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J Immunol 2002; 169:3120-3130; doi: 10.4049/jimmunol.169.6.3120
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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 Hiscox‡*†

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-xB and NF-AT pathways, resulting in the binding of p50, p65, and c-Rel to the xB1 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 xB1 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 E125–1 (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/sodium 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). Disregulation 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-kB, 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., MD). 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 CD4+ cells were CD4+CD8−CD25+. PMA and ionomycin (Calbiochem, San Diego, CA) were used at a final concentration of 10 ng/ml and 200 mM, respectively, for immunoblot analysis and PMA was used at a final concentration of 50 ng/ml for in vitro culture. CEM, MT2, and Jurkat T cells were cultured in RPMI 1640 medium (CLT) supplemented with 10% 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% 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, MD). 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 CD4+ cells were CD4+CD8−CD25+. PMA and ionomycin (Calbiochem, San Diego, CA) were used at a final concentration of 10 ng/ml and 200 mM, respectively, for immunoblot analysis and PMA was used at a final concentration of 50 ng/ml for in vitro culture. CEM, MT2, and Jurkat T cells 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., MD). 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 CD4+ cells were CD4+CD8−CD25+. PMA and ionomycin (Calbiochem, San Diego, CA) were used at a final concentration of 10 ng/ml and 200 mM, respectively, for immunoblot analysis and PMA was used at a final concentration of 50 ng/ml for in vitro culture.

Plasmid construction and mutagenesis

1.2-kbIRF-4P-3PLGB3 luc was generated by cloning the human promoter of IRF-4 (27) into SacI/BglII digested luciferase reporter plasmid pGL3 basic (pGL3B) using specific primers (upstream primer 5'-GAGCTCAT GAATAACCCGTGTCAC-3' and downstream primer 5'AGATCTT GAAGCGAGCCTTGTC-3') to amplify the 1.2-kbIRF-4P-3PLGB3 fragment from SacI/BglII digested pGL3B. Primer annealing and ligation conditions are as detailed by manufacturer (Promega). All firefly luciferase values were normalized to Renilla luciferase to control for transfection efficiency, and results shown represent the average of at least three independent experiments.

Immunoprecipitations and kinase assay

For in vitro kinase assay, whole cell lysates were immunoprecipitated with IkB kinase (IKK) γ-specific, rabbit polyclonal Ab (Santa Cruz Biotechnology). Immunoprecipitated IKK complexes were incubated in kinase reaction buffer consisting of 10 μCi [γ-32P]ATP, 1 mM ATP, 5 mM MgCl2, 1 mM dithiothreitol, 100 mM NaCl, and 50 mM Tris-HCl (pH 8) at 30°C for 30 min with a GST-IκBαS35–55 or GST-2NkBαS35–55 substrate, which contains two point mutations of serines 32 and 36 to alanine. Following fractionation of samples by SDS-PAGE on a 10% polyacrylamide gel, the upper half of the gel was transferred to a nitrocellulose membrane and blotted with IKK-β-specific mouse monoclonal antiserum (Alescos Biochemicals, San Diego, CA) at a final concentration of 1 μg/ml; the lower half of the gel was stained with Coomassie blue for 30 min, destained in 10% methanol/10% acetic acid for 1 h, dried, and exposed to Biomax XR film (Kodak, Rochester, NY) for 2 h at room temperature. For IKK-γ coimmunoprecipitation assays, cell extracts were immunoprecipitated with rabbit polyclonal Tax Ab (kind gift from Dr. W. C. Greene) cross-linked to protein A-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) using dimethyl pimelidimide (Sigma-Aldrich). Incubations were harvested by 30 μl of beads cross-linked to 1 μg anti-Tax or no Ab for 4 h at 4°C. Immunocomplexes were washed four times in Nonidet P-40 lysis buffer, eluted, resolved by SDS-PAGE, and blotted with anti-Tax and anti-IKK-γ.

