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Regulation of IFN Regulatory Factor 4 Expression in Human T Cell Leukemia Virus-I-Transformed T Cells

Sonia Sharma,*† Nathalie Grandvaux,* Yael Mamane,*† Pierre Genin,* Nazli Azimi,‡ Thomas Waldmann,* and John Hiscott*‡†

IFN regulatory factor (IRF)-4 is a lymphoid/myeloid-restricted member of the IRF transcription factor family that plays an essential role in the homeostasis and function of mature lymphocytes. IRF-4 expression is tightly regulated in resting primary T cells and is transiently induced at the mRNA and protein levels after activation by Ag-mimetic stimuli such as TCR cross-linking or treatment with phorbol ester and calcium ionophore (PMA/ionomycin). However, IRF-4 is constitutively upregulated in human T cell leukemia virus type I (HTLV-I) infected T cells as a direct gene target for the HTLV-I Tax oncoprotein. In this study we demonstrate that chronic IRF-4 expression in HTLV-I-infected T lymphocytes is associated with a leukemic phenotype, and we examine the mechanisms by which continuous production of IRF-4 is achieved in HTLV-I-transformed T cells. IRF-4 expression in HTLV-I-infected cells is driven through activation of the NF-κB and NF-AT pathways, resulting in the binding of p50, p65, and c-Rel to the κB1 element and p50, c-Rel, and NF-ATp to the CD28RE element within the −617 to −209 region of the IRF-4 promoter. Furthermore, mutation of either the κB1 or CD28RE sites blocks Tax-mediated transactivation of the human IRF-4 promoter in T cells. These experiments constitute the first detailed analysis of human IRF-4 transcriptional regulation within the context of HTLV-I infection and transformation of CD4+ T lymphocytes. The Journal of Immunology, 2002, 169: 3120–3130.

Although the best-characterized members of the IRF family—IRF-1, IRF-2, and IRF-3—display a fairly ubiquitous pattern of expression in mammalian cells, expression of IRF-4 is specific to the lymphoid and myeloid compartments of the immune system (9–13). Furthermore, the function of IRF-4 with respect to gene regulation appears to be as cell type specific as its expression pattern. IRF-4 is expressed during all stages of B lymphocyte development, where it binds and transactivates the murine IgL chain enhancer Eκ2–4 (9) and the IL-1β promoter (14) in association with the Ets protein PU.1. In B cells, IRF-4 expression is upregulated by stimulation through CD40 and treatment with IL-4, resulting in IRF-4-mediated transactivation of IL-4-inducible genes in conjunction with STAT6 (15). Constitutive IRF-4 expression in macrophages correlates with repression of the IRF-regulated ISG15 ISRE, in association with IFN consensus sequence binding protein (ICSBP) or IRF-8 (13). Interestingly, the regulation of IRF-4 activity in macrophages appears to occur at the level of cytoplasmic/nuclear localization rather than the level of gene expression (12).

IRF-4 expression is under stringent control in T lymphocytes; IRF-4 is induced upon T cell activation, an event mimicked by CD3 cross-linking or treatment with PMA/ionomycin and conA (10, 11, 16). Transcriptional regulation of IRF-4 in T cells has been shown to be dysregulated in only one case: IRF-4 is constitutively upregulated at the mRNA and protein levels in T cells infected and transformed with the human T cell leukemia virus type I (HTLV-I) (11, 16). Interestingly, recent studies have identified direct downstream target genes of IRF-4, notably IL-4 (17) and cyclin B1 (18), suggesting the involvement of IRF-4 in these signaling pathways.

At present, between 10 and 20 million people are infected with HTLV-I, a retroviral agent etiologically associated with ATL, an aggressive and often fatal malignancy of CD4+ T cells (19), as well as HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP), a demyelinating neurological syndrome (20). Infection by HTLV-I transforms T cells in vitro and in vivo (21, 22), a process that has been associated with up-regulation of specific cellular genes involved in T cell activation and proliferation during the course of
viral infection (23–25). Disruption of the lymphocyte gene expression pattern is attributed to the activity of the 40-kDa HTLV-I-encoded Tax oncoprotein, a key regulator of cellular and viral gene expression. Tax physically interacts with and modulates the activity of numerous host signaling components and transcription factors, resulting in the induction of regulatory genes during the early stages of HTLV-I infection and transformation (24–26). In this study, we provide the first detailed analysis of the regulation of the human IRF-4 gene within the context of HTLV-I infection. Our studies establish a link between IRF-4 expression in HTLV-I-infected cells and the presence of the ATL phenotype. We have characterized several transcriptional regulatory elements within the human IRF-4 promoter that interact with NF-κB, NF-AT, and stimulating factor 1 (Sp-1) transcription factors to drive IRF-4 production in HTLV-I-transformed T-cells.

