activated approximately 7- to 24-fold by Tax, underlining that these two regions of the IL17 promoter include Tax-responsive elements (Fig. 4, black boxes). The CAT activity reached a maximum value with the two shortest deletion constructs (−135 and −85), delineating a minimal Tax-responsive region upstream of the transcription initiation site. Furthermore, with the −461/CAT construct, a large decrease in CAT activity was observed, underscoring the fact that the 110 bp sequence from −461 to −351 might contain inhibitory elements. Collectively, these data revealed that Tax expressed in HeLa cells activates the transcription

Fig. 2. Induction of IL17 mRNA expression in JPX-9 cells treated with CdCl₂. (A) RT-PCR analysis of IL17 mRNA in JPX-9 cells treated with 20 μM CdCl₂. PCR analysis of AMV-retrotranscribed mRNA (2 μg) in JPX-9 cells cultured in medium alone (left panel) or medium containing CdCl₂ (right panel) for 24 h was performed with the appropriate oligonucleotide primer pairs described in Methods. Different dilutions (1:1, 1:2, 1:10, 1:20, 1:50, 1:100 and 1:200; lanes 2–8, respectively) of the amplified products were electrophoresed, blotted and hybridized with an α-32P-labelled IL17 probe. Labelled DNA products were visualized by autoradiography. Autoradiograms of PCR amplification of retrotranscribed Tax, IL6, IL8 and actin mRNAs are shown as controls. Lane 1 represents a control in which an RNA-free sample was treated like the extracted samples. (B) Densitometric analysis of RNA levels of JPX-9 cells, either CdCl₂ treated (black boxes) or not (white boxes). The relative ratio of each mRNA tested was calculated as described in Methods.
Fig. 3. The regulatory region of the human IL17 gene. (A) Tax-dependent promoter activity of the IL17 gene. HeLa cells were transfected with CAT reporter plasmids (−4014, −1663, −1043, −1283 and −814 bp fragments) without (white bars) or with a Tax expression plasmid (black bars) and examined for CAT activity after 36 h of culture. Restriction sites in the 5'-flanking region, first exon and translation initiation codon are shown. (B) Mapping of the transcription initiation site of the IL17 mRNA, determined by 5' RACE PCR analysis of total mRNAs isolated either from human PBLs activated with PMA and Ca²⁺ ionophore (lane 2) or from HUT-102 cells (lane 3). After reverse transcription and PCR amplification (see Methods), PCR products were separated on agarose gels. (C) Nucleotide sequence of the 5'-flanking region of the human IL17 gene. The nucleotide sequence from the BamHI site to the translation initiation codon (double-underlined) is shown in capital letters. The TATA sequence is underlined. The first 22 nt of exon 1 are shown in lower-case letters. The potential transcription factor binding sites (indicated by boxes) were identified by the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH) based on the TRANSFAC databases (Heinemeyer et al., 1998) with a threshold of over 80. The corresponding transcription factors have been shown to be involved in the modulation of gene expression by Tax. CREB, cAMP-responsive element (CRE)-binding protein; C/EBP, CCAAT/enhancer binding protein; E-box, bHLH binding site; AP1, activator protein 1; c-Ets, c-E twenty-six.
of the IL17 gene, through cooperation with transcription factors that bind to specific sites within two regions, one of 153 bp from −614 to −461, the other of 135 bp upstream of the transcription initiation site. Next, the transcriptional activity of the IL17 promoter was evaluated in Jurkat T cells. In the absence of Tax, a weak CAT activity was observed with two of the upstream reporter constructs, −814 and −614 (Fig. 5A, white boxes). In the presence of Tax, no significant enhancement of the CAT activity could be observed (Fig. 5A, black boxes). Consequently, we next investigated whether activation of the IL17 promoter by Tax in Jurkat T cells requires the addition of PMA and Ca\(^{2+}\) ionophore. When Jurkat cells transfected with each of the reporter plasmids were then stimulated with PMA and Ca\(^{2+}\) ionophore (in the absence of Tax), a significant increase of CAT activity was observed only with three upstream constructs as well as with the −135 reporter plasmid (Fig. 5B, white boxes). When Jurkat cells, co-transfected with the Tax vector and the serial deletion constructs, were then stimulated with PMA and Ca\(^{2+}\) ionophore, a 7-5- to 12-5-fold increase in CAT activity was observed with these three upstream constructs (black boxes).

The CAT activity, which significantly decreased with the −461 reporter plasmid, increased thereafter to reach a maximal value with the −135 construct. These observations indicated that, in Jurkat T cells, stimulation with both PMA and Ca\(^{2+}\) ionophore together with Tax markedly upregulate IL17 promoter activity. They further showed that the 614 bp fragment of the IL17 5′-flanking region contributes to an efficient transactivation of IL17 gene expression.

