Upstream Stimulating Factors Regulate the Expression of RORγT in Human Lymphocytes

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Upstream Stimulating Factors Regulate the Expression of ROR\(\gamma\)T in Human Lymphocytes

Marcin Ratajewski,* Aurelia Walczak-Drzewiecka,† Anna Sałkowska,† and Jarosław Dastych†

Retinoic acid-related orphan receptor \(\gamma\)T (ROR\(\gamma\)T) is the orphan nuclear receptor that regulates the development of Th17 cells and the expression of IL-17. The differentiation of Th17 cells is associated with the upregulation of ROR\(\gamma\)T mRNA, and the mechanisms regulating that process in human cells are not well understood. We investigated the transcriptional regulation of ROR\(\gamma\)T in a human lymphocytic cell line and Th17 differentiated from naive CD4\(^+\) cells from human peripheral blood. A series of experiments, including 5′ deletion and in situ mutagenesis analysis of the human ROR\(\gamma\)T promoter, chromatin immunoprecipitation, and overexpression of selected transcription factors, revealed that the transcription factors upstream stimulatory factor 1 (USF-1) and USF-2 are indispensable for the transcription of ROR\(\gamma\)T in human lymphocytes. There was also upregulation of USF-1 and USF-2 during the differentiation of Th17 cells from naive CD4\(^+\) cells. In this article, we report the first analysis, to our knowledge, of the human ROR\(\gamma\)T promoter and demonstrate the role of the USF-1 and USF-2 transcription factors in regulating the expression of ROR\(\gamma\)T in human lymphocytes. Thus, USFs are important for the molecular mechanisms of Th17 differentiation, and possible changes in the expression of USFs might be of interest for inflammatory conditions with a Th17 component. Furthermore, these observations suggest a possible link between metabolic disorders in which the role of glucose-induced USF expression has already been established and autoimmune diseases in which the upregulation of ROR\(\gamma\)T is frequently detected. The Journal of Immunology, 2012, 189: 000–000.

The retinoic acid-related orphan receptors \(\gamma\) (ROR\(\gamma\)) and \(\gamma\)T (ROR\(\gamma\)T) are products of the ROR\(\gamma\) gene transcribed from different transcription start sites under the control of independent promoters (1–4). These two nuclear hormone receptors differ in their amino acid sequences by 13 aa residues in the N-terminal region. The basic ROR\(\gamma\) variant (ROR\(\gamma\)) is expressed ubiquitously and plays an important function in the regulation of development and metabolism (5). Recently, the 7-oxygenated sterol was identified as an endogenous ligand modulating the function of this transcription factor (6). The expression of ROR\(\gamma\)T (also termed RORC2) has a very restricted pattern, and its product is predominantly found in immune organs, such as thymus, and in particular lineages of immune cells, such as immature double-positive thymocytes and Th17 lymphocytes (2, 7), where this transcription factor plays important functions in the maturation of \(\alpha/\beta\) T cells and the differentiation of Th17 cells, respectively (2, 8). Th17 cells are a subset of Th cells capable of expressing the proinflammatory cytokine IL-17 (9–11). This cytokine, which is also expressed by several other immune cells such as \(\gamma\delta\) T cells (12) and mast cells (13), exercises its function by triggering the IL-17RA, which is ubiquitously expressed in multiple cell types (14). IL-17RA engagement triggers a signaling cascade leading to the activation of the transcription factors NF-\(\kappa\)B and API, which are major regulators that mediate changes in the expression of the genes responsible for initiating and maintaining an inflammatory state (14). The genes upregulated by IL-17 include cytokines, such as IL-6, and chemokines, such as IL-8, Rantes, and GRO (15–17). IL-17 expression is a major phenotypic marker of Th17 cells that are capable of initiating an immune response that is distinct from those driven by Th1 and Th2 (18, 19). The Th17-driven immune response provides effective protection against certain extracellular pathogens (20, 21) and is also responsible for the development of inflammatory processes associated with several human diseases, including autoimmunity and allergic conditions (22, 23), atherosclerosis (24), liver dysfunctions (25), and tumors (26). Differentiation of naive CD4\(^+\) lymphocytes into Th17 cells is supported by the coordinated action of several cytokines, including IL-6, IL-23, and TGF-\(\beta\) (23). ROR\(\gamma\)T mRNA levels increase upon Th17 expansion in vitro, and increased ROR\(\gamma\)T mRNA levels in tissues positively correlate with Th17 activity observed in different inflammatory diseases (27–29). Mechanisms regulating the tissue-specific expression of ROR\(\gamma\)T are not well understood. Studies with knockout mice revealed the involvement of the transcription factors STAT3, IFN regulatory factor 4, and v-rel reticuloendotheliosis viral oncogene homolog (c-Rel) in the up-regulation of ROR\(\gamma\)T (4, 30–32). The mechanism by which ROR\(\gamma\)T transcripts are transcriptionally regulated in human cells is largely unknown. Our investigation of the transcriptional regulation of ROR\(\gamma\)T in human lymphocytes revealed that the transcription factors upstream stimulatory factor 1 (USF-1) and USF-2 are indispensable for the transcription of ROR\(\gamma\)T, and that the upregulation of USFs occurs in differentiating Th17 cells in parallel with the upregulation of ROR\(\gamma\)T and IL-17.