Materials and Methods

Cell culture and reagents

Primary human T lymphocytes, isolated by Ficoll-density centrifugation from whole blood, were maintained in RPMI 1640 medium (Canadian Life Technologies, Burlington, Ontario, Canada) supplemented with 15% non-heat-inactivated FBS and antibiotics. HTLV-I-infected T cell lines MT2, C8166, and MT4 and T cell lines T4, Jurkat, and CEM were cultured in RPMI 1640 medium (CLT) supplemented with 10% heat-inactivated FBS and antibiotics. PBL extracts from HAM/TSP, ATL patients, and normal donors were obtained from N. Azimi (National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). ATL is characterized by a monoclonal expansion of CD4+ T cells (21). All ATL patients possessed high leukemic cell counts (5- to 6-fold higher PBMC levels compared to normal donors) and at least 50% of peripheral T lymphocytes were leukemic. Leukemic cells were CD4+CD8+CD25+. PMA and ionomycin (Calbiochem, San Diego, CA, USA) were used at a final concentration of 10 ng/ml and 200 nM, respectively, for immunoblot analysis and PMA was used at a final concentration of 50 ng/ml for in vitro RNA cell assay. FK506 (Sigma-Aldrich, Oakville, Ontario, Canada) was used at a final concentration of 1 μM.

Plasmid construction and mutagenesis

1.2-kb IRF4-PRO-PGL3B luc was generated by cloning the human promoter of IRF-4 (27) into SacI/BlII digested luciferase reporter plasmid pGL3 basic (pGL3B) using specific primers (upstream primer 5′-GAGGCTCAT GAAAATCCTGGTCAC-3′ and downstream primer 5′-AGATCTTGAC AAGGGCAGCGGTGGGTCCC-3′) to amplify the 1.2-kb PGL3B fragment. Subcloning of PCR-amplified fragments into SacI/BlII digested pGL3B was followed by NotI digestion to obtain a 0.6-kb fragment. The XbaI site was mutated to 5′-CTCTGGACAGGCAGGCCTCCTGCCACAGC-3′ by using T4 DNA polymerase.

Immunoprecipitations and kinase assay

MT2 and Jurkat T cells were transfected with human IRF4-pGL3B-luc reporter plasmids and assayed for luciferase activity. Exponentially growing MT2 and Jurkat cells (106) were transfected with 2 μg pGL4.33-IRF4 probe and secondary antibody. After incubation for 2 min at room temperature with anti-IRF-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1/500 or for 30 min at room temperature with anti-actin (Chemicon, Temecula, CA, USA) at a dilution of 1/10,000. After four 5-min washes in PBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse 1:4000 dilution of 1/5,000 for IRF-4 and secondary goat anti-mouse at a dilution of 1/15,000 for actin. Reaction was visualized with an ECL detection system as detailed by manufacturer (Amersham). Transfections and transient coexpression assays

MT2 and Jurkat T cells were transfected with human IRF4-pGL3B-luc reporter plasmids and assayed for luciferase activity. Exponentially growing MT2 and Jurkat cells (106) were transfected with 2 μg pGL4.33-IRF4 probe and secondary antibody. After incubation for 2 min at room temperature with anti-IRF-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1/500 or for 30 min at room temperature with anti-actin (Chemicon, Temecula, CA, USA) at a dilution of 1/10,000. After four 5-min washes in PBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse 1:4000 dilution of 1/5,000 for IRF-4 and secondary goat anti-mouse at a dilution of 1/15,000 for actin. Reaction was visualized with an ECL detection system as detailed by manufacturer (Amersham).

For preparation of whole-cell lysates, cells were washed twice in PBS and lysed at 4°C in a buffer containing 10 mM Tris-HCl (pH 8), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin (Nonidet P-40, Sigma Chemical Co.) in the presence of whole-cell lysates (25–100 μg protein) were fractionated by SDS-PAGE on a 10% polyacrylamide gel. After transfer, the Hybrid membrane (Amersham, Cleveland, OH, USA) was blocked in 5% milk in PBS for 1 h and probed overnight at 4°C with anti-IRF-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1/500 or for 30 min at room temperature with anti-actin (Chemicon, Temecula, CA, USA) at a dilution of 1/10,000. After four 5-min washes in PBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse 1:4000 dilution of 1/5,000 for IRF-4 and secondary goat anti-mouse at a dilution of 1/15,000 for actin. Reaction was visualized with an ECL detection system as detailed by manufacturer (Amersham).
GACAGTTATTTG-3', Tm 65°C; for analysis of the noncoding strand of the –460 to –260 region of the IRF-4 promoter, primer 4, 5'-GTTAGTGCCTGTGGCGC-3', Tm 60°C; primer 5, 5’-GACAACCTCACCCTCAGTCTCTTCG-3', Tm 63°C; primer 6, 5’-ACCTCGAGTTCCCTCTCTTCGTGGACCATCTTCCC-3', Tm 66°C. Results shown represent at least three independent experiments.

**EMSA**

Whole-cell extracts prepared from primary T cells as detailed above and nuclear extracts prepared from Jurkat and HTLV-I-infected cells as previously described (35) were subjected to EMSA using 32P-labeled probes. The binding reaction was carried out in a 20-μL final volume in binding buffer containing 20 mM HEPES (pH 7.5), 50% glycerol, 0.1 M KCl, 0.2 mM EDTA (pH 8), 0.2 mM EGTA (pH 8), and 1 μg of poly(dI-dC) using 5–10 μg protein extract from Jurkat, T4, MT2, or ATL-derived T cells. The following oligonucleotide probes were used: NF-AT consensus, 5’-CGC CCAAAAGGAAAATTTGTTCTACA-3’; NF-AT mutant, 5’-GGCCTAAATTGTGTTCTACA (mutations are indicated in bold); Sp-1 consensus, 5’-ATTCGGATCCCTTGCGGCGACG-3’; Sp-1 control, 5’-GTCGACCGGATTACGCTCTCGACG-3’; Sp-1 mutant, 5’-ATTCGGATCCCTTGCGGCGACG-3’; Sp-1 control, 5’-GTCGACCGGATTACGCTCTCGACG-3’; Sp-1 mutant, 5’-ATTCGGATCCCTTGCGGCGACG-3’. Protein/DNA complexes were resolved on 5% polyacrylamide-0.5 M urea gel and exposed to Biomax XR film (Kodak) overnight at –80°C. Supershift analysis was performed for 15 min at room temperature using 1 μg anti-p65, anti-p50, anti-c-Rel, anti-Sp-1, or anti-NF-ATC3 Ab and overnight with 1 μg of a 1/10 dilution of anti-NF-ATp Ab (Upstate Biotechnology, Lake Placid, NY).