In vivo genomic footprinting

For in vivo methylation by dimethyl sulfate (DMS; Aldrich Chemical, Milwaukee, WI), exponentially growing MT2 or Jurkat cells (106) were harvested and resuspended in RPMI 1640/10% heat-inactivated FBS supplemented with 20 mM HEPEs (pH 7.3). Methylation treatment was performed in the presence of 10 μl/ml concentrated DMS for 1 min at room temperature. Reaction was stopped by two washes with ice-cold PBS containing 2% 2-ME. Genomic DNA extraction was performed as previously described (32). To obtain naked DNA, cells were first lysed to extract genomic DNA and then submitted to DMS treatment for 30 min at room temperature. Genomic DNA resuspended in 200 μl H2O was treated with 20 μl of piperidine (Aldrich) for 30 min at 90°C to cleave methylated G (or A) residues. For genomic footprinting, 5 μg of DNA was digested with 70 μl of restriction enzyme, and ligated to an excess of pGL3B. Ligated DNA was used for ligation-mediated PCR using Vent DNA polymerase (New England Biolabs, Mississauga, Canada), as described elsewhere (33, 34). To ensure elongation of different fragment sizes, the PCR amplification step was 2 min for the first cycle and progressively increased to 10 min in the last cycle, with a total of 18 cycles. A third primer was radiolabeled by end labeling using T4 polynucleotide kinase (Amerham) and [γ-32P]ATP (ICN Pharmaceutica, San Francisco, CA). Two more PCR cycles were performed to labeled elongated DNA. Each reaction product was phenol-chloroform extracted and ethanol precipitated prior to electrophoresis on a 7.5% Sequencing gel (Baker, Phillipsburg, NJ) in 1x TBE at 65 W. Reactions were visualized by autoradiography using Biomax MR films (Kodak). For the ligation-mediated PCR, the following sets of primers were used: for analysis of the noncoding strand of the −600 to −400 region of the 4-IRF-4 promoter, primer 1 5'-GTCATCACTTACCTCACGACGC-3', Tm 58°C; primer 2 5'-GCAAAGGATGTATAGCTGATC-3', Tm 63°C; primer 3 5'-GTAAGCATGTACGACGCA-3'.
GACAGTTATTGTG-3', Tm 65°C; for analysis of the noncoding strand of the —460 to —260 region of the IRF-4 promoter, primer 4, 5'-GTGATGCTCTGGCCGGA-3', Tm 60°C; primer 5, 5'-GCAAACCTCCACCTCTCAATTTCCTC-3', Tm 63°C; primer 6, 5'-ACCTCGAGTCCTCCTTGGACCAT-3', Tm 65°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 7.9), 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'-CCAAAGAGGAAAATTTGTTTCATA-3'; NF-AT mutant, 5'-GGCTCCAATTTGTTTCATA-3'; NF-ATp (Upstate Biotechnology), rabbit polyclonal anti-IRF-3 (Santa Cruz Biotechnology), rabbit polyclonal anti-Sp-1 (Santa Cruz Biotechnology), rabbit polyclonal anti-cRel, anti-NF-ATc3 (Santa Cruz Biotechnology), anti-NF-κB, anti-p50, anti-p65, anti-p52, anti-IRF-3, anti-Sp-1, anti-NF-ATc3 (Santa Cruz Biotechnology), or no Ab overnight at 4°C. Prior to dilution, 20% glycerol was added directly to the growth medium at a final concentration of 200 mM and incubation at 65°C, Tm6 6°C. Induction of IRF-4 protein in primary T lymphocytes was inhibited by the immunosuppressive drug FK506 (Fig. 1A, lane 8). IRF-4 protein expression has been shown to be constitutively produced in a number of EBV-infected, transformed B cell lines and HTLV-I-infected, transformed T cell lines (16). Fig. 1B demonstrates IRF-4 overexpression in the HTLV-I-transformed MT2, C8166, and MT4 cell lines (Fig. 1B, lanes 1, 2, and 3), whereas IRF-4 is not detected in noninfected Jurkat, CEM, or T4 cells (Fig. 1B, lanes 4, 5, and 6).