*Laboratory of Transcriptional Regulation, Institute of Medical Biology, Polish Academy of Sciences, 93-232 Lodz, Poland; and Laboratory of Cellular Immunology, Institute of Medical Biology, Polish Academy of Sciences, 93-232 Lodz, Poland

†Laboratory of Transcriptional Regulation, Institute of Medical Biology, Polish Academy of Sciences, 93-232 Lodz, Poland

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Address correspondence and reprint requests to Prof. Jaroslaw Dastych, Laboratory of Cellular Immunology, Institute of Medical Biology, Polish Academy of Sciences, Lodowa 106, 93-232 Lodz, Poland. E-mail address: jdstych@cbm.pan.pl

Abbreviations used in this article: GDAP1, ganglioside-induced differentiation-associated protein; HMBS, hydroxymethylbilane synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; ROR, retinoic acid-related orphan receptor; ROR\(\gamma\), ROR\(\gamma\)T, ROR\(\gamma\)C, ROR\(\gamma\)T; ROR\(\gamma\)T, ROR\(\gamma\)T; RPL13A, ribosomal protein L13a; siRNA, small interfering RNA; USF, upstream stimulatory factor.
Materials and Methods

Cell culture
Jurkat (human T cell lymphoblast-like), HeLa (cervix carcinoma), and HepG2 (hepatocellular carcinoma) cell lines were obtained from American Type Culture Collection (Manassas, VA). They were maintained under standard conditions in RPMI 1640 or DMEM containing 10% FBS at 37°C in a 5% CO₂ atmosphere.

Real-time RT-PCR
RNA was isolated from cells using TRI Reagent (Molecular Research Center, Cincinnati, OH) and reverse-transcribed with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/ Fermentas, Vilnius, Lithuania). Real-time RT-PCR amplification was carried out on a LightCycler 480 (Roche, Basel, Switzerland) using SYBRGreen I Master Mix (Roche) as follows: 5 min at 95°C and then 45 cycles each at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Sequence of GATA-3 primers were published by Hoene et al. (33). Other intron-spanning primers used to detect cDNA sequences were designed using Primer3 software: RORγT forward, 5′-CTGCTGAGAAGAACAGGGGAG-3′; RORγT reverse, 5′-CACAAGAGAAGACCAGGC-3′; RORγT/RORγ reverse (same for both isoforms), 5′-AGTTCTCTGTAGGCTGGTG-3′; USF-1, 5′-AATGTCAGTCTAGACTACCC-3′ (forward) and 5′-CAACAGGTTGTTACCTGTCGCATGTGGTTT-3′ (reverse); USF-2, 5′-TCTTCTCCTCTCATCTCGGG-3′ (reverse) and 5′-CTTTCCCTACCTCATCCTGGG-3′ (reverse); and IL-17A, 5′-AACACACAGTGATCCCTGCGG-3′ (forward) and 5′-CCTTGCCATGAGTTCCATT-3′ (reverse). Δ cycle threshold values were transformed into relative copy number values (the number of copies of cDNA per RNA per housekeeping gene index, calculated as the averaged cycle threshold of the housekeeping genes hypoxanthine phosphoribosyltransferase 1 (HPRT1), and hydroxymethylbilane synthase (HMBS), and ribosomal protein L31a (RPL31A) as described in our previous study (34).