**Formaldehyde cross-linking and chromatin immunoprecipitation (chIP) assays**

Formaldehyde cross-linking and chIP procedure was modified from the protocol reported by Boyd et al. (36). Exponentially growing Jurkat or MT2 cells (2 × 10^6) were treated with formaldehyde (Fischer Scientific), which was added directly to the growth medium at a final concentration of 1% at room temperature with stirring. The reaction was stopped after 10 min by addition of glycine at a final concentration of 0.125 M. Suspension cells were then washed twice in ice-cold PBS, and lysed in SDS lysis buffer (Upstate Biotechnology) on ice for 10 min. DNA was sonicated to an average length of 600 bp using 5 sets of 10-s pulses at 30% efficiency, followed by centrifugation at 13,000 rpm at 4°C. Prior to dilution, 20% glycerol was added to the c-Rel sample 2 h before collection. The binding reaction was carried out in 20 μL binding buffer containing 5 mM EDTA (pH 8), 0.2 mM EGTA (pH 8), and 1 μg of poly(dI-dC). Immune complexes were further increase IRF-4 levels in HTLV-I-transformed cell lines (data not shown), demonstrating that IRF-4 was maximally expressed in these cells. Analysis of PBL isolated from HTLV-I-infected patients (Fig. 1C) revealed that IRF-4 expression was detected in samples from patients with the leukemic ATL phenotype (Fig. 1C, lanes 2–6), whereas IRF-4 was absent from nonleukemic HAM/TSP patient samples (Fig. 1C, lane 1). Although a single HAM/TSP sample was shown in Fig. 1, a total of 11 HAM/TSP PBL samples were evaluated and all were negative for IRF-4 expression (data not shown).

**HTLV-I responsive domains within the IRF-4 promoter**

To examine the functional domains of the 1189-bp upstream nucleotides corresponding to the human IRF-4 promoter (27) involved in HTLV-I-induced IRF-4 activation, full-length and 5’ promoter deletions were transfected into MT2 cells to analyze relative promoter activity within the context of HTLV-I-transformed human PBL isolated from HTLV-I-infected patients. Fig. 1A demonstrates that IRF-4 protein expression was absent in resting primary T lymphocytes (Fig. 1A, lane 1), but induced after treatment with PMA/
cells (Fig. 2A). Deletion of the −617 to −367 nucleotides of the IRF-4 promoter resulted in a decrease of luciferase activity that represented 55% of full-length 1.2-kb promoter activity in MT2 cells (Fig. 2A, 0.4 kb). Removal of the adjacent −367 to −209 nucleotides abrogated IRF-4 promoter luciferase activity in MT2 cells compared to full-length promoter construct (Fig. 2A, 0.2 and 0.08 kb, respectively); these results suggest that activation of the IRF-4 gene in HTLV-I-infected MT2 cells is mediated through the −617 to −209 region of the human IRF-4 promoter. Sequence evaluation within the −700 to −200 region of the IRF-4 promoter revealed the presence of multiple consensus binding sites for NF-κB, NF-AT, and several other transcription factors, as represented schematically in Fig. 2B.

NF-κB activation in HTLV-I-transformed cells

It has previously been shown that Tax-induced NF-κB activation in HTLV-I-infected T cells occurs at the level of the IKK complex (37, 38). To compare IKK kinase activity in Jurkat and HTLV-I-infected T cell lines, in vitro kinase assay was performed using the N-terminal 55 aa of IκBα (GST-IκBα) (Fig. 3A, lanes 1–5) or S32/36A-substituted IκBα (GST-2NIκBα) (Fig. 3A, lanes 6–10) as substrates for endogenous IKK immunoprecipitated from Jurkat, PMA-treated Jurkat, and HTLV-I-transformed T cells. As shown in Fig. 3A, IKK activity was activated in Jurkat PMA and untreated MT2, C8166, and MT4 cells (Fig. 3A, lanes 2–5) but not in control Jurkat cells (Fig. 3A, lane 1). Whole-cell extracts from primary ATL cells were assayed for NF-κB activation by EMSA analysis using an NF-κB consensus probe, and the results also demonstrated constitutive, NF-κB-specific DNA binding activity (data not shown). Coimmunoprecipitation assays using anti-Tax Ab were performed in control Jurkat and HTLV-I-transformed MT2 cells (Fig. 3B). As previously demonstrated (38), Tax immunoprecipitation coprecipitated IKK-γ/NEMO in MT2 cells (Fig. 3B, lane 4) but not in Jurkat cells (Fig. 3B, lane 2).