**The Tax transactivation of the IL17 gene is mediated through the CREB/ATF pathway**

As shown in Fig. 3(C), transcription factors involved in the Tax-induced upregulation of several cellular genes were identified by their binding sites on the 1043 bp region of the IL17 promoter (Blumenthal et al., 1999; Hiraïwa et al., 2003; Li-Weber et al., 2001; Yamagata et al., 1997). In particular, the presence of several CREB binding sites suggested that these transcription factors may be linked to the transactivation of the IL17 gene by Tax. To investigate their potential role in Tax-induced IL17 transactivation, we used two Tax mutants, M22 and M47. M22 is unable...
to activate gene expression via the NF-κB, but not the CREB/ATF pathway. M47 is defective in activation of gene expression via the CREB/ATF but not the NF-κB pathway. We first verified the transcriptional activity of these Tax mutants. Thus, HeLa cells were transfected with an HTLV-1 LTR–CAT reporter, which contained three unique CRE sites known as 21 bp repeats, together with either the wild-type or each Tax mutant. As expected, the wild-type Tax as well as the M22 mutant could activate CAT expression from the HTLV-1 LTR, whereas the M47 mutant was found to be unable to activate gene expression from the HTLV-1 LTR (Fig. 6, left panel). Next, HeLa cells were transiently transfected with the −614 IL17–CAT reporter construct together with either the wild-type or each Tax mutant (Fig. 6, right panel). Whereas the wild-type Tax and the M22 mutant were found to activate gene expression equally, a significant decrease in CAT activity was observed with the M47 mutant, clearly underlining that the transcriptional ability of Tax to transactivate the IL17 promoter might be dependent on the CREB/ATF pathway.

IL17 expression in cells from HAM/TSP patients

It has been previously shown that fresh peripheral blood mononuclear cells from HTLV-1-infected patients expressed detectable levels of Tax after in vitro culture. To confirm and extend the above observations, we quantified Tax and IL17 mRNA expression patterns by real-time RT-PCR in T lymphocytes from a HAM/TSP patient and from an HTLV-negative healthy individual (Table 1). As expected, Tax expression was not detected in T lymphocytes from the uninfected donor, cultured for 3 days in medium containing either IL2 or PMA and Ca^{2+} ionophore. By contrast, Tax expression was readily observed in T lymphocytes from the HAM/TSP patient cultured for 3 days in medium containing IL2. IL17 expression was very low in unstimulated T lymphocytes, but was induced after addition of PMA and Ca^{2+} ionophore (a 212-5-fold induction within 4 h) and then declined after 20 h. By contrast, IL17 mRNA expression in T lymphocytes from the HAM/TSP patient was found to be 17-fold higher than that observed in unstimulated HTLV-1-negative T lymphocytes cultured in presence of IL2. Interestingly, the IL17/Tax mRNA ratio in lymphocytes from the HTLV-1-infected patient was found to be quite similar to that in HTLV-1-infected HUT-102 cells (6-8 and 7-3, respectively). In conclusion, these results indicated that an increase in IL17 mRNA expression in cells from HAM/TSP patients correlated with Tax expression.

DISCUSSION

After cloning the human IL17 cDNA, Northern blot analysis has revealed that the IL17 gene is expressed as a
1.9 kb mRNA only in activated CD4<sup>+</sup> T cells and that this transcript could not be found in any human tissue. In this study, we showed that IL17 mRNA was detected in HTLV-1-infected T cells and that Tax expression was associated with the expression of this cytokine gene in these cells. Indeed, IL17 mRNA was not detectable in uninfected T cell lines, such as Jurkat cells. Conversely, in the JPX-9 cell line, a derivative of Jurkat cells in which a tax cDNA is placed under the control of an inducible promoter, the induction of Tax expression correlated with the detection of IL17 mRNA. These observations were clinically relevant as IL17 expression was related to the level of Tax expression in T lymphocytes obtained from a HAM/TSP patient after in vitro culture. Furthermore, to understand the regulatory mechanisms that control IL17 expression, we proceeded to clone the sequences upstream of the ATG site and analyse the promoter activity of this region in HeLa cells as well as in Jurkat cells. Whereas the human IL17 gene was originally mapped, by in situ hybridization, to human chromosome 2q31 (Fossiez et al., 1996), we observed that both the IL17 sequences and the upstream region were found in chromosome 6p12.1–21 (GenBank accession no. CAB 75300). Furthermore, RACE analysis, performed with activated T cells and one HTLV-1-infected T cell line, revealed the presence of a transcriptional start site, located 57 bp upstream of the ATG codon. By using serial deletion mutants, we then defined the IL17 gene promoter region that is transactivated by Tax. In HeLa cells, we found that a 1043 bp region upstream of the transcriptional start site contained two domains responsive to Tax. The upstream domain was located between −1043 and −614, while the downstream domain extended from −351 to −85. Inside each domain, a minimal Tax-responsive

**Table 1. Quantification of IL17 gene expression in human T lymphocytes**

T lymphocytes were isolated from peripheral blood of an HTLV-1-negative healthy control and cultivated in complete medium with IL2 alone for resting cells or stimulated with PMA and Ca<sup>2+</sup> ionophore (PI) for 4 and 20 h. T lymphocytes from a HAM/TSP patient (CJ) were cultivated in complete medium with IL2 alone and without any added stimuli.