RORγT promoter constructs and transfection
Fragments of RORγT spanning its putative promoter region were prepared by PCR amplification of human genomic DNA and cloned into a pUC18 vector using HincII, a blunt restriction endonuclease. The following primers were used for amplification: reverse primer, 5′-CTGTTAAGCCTA- CATTCCCTTTCCAGAGGG-3′ (position +78); all nucleotide locations are reported relative to the A in the ATG translation initiation codon) and forward primers: 5′-AGTTCTCTGTAGGCTGGTG-3′ (position +1); 5′-CTTTCCCTACCTCATCCTGGG-3′ (reverse); and IL-17A, 5′-AACACACAGTGATCCCTGCGG-3′ (forward) and 5′-CCTTGCCATGAGTTCCATT-3′ (reverse). Δ cycle threshold values were transformed into relative copy number values (the number of copies of cDNA per RNA per housekeeping gene index, calculated as the averaged cycle threshold of the housekeeping genes hypoxanthine phosphoribosyltransferase 1 (HPRT1), and hydroxymethylbilane synthase (HMBS), and ribosomal protein L31a (RPL31A) as described in our previous study (34).

Site-directed mutagenesis
Mutagenesis was performed directly on plasmids (pUC18) containing sequences −589/+78 and −341/+78 of RORγT promoter using the PCR-based method followed by removal of the template by DpnI digestion. Introduction of mutation to the E-BOX sequence was based on a previous work of Ciccone et al. (35). For mutagenesis, the following primer pairs were used: 5′-TCCACAGGGTTGAGTCATAGCAACATCCACCACCC-3′ (m48) and 5′-GCTGGTACCTACATCGCCACCTCATCCAC-3′ (m48r) (100 ng/ml), IL-6 (30 ng/ml), IL-23 (10 ng/ml), GATA-3 (m1r). The mutated sequences were verified by sequencing and then recloned into pGL3-Basic vector.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation with normal mouse IgG (Millipore, Billerica, MA), anti-USF-1 (H-86; Santa Cruz; Santa Cruz, CA), anti–USF-2 (H-100, Santa Cruz), and anti–c-Myc (9E11; Santa Cruz; Santa Cruz, CA) Abs were used for supershift experiments. Analysis of IL-17 production

For the analysis of IL-17 production, IL-23+ T cells and CD4+ T cells stimulated with the T Cell Activation Kit (Millenyi Biotec GmbH Bergisch, Gladbach, Germany). Naive CD4+ T cells were maintained in Yssel’s media containing human AB serum for 5 d in Th17 polarizing conditions with beads coated with anti-CD2, anti-CD3, and anti-CD28 (Caltag) and a cytokine mixture containing human IL-1β (50 ng/ml), human IL-6 (30 ng/ml), human IL-21 (10 ng/ml), and human TGF-β (10 ng/ml). The cytokines were purchased from R&D Systems (Minneapolis, MN).

EMSA
Nuclear extracts were prepared as described elsewhere (37) and subsequently used in EMSA. Infrared dye-labeled annealed oligonucleotides were incubated with 1 μg nuclear extract in binding buffer containing 10 fmol infrared dye-labeled probes, 5 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM DTT, 5 mM MgCl₂, 0.5% Igepal, 25% glycerol, 100 μM ZnCl₂, and 0.2 μg salmon testis DNA in a 20-μl reaction volume. For the competition assay, 10- and 50-fold molar excesses of unlabeled oligonucleotides (wt, m3, m2, or m1) were added to the reaction mixture. The reactions were incubated on ice for 60 min before probe addition, followed by an additional 30-min incubation. The binding reactions were resolved by electrophoresis on a 5% polyacrylamide gels in 0.5% Tris-acetate EDTA buffer. The products were then imaged on an Odyssey (LiCor, Lincoln, NE) infrared fluorosence scanner. Anti–USF-1 (H-86; Santa Cruz) and anti–USF-2 (H-100; Santa Cruz) Abs were used for supershift experiments.