![FIGURE 2](image)

**FIGURE 2.** Analysis of regulatory domains within the human IRF-4 promoter. A, Full-length (1.2 kb) and deletion mutants of the human IRF-4 promoter used in luciferase analysis of IRF-4 promoter activity in HTLV-I-transformed MT2 cells are represented. MT2 cells (10^6) were transfected by Fugene 6 with 0.2 μg of each reporter construct and 25 ng of pRLTK normalizing vector. Empty pFlag-CMV-2 vector was added to each sample to bring the total amount of DNA to 2 μg per sample. Cells were assayed for luciferase activity at 46 h post-transfection; percentages represent firefly luciferase units corrected for transfection efficiency by Renilla luciferase activity relative to luciferase activity of the full-length 1.2-kb promoter in MT2 cells. Results presented are representative of at least three independent experiments. B, Schematic representation of the IRF-4 promoter. The sequence of the proximal −200 to −700 region of the IRF-4 promoter, major consensus binding sites for NF-κB (κB1 and κB2), NF-AT (NF-1, NF-2, and NF-3), AP-1, and Ets-1 transcription factors and CD28 response element (CD28RE) are shown in boxes. Transcription factor binding sites were determined by searching against the TRANSFAC database using MatInspector algorithm. Sequences used as probes for EMSA are indicated in bold. Arrows correspond to primers used in genomic footprinting experiments. Primers 1–3 and 4–6 were designed to analyse the −600 to −400 and −460 to −260 regions of the promoter, respectively.

![FIGURE 3](image)

**FIGURE 3.** NF-κB activation in HTLV-I-infected cells. A, Whole-cell lysates (500 μg) from control Jurkat or PMA-treated Jurkat (50 ng/ml for 15 min) and HTLV-I-infected MT2, C8166, and MT4 T cell lines were immunoprecipitated with rabbit polyclonal Ab directed against IKKγ. Immunoprecipitates were incubated in kinase reaction buffer with 10 μCi [γ-^32P]ATP for 30 min at 30°C. Substrates for the kinase reactions were GST-IκBα (aa 1–55) (lanes 1–4) or GST-IκBα (aa 1–55) with Ser32/36 Ala substitutions, which cannot be phosphorylated by IKK (lanes 5–8). B, Jurkat (lane 2) and MT2 (lane 4) whole cell lysates (1 mg) were immunoprecipitated with rabbit polyclonal anti-Tax Ab. Immunoprecipitated complexes were resolved by 8% SDS-PAGE and immunoblotted with anti-IKKγ (lower panel) or anti-Tax (upper panel).
NF-AT activation in HTLV-I-transformed cells

NF-AT transcription factors play a pivotal role in inducible gene expression during the immune response. At least five structurally related subsets of NF-AT proteins differentially expressed in various classes of immune and nonimmune cells (39) have been implicated in the direct regulation of cytokines, cell surface receptors, and transcription factors (39–41). HTLV-I-transformed and Tax-expressing T cell lines are characterized by constitutively dephosphorylated, active NF-AT proteins in the nucleus (42); a role for NF-AT proteins in mediating inducible gene expression during the course of HTLV-I infection in T lymphocytes has been established through analysis of the IL-2 CD28RE enhancer element, which identified constitutive protein-DNA complexes containing NF-AT proteins (43). To assess NF-AT DNA binding activity in HTLV-I-infected MT2 and ATL cells (ATL2, Fig. 1C, lane 3), EMSA was performed using a consensus NF-AT oligonucleotide probe. An inducible protein-DNA complex was detected in MT2 and ATL2 cells (Fig. 4, A and B, lane 2) but not in control Jurkat cells (Fig. 4, A and B, lane 1). Supershift analysis using NF-AT-specific Abs revealed that inducible complexes were composed of NF-ATp (NF-AT1/NF-ATc2) in MT2 extracts (Fig. 4A, lane 4) and NF-ATp/NF-ATc (NF-AT2/NF-ATc1) in ATL2 extracts (Fig. 4B, lanes 3 and 4). Binding specificity of these complexes was confirmed by competition using 100-fold molar excess of NF-AT consensus oligonucleotide (Fig. 4, A and B, lane 11) and the corresponding mutant NF-AT probe (Fig. 4, A and B, lane 12). Thus, in HTLV-I-transformed MT2 and ATL2 cells, NF-AT DNA binding activity is constitutive.

Analysis of in vivo occupancy of the κB1 and κB2 sites in Jurkat and MT2 cells

Activation of IRF-4 expression by NF-κB was studied by in vivo genomic footprinting in HTLV-I-transformed cells. Primers were designed to analyze in vivo protein-DNA interactions in the −600 to −400 region of the IRF-4 promoter containing the κB1 and κB2 binding sites (primers 1, 2, and 3, Fig. 2B). Analysis was performed on the noncoding strand because the κB1 and κB2 sites are G rich on this strand. Protection of the κB1 site was observed in MT2 cells but not in control Jurkat cells (Fig. 5A, compare lanes 1 and 3) and comparison of the in vivo and naked DNA pattern obtained from MT2 cells revealed decreased methylation of −429G, −430G, and −431 G residues (Fig. 5A, lanes 2 and 3). Modifications with respect to the methylation pattern within the κB1 site of the IRF-4 promoter are represented by densitometric scanning in Fig. 5A, upper right panel, with arrows indicating the position of the three G residues showing decreased methylation in MT2 cells. In contrast, no occupancy of the κB2 site was observed by in vivo genomic footprinting or EMSA (data not shown) in Jurkat or MT2 cells. Interestingly, analysis of this region also showed significant modification at the Ets-1 binding site (−483 to −492). Significant hypermethylation of −487G and −493G residues were observed specifically in MT2 cells, suggesting protein-DNA binding at this site in MT2 cells (Fig. 5A, compare lanes 2 and 3). Densitometry scanning of in vivo footprinting within the Ets-1 site of the IRF-4 promoter is represented in Fig. 5A, lower right panel, where the arrows represent G residues showing increased methylation in vivo in MT2 cells.