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<td>PI stimulated for 20 h</td>
<td>–</td>
</tr>
<tr>
<td>HTLV-1-positive</td>
<td></td>
</tr>
<tr>
<td>HAM/TSP (CJ)</td>
<td>10 ± 1.1</td>
</tr>
<tr>
<td>HUT-102</td>
<td>134 ± 11.3</td>
</tr>
</tbody>
</table>

*Amount of mRNA assessed by real-time quantitative PCR was defined as the number of either Tax or IL17 copies relative to the number of standard gene (PBGD) copies. Each value represents the mean ± SD of at least two determinations performed in duplicate.
element could be defined: a 153 bp element (from −614 to −461) in the upstream domain and a 135 bp element in the downstream domain. This promoter activity analysis allowed us to identify an element (located between −461 and −351) that suppresses the transcriptional activation exerted by the Tax-responsive downstream domain. The suppressive activity of this negative element is, in turn, inhibited by the transcriptional activity of the Tax-responsive upstream domain. When the analysis of the IL17 promoter activity was performed in Jurkat cells using the same deletion mutants, the same Tax-responsive domains as well as the negative element could be characterized, providing that the Jurkat T cells were also stimulated with PMA and Ca\(^{2+}\) ionophore, which mimic activation through the antigen-specific T cell receptor. Consequently, these data suggest that Tax transactivation alone may not be sufficient for complete T cell activation and that both signals may be required for sufficient IL17 expression. Our results therefore indicate that Tax functions as an efficient co-stimulator of IL17 promoter activity in cooperation with T cell activation signals. Such a situation is reminiscent of that observed previously with the IL5 promoter (Blumenthal et al., 1999; Yamagata et al., 1997). Indeed, most of the other cytokine promoters have been shown to be fully activated either with Tax alone, such as IL3, IL6 and GM-CSF (Yamashita et al., 1994), or with both Tax and PMA, such as IL2 (Curtiss et al., 1996). The question remains as to why Tax is able to activate the transcription of the IL17 gene in HeLa cells, independently of signals provided by PMA and Ca\(^{2+}\) ionophore. It is plausible that HeLa cells express transcription factors needed for the full activation of the IL17 gene promoter. The same possibility might occur in the case of either HTLV-1-transformed T cells or Jurkat cells stably expressing Tax, such as the JPX-9 cell line.

Nevertheless, based on these results, we consider that the region up to position −614 upstream of the transcription initiation site contains major cis regulatory elements and is therefore needed for an efficient upregulation of the IL17 promoter activity by Tax. Interestingly, the use of Tax mutants that discriminate the activation of gene expression by Tax between the CREB/ATF and the NF-kB pathways demonstrated that Tax might activate the IL17 promoter via the former. The presence of several CREB binding sites in the Tax-responsive region of the IL17 promoter pleads for such a possibility.

How is the induction of the IL17 expression by Tax related to the pathology associated with HTLV-1 infection? The link with inflammatory diseases, such as HAM/TSP and arthropathy, may be explained by the pro-inflammatory properties of this cytokine. Despite the fact that expression of IL17 is restricted to activated T cells, expression of the IL17 receptor has been detected in all cells and tissues examined by Northern blot analysis (Fossiez et al., 1996; Yao et al., 1995b, 1997). Signalling by the IL17 receptor induces the activation of NF-kB and regulates the activity of extracellular-regulated kinase 1 (ERK1), ERK2, c-jun terminal kinase and p38 mitogen-activated protein kinases (Hsieh et al., 2002; Shalom-Barak et al., 1998). Consequently, IL17 has the ability to stimulate the production of other inflammatory cytokines and chemokines, including IL6, IL8, GM-CSF and MCP-1 (Aggarwal & Gurney, 2002; Fossiez et al., 1996; Jovanovic et al., 1998). As mentioned in the Introduction, IL17 is believed to play an important role in the induction and perpetuation of immunopathological processes (Aggarwal & Gurney, 2002). Indeed, high levels of IL17 are detected in rheumatoid arthritis and in other chronic inflammatory diseases, such as multiple sclerosis and psoriasis. Finally, IL17 has recently emerged as a CD4 T cell-derived cytokine that stimulates the migration of vascular endothelial cells and upregulates elaboration of a variety of pro-angiogenic factors by fibroblasts as well as tumour cells (Numasaki et al., 2003). Therefore, the biological functions of IL17 deserve further examination to unravel their impact on the course and severity of inflammatory and degenerative HTLV-1-associated diseases.

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REFERENCES


Tax-induced IL17 expression by T cells