Naive CD4+ T cell isolation and differentiation

For the isolation of IL-17 producing cells, CD4+ T cells and CD4+ T cells stimulated with the T Cell Activation Kit, in the presence or absence of the cytokine mixture, were cultured for 5 d. The cells and supernatants were collected for RNA isolation and ELISA assays, respectively. ELISA was performed with the Quantikine Human IL-17 Immunoassay kit (R&D Systems) according to the manufacturer’s protocol. Measurements were performed at 450 nm using a Sunrise microplate reader (Tecan).

Small interfering RNA and nucleofection

Duplexes of Stealth small interfering RNA (siRNA) against the USF-1 (USF1HSS144431) and USF-2 (USF2HSS144434) transcripts were purchased from Invitrogen (Carlsbad, USA). Negative control siRNA–A with a scrambled sequence was purchased from Santa Cruz. Jurkat cells were nuleofected with 100 pmol of each target siRNA duplex using Amaxa SE Cell Line for 4-DNucleofector X Kit L (Lonza, Basel, Switzerland) according to the manufacturer’s protocol. After nuleofection, the cells were cultured for 96 h and then collected for RNA extraction. Th17 cells differentiated in vitro from naive CD4+ were nuleofected with the same siRNA duplexes as described earlier using Amaxa P3 Primary Cell 4D-Nucleofector X Kit (Lonza) according to the manufacturer’s instruction. After nuleofection, the Th17 cells were cultured in the Yssel’s medium without cytokines for 16 h. This was followed by addition of cytokine mixture containing IL-1β (50 ng/ml), IL-6 (30 ng/ml), IL-23 (10 ng/ml), and TGF-β (10 ng/ml), and beads coated with anti-CD2, anti-CD3, and anti-CD28 and culture for an additional 5 d.

Computational analysis and statistics

Putative transcription factor elements within the RORγT gene promoter were identified using MatInspector software (38). Alignment of the RORγT promoters from various mammalian species was performed using the CLUSTALW program (39). Genomic sequences were downloaded from the University of California Santa Cruz genome browser (40). Statistical analysis was performed using paired t test or one-way ANOVA, followed by Dunn’s post hoc test. A p value ≤0.05 was considered statistically significant.
Results

Tissue-specific expression of RORC gene variants

The expression of two known variants of the RORC gene was first analyzed in selected cell lines. HepG2 hepatocytes and cervix cell carcinoma HeLa cells almost exclusively expressed ROR\(_g\), whereas Jurkat lymphocytes predominantly expressed ROR\(_g\)T (Fig. 1A).

Analysis of the human ROR\(_g\)T promoter activity

Next, a series of 5' -deletion mutants of the human ROR\(_g\)T promoter cloned into a luciferase reporter construct was used to analyze promoter activity in Jurkat lymphocytes and identify the responsible sequences. The transfection of Jurkat lymphocytes with ROR\(_g\)T promoter constructs resulted in significant promoter activity, with the highest activity observed for constructs containing the sequences spanning from −2340 to +78 (Fig. 1B). The shortest analyzed sequence (−2180/+78) and constructs representing sequences further 5' upstream of the ROR\(_g\)T translation start site also exhibited significant activity compared with the promoterless pGL3-basic vector. The promoter activities in nonlymphatic cells (HepG2 hepatocytes and HeLa cells) were several fold lower than that observed in Jurkat lymphocytes. The longest promoter construct (−2869/+78) had the highest activity.

Screening for transcription factors interacting with human ROR\(_g\)T promoter

Several transcription factors were then overexpressed in HeLa cells cotransfected with the longest reporter construct. Overexpression of USF-1 and USF-2, but not the other tested transcription factors, resulted in a significant (2.1- and 2.9-fold, respectively) increase in human ROR\(_g\)T promoter activity (Fig. 2). Analysis of the 5' -upstream sequences of the human ROR\(_g\)T gene revealed four binding sites for USF-1 and USF-2 (E-BOX 1–4) that are preserved among mammalian species (Fig. 3).