To identify proteins occupying the IRF-4 κB1 site in HTLV-I-infected cells, EMSA experiments were performed using a probe corresponding to the −467 to −421 region of the human IRF-4 promoter (Fig. 2B). Analysis of DNA binding activity in nuclear extracts from MT2 cells and whole-cell extracts from ATL2 cells revealed subtle differences in complex composition. Anti-c-Rel Abs supershifted the majority of the upper complex detected in HTLV-I-infected MT2 cells (Fig. 5B, upper panel, lane 3). Supershift using anti-p50 revealed the upper complex to be a p50/c-Rel heterodimer (Fig. 5B, upper panel, lane 5). In ATL2, the upper complex did not react with anti-c-Rel Abs (Fig. 5B, lower panel, lane 3) but shifted with anti-p65 and p50 Abs (Fig. 5B, lower panel, lanes 5 and 6), indicating that the upper complex was a heterodimer of p65/p50 and the middle complex was a p50 homodimer. Complex NS was designated nonspecific, based on the failure of 100-fold molar excess of cold κB1 probe or antisera and consensus probes to NF-κB, NFAT, Sp-1, Ap-1, or Ets proteins to specifically compete for formation of this complex (data not shown). κB1 EMSA was also performed using whole-cell extracts derived from primary human T lymphocytes, which demonstrated inducible, specific binding to the κB1 site upon T cell stimulation with PMA/ ionomycin (data not shown).

The requirement for NF-κB in Tax-mediated transactivation of the IRF-4 promoter was further investigated through luciferase analysis performed in Jurkat T cells (Fig. 5C). Overexpression of NF-κB-inducing kinase resulted in a 3-fold induction of the 0.6-kb IRF-4 promoter construct (Fig. 5C). The mutant Tax M47, deficient in the activation of the CREB/ATF pathway, retained the ability to transactivate 0.6-kbIRF4PRO-LUC similar to wild-type levels (Fig. 5C), whereas Tax M22, which is defective in NF-κB
Activation, failed to stimulate the 0.6-kb IRF-4 promoter. Together, these results implicate NF-κB signaling as a necessary component of Tax-mediated activation of the IRF-4 promoter, as demonstrated through constitutive in vivo occupancy of the κB1 site in HTLV-I-infected cells.

Analysis of the Sp-1/NF-2 site in MT2 cells
A second set of primers for genomic footprinting was designed to analyze the noncoding strand of the −660 to −400 region of the IRF-4 promoter containing the CD28RE/NF-1, Sp-1/NF-2, and Ap-1 consensus binding sites (primers 4, 5, and 6, Fig. 2B). Using

FIGURE 5. Binding to the κB1 site of the IRF-4 promoter in HTLV-I-infected cells. A, In vivo genomic footprinting of the noncoding strand of the −660 to −400 region of the IRF-4 promoter was carried out as described in Materials and Methods. Putative NF-κB binding sites, κB1 and κB2, are indicated as well as the Ets-1. Arrows indicate G residues that are protected or hypermethylated in vivo in MT2 cells compared to control lanes. Methylation patterns observed in the −660 to −400 region of the IRF-4 promoter were analyzed by densitometric scanning, using a Hewlett-Packard Scan Jet 4c scanner and NIH Image 1.60 software. Arrows pointing up or down represent increased or decreased in vivo methylation on G residues in MT2 cells. The sequence of the scanned region and position of the methylated G residues in capital letters are indicated below each graph. B, Nuclear extracts (3 μg) from control Jurkat (lane 1) and HTLV-I infected MT2 (lanes 2–6) cells (upper panel) and whole-cell extracts (5 μg) from control (lane 1) and ATL-derived (lane 2–6) PBL (lower panel) were incubated with radiolabeled oligonucleotides corresponding to the κB1 site of the IRF-4 promoter. Arrows indicate the positions of the inducible, HTLV-I-specific and constitutive, nonspecific (NS) complexes. Supershift analysis was carried out with 1 μg of c-Rel (lane 3), p65 (lane 4), p50 (lane 5), or control (lane 6) Abs. C, Jurkat T cells (10⁶) were transfected with 0.2 μg of 0.6-kbPro-pGL3B, 25 ng pRLTK, and a 3-fold molar excess of NIK (lane 2), wild-type Tax (lane 3), M47 Tax (lane 4), or M22 Tax (lane 5). Empty pFlag-CMV-2 vector was added to each sample to bring the total amount of DNA to 2 μg per sample. Cells were assayed for luciferase activity at 48 h post-transfection. Fold induction was calculated relative to the basal level for the reporter gene in the presence of empty vector after correction for transfection efficiency by Renilla luciferase. Results presented are representative of at least three independent experiments.
these primers, protection of several G residues within and adjacent to the Sp-1/NF-2 site (−385G to −413G) was observed in MT2 cells compared to the DNA control (Fig. 6A, lanes 2 and 3). Protection of the −388, −396 to −398, −402, −410, −412, and −413 G residues within and around the Sp-1/NF-2 site was specific to HTLV-I-transformed cells and was not detected in control Jurkat cells (Fig. 6A, compare lanes 1 and 3). Changes in methylation within this site of the IRF-4 promoter in MT2 cells was further analyzed by densitometric scanning (Fig. 6A, right panel), with arrows indicating the eight G residues within and adjacent to NF-2/Sp-1 that exhibit a decrease in methylation in MT2 cells.