Results

IRF-4 expression patterns in T lymphocytes

Initial experiments sought to analyze the pattern of IRF-4 expression in primary T lymphocytes, HTLV-I-infected cell lines, and PBL isolated from HTLV-I-infected patients. Fig. 1A demonstrates IRF-4 expression in T lymphocytes (Fig. 1A, lane 1), but induced after treatment with PMA/ ionomycin (Fig. 1A, lanes 2–6). Induction of IRF-4 protein in primary T lymphocytes was inhibited by the immunosuppressive drug FK506 (Fig. 1A, lane 8). IRF-4 protein expression has been shown to be constitutively produced in a number of EBV-infected, transformed B cell lines and HTLV-I-infected, transformed T cell lines (16). Fig. 1B demonstrates IRF-4 overexpression in the HTLV-I-transformed MT2, C8166, and MT4 cell lines (Fig. 1B, lanes 1, 2, and 3), whereas IRF-4 is not detected in noninfected Jurkat, CEM, or T4 cells (Fig. 1B, lanes 4, 5, and 6). Treatment with anti-CD3/CD28 mAbs, PMA/ionomycin, or Con A did not further increase IRF-4 levels in HTLV-I-transformed cell lines (data not shown), indicating 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

FIGURE 1. IRF-4 expression in T lymphocytes. Whole cell extracts were prepared from (A) primary human T lymphocytes (A), HTLV-I-infected MT2, C8166, and MT4 or control Jurkat, CEM, and T4 T cell lines (B), and PBL isolated from patients with ATL or HAM/TSP (C). Primary T lymphocytes were stimulated for various times with 10 ng/mL PMA and 200 nmol ionomycin. Treatment with FK506 was carried out at a final concentration of 1 μM for 1 h before PMA/ionomycin stimulation. Whole cell extracts (A, 25 μg; B, 40 μg; and C, 100 μg) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-IRF-4 and anti-actin Abs, as described in Materials and Methods.
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, PMTA-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 immunoprecipitated coreprecipitated IKK-γ/NEMO in MT2 cells (Fig. 3B, lane 4) but not in Jurkat cells (Fig. 3B, lane 2).

![Image](http://www.jimmunol.org/DownloadedFrom/Image)
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 phosphorylated, 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-AT-c2) in MT2 extracts (Fig. 4A, lane 4) and NF-ATp/NF-ATc (NF-AT2/NF-AT-c1) 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 −431G 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 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 −447 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). Supernatant anti-p50 revealed the upper complex to be a p50/κB dimer. 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/A23187 (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-kb IRF-4PRO-LUC similar to wild-type levels (Fig. 5C), whereas Tax M22, which is defective in NF-κB activity, showed no transactivation.

**FIGURE 4.** NF-AT activation in HTLV-I-infected cells. Nuclear extracts (3 μg) from control T4 (lane 1) and MT2 (lanes 2–12) cells (A) and whole-cell extracts (5 μg) from control (lane 1) and ATL-derived PBL (lanes 2–12) (B) were incubated with radiolabeled oligonucleotides corresponding to a consensus NF-AT binding site, as described in Materials and Methods. Arrows indicate the positions of the inducible, HTLV-I-specific complex. Supershift analysis was carried out with 1 μg of NF-ATc Ab (lanes 3, 6, 8, and 9), NF-ATx Ab (lanes 5 and 7–9) or control (lane 10) and 1 μL of a 1/10 dilution of NF-ATp Ab (lanes 4, 6, 7, and 9). Competition assays were carried out using 100-fold molar excess of NF-AT-wt (lane 11) or NF-AT-mutated (lane 12) oligonucleotide as competitor.
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 analyse the noncoding strand of the −600 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
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. 2B). 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).