Effect of USF-1 and USF-2 overexpression on activity of human ROR\(_g\)T promoter

Next, the effect of USF-1 and USF-2 overexpression on the activities of different human ROR\(_g\)T promoter constructs was assessed.
Overexpression of USF-1 and USF-2 did not change the activity of the shortest promoter construct (−180/+78), which does not contain a USF binding motif (Fig. 4A). In contrast, the activity of the promoter construct containing sequences −340 to +78, which overlap the E-BOX 1 and 2 sites, was significantly upregulated. The activities of the two longer promoter constructs (−869/+78 and −589/+78), which overlap all four USF binding sites (E-BOX 1–4), were upregulated slightly higher than the activity of phRORγT (−341/+78)Luc. To gain further insights into the role of USF in the regulation of RORγT, we used additional promoter constructs (−589/+78) with mutations in E-BOX 3 alone, E-BOX 4 alone, or both E-BOX 3 and E-BOX 4 in a series of transfection experiments in HeLa cells. Mutation of E-BOX 4, the most distal site, enhanced the responses elicited in HeLa cells by overexpressed USF-2 but did not change the response caused by overexpressed USF-1 (Fig. 4B). In contrast, mutation of E-BOX 3 resulted in
a small but significant decrease in response to USF-1 overexpression and did not alter the level of upregulation caused by USF-2 overexpression. The construct containing both mutations enhanced the response to USF-2 overexpression and caused a small decrease in response to USF-1 overexpression. When a similar analysis was performed using the construct phRORγT (−341/+78)Luc containing E-BOX 1 and E-BOX 2 and additional constructs with mutations in E-BOX 1 alone, E-BOX 2 alone, or both, the data showed a different pattern of effects on RORγT promoter activity following the overexpression of USF-1 and USF-2 (Fig. 4C). Thus, mutation of each of these two binding sites resulted in a significant decrease in promoter activity following the overexpression of USF-1 and USF-2. Mutation of both E-BOXes resulted in promoter activity that was significantly lower than that observed in the presence of either alone. The promoter activities of mutated and wild type phRORγT (−341/+78)Luc and phRORγT (−589/+78)Luc promoter constructs were compared in Jurkat lymphocytes. In the longer construct (−589/+78), mutation of E-BOX 3 alone or E-BOX 3 and E-BOX 4 (but not E-BOX 4 alone) resulted in a small but detectable decrease in promoter activity in the Jurkat lymphocytes (Fig. 4D). Mutations in phRORγT (−341/+78)Luc, which depleted this construct of the more distally located E-BOX 2 USF binding site, strongly (>2-fold) decreased promoter activity. Mutation of E-BOX 1 resulted in a significant but small (10%) decrease in promoter activity, and double mutation of E-BOX 1 and E-BOX 2 resulted in promoter activity comparable with those observed with the single E-BOX 2-deficient mutant.

FIGURE 5. Binding of USF-1 and USF-2 to the human RORγT gene promoter. (A–D) Nuclear extracts were prepared from resting Jurkat cells as described in Materials and Methods. EMSAs were performed using infrared (IR) fluorescently labeled oligonucleotide probes and unlabeled “cold” oligonucleotides of indicated sequences. For the competition assay, the “cold” competitor oligonucleotides with wild-type (wt) or mutated (m4, m3, m1, or m2) sequences were added at indicated molar fold excess over the amount of the labeled probe. For supershift assay, anti–USF-1 and anti–USF-2 Abs were added to the reaction mixture as indicated. (E) Results of chromatin immunoprecipitation performed on living Jurkat cells. Chromatin was isolated from resting Jurkat cells fragmented and immunoprecipitated with normal mouse IgG, anti–USF-1, anti–USF-2, and anti–c-Myc Abs. Immunoprecipitated DNA sequences were amplified by PCR with primers specific to the RORγT promoter and GDAP1 promoter (negative control), and visualized on agarose gel stained with ethidium bromide. NS, Nonspecific; S, shift; SS, supershift.
Analysis of interaction of USFs with human RORγT promoter sequences