To identify the complex(es) inducing strong methylation protection at the Sp-1/NF-2 site, EMSA was performed using an oligonucleotide corresponding to the −413 to −378 region of the IRF-4 promoter (Fig. 6B). EMSA analysis revealed two inducible complexes binding to the Sp-1/NF-2 probe in MT2 cells compared to control T4 cells (Fig. 6B, left panel, lanes 1 and 2). Competition analysis demonstrated that inducible complexes were diminished by a consensus Sp-1 oligonucleotide (Fig. 6B, left panel, lane 6) but not with the corresponding mutant oligonucleotide (Fig. 6B, left panel, lane 7); furthermore, supershift with Sp-1 Ab demonstrated that the upper complex was specifically displaced (Fig. 6B, left panel, lanes 3 and 4). MT2-specific complexes were not removed by competition with heterologous NF-AT or Ets oligonucleotides, nor were they supershifted with NF-AT or Ets specific Abs (data not shown). EMSA with Sp-1/NF-2 was also performed with primary ATL2 cell extracts (Fig. 6B, right panel) and primary T lymphocytes treated with PMA/ionomycin (data not shown) with the same results. These data indicate that occupancy of the Sp-1/NF2 region of the IRF-4 promoter in HTLV-I cells is mediated through Sp-1 binding to at least two adjacent sites.

**Analysis of in vivo and in vitro occupancy of the CD28RE site in MT2 cells**

The CD28-response enhancer element (CD28RE) plays an important role in the regulation of gene expression during the T cell activation response. CD28RE mediates activation of several lymphoid-specific genes such as IL-2, GM-CSF, and CD40 ligand (44–46), through binding of numerous transcriptional activators, including Fos/Jun (AP-1), NF-κB, and NF-AT proteins. Furthermore, the IL-2 CD28RE is a direct target for HTLV-I Tax-mediated trans-activation (39, 43, 47). IRF-4 promoter deletion analysis in MT2 cells indicated that the CD28RE element may function in activation of IRF-4 expression in HTLV-I-infected cells (Fig. 2A).
Consistent hypermethylation of −270G residue adjacent to the CD28RE site was observed in MT2 cells but not in Jurkat cells, suggesting binding of one or several transcription factors involved in IRF-4 regulation near this site (Fig. 7A, lane 3). Hypermethylation of the IRF-4 promoter adjacent to the CD28RE site was quantified by densitometric scanning (Fig. 7A, right panel); the arrow represents the −270 G residue and demonstrates a reproducible increase in methylation that is specific to MT2 cells. Further analysis of the coding strand would have been necessary to provide a detailed characterization of promoter occupancy in the CD28RE region (including the NF-1 site) of the IRF-4 promoter by in vivo genomic footprinting. However, PCR amplification was not successful using antisense primers because of the fact that the highly GC-rich region impaired PCR-mediated elongation. Because of the low abundance of G residues in the noncoding strand of the CD28RE site, no modification of the methylation pattern was detected in either Jurkat cells or in MT2 cells (Fig. 7A, lanes 1 and 3) compared to naked DNA.

EMSA analysis using the CD28RE (−305 to −263) demonstrated specific protein-DNA complexes in nuclear extracts from HTLV-I-infected MT2 cells (Fig. 7B, lane 2). Supershift analysis with NF-κB Abs identified c-Rel and p50 (Fig. 7B, lanes 3 and 4) but not p65 (Fig. 7B, lane 5) binding in MT2 cells. Because the CD28RE binds several transcription factors, other competition analyses were performed, demonstrating that the NF-AT-specific oligonucleotide (Fig. 7B, lane 6) but not the mutant oligonucleotide removed a specific complex on the CD28RE element in HTLV-I-infected MT2 nuclear extracts (Fig. 7B, lane 7). Supershift with NF-AT-specific Abs identified NF-ATp bound to the CD28RE in MT2 cells (Fig. 7B, lane 8). However, complexes bound to the IRF-4 CD28RE did not react with Abs to AP-1, Sp-1, or Ets (data not shown), indicating that the IRF-4 CD28RE binds both NF-κB (c-Rel and p50) and NF-AT (NF-ATp) in HTLV-I-infected cells. Using whole cell extracts from primary human T lymphocytes, CD28RE-specific EMSA demonstrated inducible and specific binding in whole-cell extracts stimulated with PMA/ionomycin, suggesting that the IRF-4 CD28RE is occupied in primary, activated T lymphocytes (data not shown). To complement footprinting studies, EMSA experiments were performed using DNA probes corresponding to each potential transcription factor consensus sequence identified within the −367 to −209 region of the IRF-4 promoter. In accordance with in vivo footprinting data,
In HTLV-I-infected cells, formaldehyde cross-linking and chIP assays (33) were performed in IRF-4-expressing, HTLV-I-infected MT2 cells, confirming that de novo IRF-4 induction in response to Tax expression (Fig. 8A), requiring both the κB1 and Sp-1 elements while retaining the CD28RE element. Wild-type and mutated IRF-4 promoter fragments were analyzed by PCR amplification from DNA immunoprecipitated with Abs to NF-ATp, c-Rel, p50, and Sp-1 elements, respectively (Fig. 8C). These data indicate that de novo IRF-4 induction in response to Tax expression requires both the κB1 and CD28RE sites of the human IRF-4 promoter.