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**FIGURE 6.** Binding to the Sp-1/NF-2 site of the IRF-4 promoter in HTLV-I-infected cells. A. In vivo genomic footprinting of the noncoding strand of the −460 to −260 region of the IRF-4 promoter was carried out as described in Materials and Methods. Putative NF-AT binding sites, NF-1, NF-2, and NF-3, are indicated. Methylation patterns observed in the −460 to −260 region of the IRF-4 promoter were analyzed by densitometry scanning. Arrows pointing down represent decreased in vivo methylation on G residues in MT2 cells. The sequence of the scanned region and the position of methylated G residues in capital letters are indicated below each graph. B. Nuclear extracts (1 μg) from Jurkat (lane 1) and HTLV-I-infected MT2 (lanes 2–7) cells (right panel) and whole-cell extracts from noninfected cells (lane 8) and primary ATL2 cells (lanes 9–13) (left panel) were incubated with radiolabeled oligonucleotides corresponding to the Sp-1/NF-2 site of the IRF-4 promoter. Arrows indicate the positions of the inducible, HTLV-I-specific complexes. Supershift analysis was carried out with 1 μg of Sp-1 (lanes 4 and 12) or control (lanes 3 and 13) Abs, and competition assays were carried out using 100-fold molar excess of cold probe (lane 5), Sp-1-wt (lanes 6 and 10), or Sp-1-mutated (lanes 7 and 11) consensus oligonucleotide as competitor.
Consistent hypermethylation of $-270 G$ 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 to the CD28RE 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-kB (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, 

FIGURE 7. Binding to the CD28RE site of the IRF-4 promoter in HTLV-I-infected cells. A, In vivo genomic footprinting of the noncoding strand of the $-460$ to $-260$ region of the IRF-4 promoter was carried out as described in Materials and Methods. The position of the CD28RE is indicated and arrows indicate hypermethylated G residues. Methylation patterns observed in the CD28RE region of the IRF-4 promoter are represented by densitometric scanning. Arrow pointing up represents increased in vivo methylation on G residues in MT2 cells. The sequence of the scanned region, where the methylated G residues are in capital letters, is indicated below each graph. B, Nuclear extracts (10 μg) from Jurkat (lane 1) or HTLV-I-infected MT2 (lanes 2–10) cells were incubated with radiolabeled oligonucleotide corresponding to the CD28RE site of the IRF-4 promoter. Arrows indicate the positions of the inducible, HTLV-I-specific complexes. Supershift analysis was carried out with 1 μg of c-Rel (lane 3), p50 (lane 4), p65 (lane 5), NF-ATc (lane 9), and NF-ATx (lane 10) Abs and 1 μl of a 1/10 dilution of NF-ATp (lane 8) Ab. C. Jurkat T cells (10^6) were transfected with 0.2 μg of 0.4-kb pPRL-RLuc plasmid, 25 ng pRL-TK, and a 3-fold molar excess of NIK (lane 2), NF-ATpXS (lane 3), NIK + NF-ATpXS (lane 4), wild-type Tax (lane 5), M47 Tax (lane 6), or M22 Tax (lane 7). 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.
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

FIGURE 8. κB1 and CD28RE are required for Tax-mediated trans-activation 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-gpGL3B wild type (lanes 1 and 2), 0.6-kb PROκB1-gpGL3B (lane 3), 0.6-kb PROSp1-gpGL3B (lane 4), and 0.6-kb PROκB1/CD28RE-gpGL3B (lane 5). pRLTK (25 ng) and a 3-fold molar excess of wild-type Tax (lanes 2–5) were added with 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 p65 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 oncoprotein 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 immunomodulation 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, Sp-1, 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.

Acknowledgments
We thank the following researchers: Dr. Warner C. Greene (Gladstone Institute, University of California) for the rabbit polyclonal Tax Ab and wild-type, M22, and M47 Tax expression vectors; Dr. S. C. Sun at the M. S. Hershey Medical Center (Pennsylvania State University) for the NF-ATpXS expression vector; and Dr. Tak Mak (AMGEN Research Institute) for the human IRF-4 promoter-containing construct. We also thank members of the Molecular Oncology Group (Lady Davis Institute, McGill University) for helpful discussions.

References