To confirm that USFs regulate RORγT promoter activity in Jurkat lymphocytes, EMSA assays were performed using Jurkat lymphocyte nuclear extracts and nucleotide probes matching the sequences of E-BOX 1–4. Incubation of the Jurkat-derived nuclear extract with all four nucleotide probes resulted in the detection of specific bands that were competed out with an excess of cold nucleotide of matching but not with mutated sequences (Fig. 5). The addition of Abs against USF-1 and USF-2 resulted in the appearance of additional bands representing supershifts. For the E-BOX 4 nucleotide (Fig. 5A), the weak but detectable supershift band appeared following the addition of anti-USF-2, but not anti–USF-1 Ab. For the E-BOX 3 nucleotide (Fig. 5B), in contrast, a supershift band appeared after the addition of anti-USF-1, but not anti–USF-2 Ab. Supershift experiments with nucleotide probes matching the sequences of E-BOX 2 and E-BOX 1 (Fig. 5C, 5D) generated similar extra bands with both anti–USF-1 and anti–USF-2 Ab. Thus, the USF-1 and USF-2 proteins in the nuclear extract of Jurkat lymphocytes interacted with all four tested USF-binding motifs in vitro. The interaction of USF-1 and USF-2 with the RORγT promoter was then investigated in intact Jurkat lymphocytes. Chromatin immunoprecipitation experiments revealed the binding of both USF-1 and USF-2 but not v-myc myelocytomatosis viral oncogene homolog (c-Myc; used as negative control) proteins to the RORγT promoter (Fig. 5E). In addition, USF-1 and USF-2 did not bind to the GDAP1 promoter, which does not contain USF binding motifs.

Effect of inhibition of USFs on RORγT expression in Jurkat cells

To confirm the functionality of USFs binding to the RORγT promoter, we used siRNAs targeting USF-1 and USF-2. The transfection of Jurkat cells with siRNAs targeting USF-1 and USF-2 resulted in a significant and targeted gene-specific decrease in the respective mRNA levels (Fig. 6A, 6B). Jurkat cells treated with siRNAs targeting USF-1 alone, USF-2 alone, and both transcripts showed RORγT mRNA (data not shown) and GATA-3 mRNA (Fig. 6C) levels similar to those seen in control cells. In contrast, Jurkat cells treated with siRNAs targeting USF-1 alone, USF-2 alone, or both transcripts showed significantly lower RORγT mRNA levels (Fig. 6D). The USF-2 siRNA inhibited RORγT expression more than the USF-1 siRNA, with the combination of both siRNAs having the strongest inhibitory effect (65% decrease).

Effect of inhibition of USFs on RORγT expression in human Th17 cells

To gain evidence of USF involvement in the regulation of RORγT expression in Th17, the standard experimental protocol for in vitro differentiation of Th17 cells from peripheral blood naive CD4+ lymphocytes was used. The culture of naive CD4+ lymphocytes in the presence of a standard cytokine mixture combined with activation by cross-linking of CD2, CD3, and CD28 resulted in the accumulation of a significant amount of IL-17 in the supernatant (2.1 ± 0.9 pg/ml for CD4+ and 403.1 ± 103.8 pg/ml for Th17, statistically significant difference at p < 0.05) in parallel with an increase in IL-17 mRNA in the cell pellet (Fig. 7A). As expected, the differentiation of Th17 from CD4+ was also associated with a significant upregulation of RORγT mRNA (Fig. 7B), but not RORγ mRNA (Fig. 7C) and GATA-3 (Fig. 7D) in these cells. Interestingly, this in vitro differentiation process was associated with significant upregulation of USF-1 and USF-2 mRNA (Fig. 7E, 7F). To further confirm the role of USFs in RORγT expression, Th17 cells obtained in vitro from CD4+ lymphocytes were nucleofected with siRNAs targeting USF-1 and USF-2, and cultured for additional 5 d under optimal conditions. As shown in Table I, this treatment inhibited USF-1 and USF-2 expression and resulted in a significant decrease in the level of RORγT mRNA. The expression of RORγ, GATA-3, and IL-17 were not significantly inhibited.