To confirm constitutive promoter occupancy of the κB1, Sp-1/ NF-2, and CD28RE elements of the IRF-4 promoter in HTLV-I-infected MT2 cells, formaldehyde cross-linking and chIP assays (33) were performed in IRF-4-expressing, HTLV-I-infected MT2 cells (Fig. 8B, upper panel) and control Jurkat (Fig. 8B, lower panel) cells. A 250-bp sequence, corresponding to the κB1 through CD28RE sites of the IRF-4 promoter, was PCR amplified from DNA immunoprecipitated with Abs to NF-ATp, c-Rel, p50, and Sp-1 (Fig. 8B, lanes 4–7), to a degree significantly above control IRF-3 or no Ab samples. Pulldown of IRF-4-specific regulatory sequences was limited to MT2 cells and was absent from control Jurkat cells, which demonstrated constitutive binding of specific transcription factors to the κB1, Sp-1/ NF-2, and CD28RE sites in HTLV-I-infected cells. These results are summarized in a schematic representation of human IRF-4 promoter occupancy in HTLV-I-infected cells (Fig. 8C).

**Discussion**

This study presents the first detailed analysis of the transcriptional mechanisms governing human IRF-4 gene activation in T cells infected and transformed with HTLV-I. Our results demonstrate that, in HTLV-I-infected cells, Tax-mediated IRF-4 expression is achieved through the recruitment of transcriptional activators to three sites within the −617 to −209 region of the human IRF-4 promoter—the κB1, Sp-1, and CD28RE enhancer elements. Transient coexpression assays indicate that two of the sites, κB1 and CD28RE, are absolutely essential for Tax-mediated trans-activation of the human IRF-4 promoter T cells.

Gene knockout analysis has demonstrated that c-Rel is essential for IRF-4 production in primary murine B and T lymphocytes (48). IRF-4 expression was completely blocked in response to Ag-mimetic CD3/CD28 stimulation in c-Rel-deficient lymphocytes, and c-Rel was shown to bind κB enhancers within the murine IRF-4

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**Figure 8.** κB1 and CD28RE are required for Tax-mediated transactivation of the IRF-4 promoter. A, Wild-type and mutated IRF-4 promoter constructs used in luciferase analysis of Tax-mediated IRF-4 activity are represented. Jurkat T cells (10⁶) were transfected with 0.2 μg of 0.6-kb PRO-pGL3B wild type (lanes 1 and 2), 0.6-kb PRO pB1−mut-pGL3B (lane 3), 0.6-kb PRO Sp1−mut-pGL3B (lane 4), and 0.6-kb PRO ΔCD28RE-pGL3B (lane 5). pRLTK (25 ng) and a 3-fold molar excess of wild-type pFlag-CMV-2 vector to bring the total amount of DNA to 2 μg per sample. Cells were assayed for luciferase activity at 48 h post-transfection. Fold induction was calculated relative to the basal level for the reporter gene in the presence of empty vector after correction for transfection efficiency by Renilla luciferase. Results presented are representative of at least three independent experiments. B, Abs to NF-ATp, c-Rel, p50, and Sp-1 enrich IRF-4 promoter sequences in chIP assay. Proteins were cross-linked to DNA by formaldehyde treatment in unstimulated Jurkat (lower panel) and HTLV-I-infected MT2 (upper panel) cells and protein-DNA complexes were isolated by immunoprecipitation using Abs (4 μg) specific to IRF-3 (lane 3), NF-ATp (lane 4), c-Rel (lane 5), p50 (lane 6), and Sp-1 (lane 7). Precipitated DNA was analyzed by PCR for the presence of the proximal IRF-4 promoter region, spanning the CD28RE to the κB1 site. Amplification products were analyzed on a 5% long-range sequencing gel and visualized by autoradiography. C, Schematic representation of the protein-DNA interactions regulating the human IRF-4 promoter. Organization of the IRF-4 regulatory domains within the −500 to −200 region of the IRF-4 promoter, including the κB1 to κB2, Ets-1, Sp-1, and CD28RE, is shown at the top. In HTLV-I-infected cells, constitutive binding is observed at the κB1, Ets-1, Sp-1, and CD28RE sites, corresponding to p50/p65 or p50/c-Rel binding to κB1, Sp-1 binding to two adjacent Sp-1 sites, and p50/c-Rel and NF-ATp binding to the CD28RE, as shown on the bottom.
promoter (48). Our data extend these studies and establish an important role for NF-κB in HTLV-I-induced activation of the human IRF-4 promoter in ATL cells. Tax-mediated activation of the IKK complex in HTLV-I-transformed cells correlates with constitutive in vivo occupancy at the κB1 site of the IRF-4 promoter that is not occupied in control Jurkat cells. κB1 is a classical NF-κB sequence that binds p65/p50 or c-Rel/p50 complexes in primary ATL and MT2 extracts, respectively. Furthermore, abrogation of IRF-4 promoter activity in response to Tax M22 expression indicates that NF-κB activity is absolutely required for Tax-mediated IRF-4 gene induction in HTLV-I-infected T lymphocytes.