Discussion

The transcriptional regulation of RORγT, one of the two RORC variants, is an important element of the regulation of Th17 differentiation. Most data regarding the tissue-specific expression of RORγT and its transcriptional regulation come from murine studies. Similar to those of the mouse, the two variants of the human RORC gene originate from different transcription start sites and are regulated by different promoters. There was a cell line-(Fig. 1A) and cell lineage-specific (data not shown) pattern of expression of RORγ and RORγT. Also like the mouse, human RORγT is preferentially expressed in selected lymphocytic lineages but is hardly detectable in nonlymphatic cells (Fig. 1A, Fig. 7B, data not shown). This preferential expression of RORγT in human Jurkat lymphocytes and peripheral blood-derived CD4+ Th17 cells was associated with a very low expression of RORγ.
that is similar to the previously reported pattern of RORγ/T expression in murine thymocytes (2) and different from the pattern observed in murine Th17 cells, in which high expression of RORγ/T was associated with comparable levels of RORγ (4). This tissue-specific pattern of expression is consistent with the results of promoter activity analysis, which showed that RORγ/T promoter constructs had significantly higher activity in Jurkat T lymphocytes than in nonlymphatic cells (Fig. 1B). Although significant promoter activity in Jurkat lymphocytes was observed with sequences spanning −180 bp 5′ upstream of RORγ/T, the highest activity was mediated by sequences further upstream, up to −340 bp relative to the RORγ/T translation start site (Fig. 1B). To our knowledge, this represents the first analysis of human RORγ/T promoter activity. Our observations are consistent with the hypothesis that preferential transcription of the RORγ/T promoter in T cells participates in the tissue-specific expression of RORγ/T in human T cells. Previously, a similar upstream sequence (−400 to +151) of murine RORγ/T showed significant promoter activity in murine EL-4 lymphocytes (4). Screening for transcription factors that upregulate the human RORγ/T promoter revealed possible roles for USF-1 and USF-2 in the regulation of human RORγ/T (4). Screening for transcription factors that upregulate the human RORγ/T promoter revealed possible roles for USF-1 and USF-2 in the regulation of human RORγ/T (4). Screening for transcription factors that upregulate the human RORγ/T promoter revealed possible roles for USF-1 and USF-2 in the regulation of human RORγ/T (4). Screening for transcription factors that upregulate the human RORγ/T promoter revealed possible roles for USF-1 and USF-2 in the regulation of human RORγ/T (4).

Additional experimental data obtained using Jurkat lymphocytes confirmed that these binding sites can bind USF-1 and USF-2 and are necessary for mediating the effects of USF-1 and USF-2 on the activity of the RORγ/T promoter. Although the proximal binding sites (E-BOX 1 and E-BOX 2) seemed to mediate a strong upregulation of promoter activity by both USF-1 and USF-2, the distal binding sites (E-BOX 3 and E-BOX 4) seemed to preferentially bind USF-1 and USF-2, respectively, and upregulate (E-BOX 4) or downregulate (E-BOX 3) promoter activity. This observation is consistent with the pattern of 5′-deletion promoter construct activities in Jurkat cells, in which the two constructs containing E-BOX 4 had significantly lower activity compared with the construct containing only E-BOX 1 and E-BOX 2 (Fig. 1B). Additional experimental data obtained using Jurkat lymphocytes strongly suggest that high RORγ/T promoter activity and high RORγ/T mRNA expression depend on USF-1 and USF-2 expression in those cells. Thus, Jurkat lymphocytes expressed 2- to 3-fold

Table I. Effect of downregulation of the USF-1 and USF-2 mRNA on the expression of selected genes in Th17 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA-A</th>
<th>siUSF-1 + 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>USF-1</td>
<td>209.3 ± 71.6</td>
<td>129.3 ± 50.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>USF-2</td>
<td>150.4 ± 44.1</td>
<td>54.8 ± 23.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RORγ</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>RORγ/T</td>
<td>46.3 ± 11.7</td>
<td>34.6 ± 9.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GATA-3</td>
<td>34.8 ± 8.4</td>
<td>46.2 ± 14.0</td>
</tr>
<tr>
<td>IL-17A</td>
<td>271.1 ± 79.2</td>
<td>223.7 ± 66.4</td>
</tr>
<tr>
<td>IL-17 secretion (pg/ml)</td>
<td>1488.4 ± 1125.2</td>
<td>570.6 ± 272.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Statistically significant difference at p < 0.05 as compared with cells nucleofected with control siRNA-A.

 badass! you are learning a lot, aren't you? how can I help you with this?