The CD28RE site of the IRF-4 promoter functions as a more complex regulatory element, and our results are consistent with previous studies examining the CD28RE within the context of the IL-2 promoter. The CD28RE is essential for IL-2 production by CD3/CD28 stimulation (43, 49) and functions as a Tax-responsive element in HTLV-I-induced IL-2 production (46, 47). CD28RE binds several different transcriptional regulatory proteins (46, 50–52). In the present study, the IRF-4 CD28RE interacted with c-Rel and p50, as demonstrated through EMSA analysis and chIP in HTLV-I-infected cells. Furthermore, we have correlated constitutive NF-AT DNA binding activity in the MT2 cell line and primary leukemic ATL cells with occupancy of the IRF-4 CD28RE by NF-ATp in HTLV-I-infected cells. Overexpression of NF-ATp in Jurkat T cells synergizes with NK to activate the CD28RE of the human IRF-4 promoter in the reporter gene assay, indicating that these two factors are involved in IRF-4 CD28RE trans-activation.

HTLV-I Tax-mediated induction of cellular gene expression is required during the early stages of viral pathogenesis for the establishment of infection. Hence, HTLV-I infection of a mature CD4$^+$ T lymphocyte is directly associated with up-regulation of numerous cellular genes involved in T cell activation and proliferation, such as IL-2, IL-2Ra, IL-15, c-fos, and Fas ligand (23, 53–55). The classical example of this phenomenon is HTLV-I-associated induction of the IL-2 gene, a hallmark of the early phase of HTLV-I infection (22, 56, 57). IL-2, as well as IL-15, induction by the Tax oncprotein is believed to initiate an autocrine/paracrine loop that drives the polyclonal proliferation of HTLV-I-infected cells (26, 58, 59). IL-dependent proliferation during the early stages of infection may facilitate the accumulation of multiple genetic mutations that contribute to the transition from immortalization to transformation (26, 58, 59).

Regulation of the IRF-4 gene in T lymphocytes displays striking similarities to that of the IL-2 promoter. IRF-4 expression is inducible through stimuli that mimic TCR engagement by Ag, such as PMA/ionomycin, con A, or anti-CD3/CD28 treatment (10, 11, 18). Induction of IRF-4 expression in primary T cells is also blocked by treatment with the immunosuppressive drug FK506, which suggests that changes in intracellular calcium concentration—an early feature of T cell activation—is required for IRF-4 induction. The parallels between IL-2 and IRF-4 expression imply that their continuous production within the context of HTLV-I infection may involve common mechanisms. Like IL-2, IRF-4 expression in HTLV-I-transformed cells is Tax dependent, and transient transfection of the tax gene induces low levels of IRF-4 expression in Jurkat T cells (11). In this study we have demonstrated that overexpression of Tax in Jurkat T cells stimulates transcriptional activity within the −617 to −209 region of the human IRF-4 promoter through the κB1, Sp1, and CD28RE sites.

Continuous production of IRF-4 in HTLV-I-transformed cells suggests that IRF-4 may contribute to virus-induced leukemogenesis. In fact, studies examining the physiological role of IRF-4 in T cells are consistent with such an interpretation. Knockout analysis revealed that the IRF-4 gene is essential for the function and homeostasis of mature B and T lymphocytes. Although mice lacking IRF-4 displayed a normal distribution of mature B and T cells in the periphery at 4–5 wk of age, the lymphocyte population was severely impaired at the level of late-stage activation events. IRF-4$^{-/-}$ T lymphocytes maintained normal early activation responses such as calcium influx and expression of CD25 and CD69, but exhibited a dramatic reduction with respect to proliferation and cytokine production in response to anti-CD3 stimulation, con A, or bacterial superantigen staphylococcal enterotoxin A treatment (60). This phenotype could not be rescued by exogenous IL-2 treatment, which suggests that IRF-4 may be involved during the later stages of the T cell activation response. A role for IRF-4 expression in the development of certain lymphomas was further emphasized through a link to multiple myeloma, a hyperproliferative disorder of terminally differentiated B cells. Multiple myeloma has been associated with a t(6;14)(p25;q32) translocation that juxtaposes the IgH chain locus regulatory region to the IRF-4 coding sequence (61). As a result, MUM/IRF-4 is overexpressed, an event that has been implicated in leukemogenesis because IRF-4 overexpression is capable of transforming Rat-1 fibroblasts in vitro (62). Tax-driven IRF-4 expression in HTLV-I-infected cells suggests a role for IRF-4 in reprogramming T cell gene expression. Constitutive IRF-4 expression in HTLV-I-infected cells may reprogram T cell-specific gene expression; further studies will identify and characterize IRF-4 target genes in an effort to further characterize the role of the IRF-4 transcription factor in HTLV-I pathogenesis.

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