

Possible Involvement of Interferon Regulatory Factor 4 (IRF4) in a Clinical Subtype of Adult T-Cell Leukemia

Yoshitaka Imaizumi,^{1,2} Tomoko Kohno,¹ Yasuaki Yamada,³ Shuichi Ikeda,⁴ Yuetsu Tanaka,⁵ Masao Tomonaga² and Toshifumi Matsuyama^{1,6}

¹Division of Cytokine Signaling, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Medical Science, ²Department of Hematology, Atomic Disease Institute, ³Department of Laboratory Medicine, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, ⁴Department of Medicine, City of Sasebo General Hospital, 9-3 Hirase-cho, Sasebo 857-8511, ⁵Department of Infectious Disease and Immunology, Okinawa-Asia Research Center of Medical Science, Faculty of Medicine, University of the Ryukyus, 207 Aza-uehara, Nishihara-cho, Nakagami-gun, Okinawa 903-0215

Interferon regulatory factor (IRF) 4 is the lymphoid-specific transcription factor that is required for the proliferation of mitogen-activated T cells. IRF4 has been suggested to be involved in tumorigenesis because the overexpression of IRF4 caused the transformation of Rat-1 fibroblasts *in vitro*. Here, we show that IRF4 is constitutively expressed in adult T-cell leukemia (ATL)-derived cell lines, which were infected with human T-cell leukemia virus type-I, but hardly expressed the trans-activator protein, Tax. Similarly, constitutive expression of IRF4 was demonstrated in freshly isolated peripheral blood mononuclear cells (PBMC) from patients with either acute or chronic ATL. However, the high-level expression of IRF4 was specifically associated with acute ATL. With mitogen-activated PBMC from healthy donors, cell cycle analyses revealed that the induction of IRF4 occurred prior to cell cycle progression and the cells that had entered the cell cycle were predominantly IRF4-positive cells. In addition, ectopic expression of IRF4 in Rat-1 fibroblasts increased the S and G2/M phase population significantly. Taken together, our results indicate that IRF4 is involved in the pathogenesis of ATL through its positive effect on the cell cycle, and that IRF4 can be used as a molecular marker of clinical subtype in ATL.

Key words: IRF4 — ATL — HTLV-I — Tax — Cell cycle

Adult T-cell leukemia (ATL) is a peripheral T cell malignancy caused by human T-cell leukemia virus type-I (HTLV-I).^{1,2} The disease is usually classified into four clinical subtypes: acute, lymphoma, chronic, and smoldering types.³ Chronic and smoldering-type patients have an indolent clinical course, whereas in acute and lymphoma-type patients, the prognosis is very poor. Thus, diagnosis of the latter two types usually becomes an indication for intensive chemotherapy. However, the differential diagnostic criteria of the subtypes of ATL are based on clinical parameters, such as serum lactic dehydrogenase activity, serum calcium level, and invasion of ATL cells to the visceral organs.³ There is no diagnostic molecular marker available at present, that is directly involved in the pathogenesis of ATL.

The development of leukemia in HTLV-I-infected individuals is preceded by a long latent period of 40 to 50 years. In addition, only about 5% of HTLV-I carriers develop ATL. Thus, multistep oncogenic processes have been predicted to be involved in the development of the full malignant phenotype in HTLV-I infected cells.⁴ There is increasing evidence that the viral trans-activating pro-

tein Tax plays a crucial role in cellular transformation. Indeed, Tax transforms fibroblast-derived cells and immortalizes primary human T cells *in vitro* in the presence of interleukin-2.^{5–8} However, as judged from clinical samples, the expression of Tax is rarely detected in most leukemic cells.^{9,10} It is speculated that the expression of viral proteins, including Tax, is not necessary for the proliferation of leukemic cells, but the precise mechanism of the deregulated proliferation of leukemic cells *in vivo* has not yet been clarified.

Interferon regulatory factor (IRF) 4 is a transcription factor whose expression is restricted to lymphoid cells.^{11,12} In peripheral T cells, only weak expression of IRF4 is detectable in the absence of stimuli. However, upon antigen-receptor-mediated stimuli, rapid and transient expression of IRF4 is induced.¹¹ The study of IRF4-deficient mice revealed the importance of IRF4 in T and B cell functions. In murine mature T cells, the deficiency of IRF4 almost abolished the proliferative response to mitogenic stimuli, and it was suggested that IRF4 is involved in late events after T cell activation, such as interleukin-2 production or response to the cytokine.¹³

A possible relationship between IRF4 and oncogenicity was demonstrated in some patients or cell lines of multiple myeloma with the chromosomal translocation

⁶To whom correspondence should be addressed.

E-mail: tosim@net.nagasaki-u.ac.jp

t(6;14)(p25;q32).^{14,15} In such cases, the immunoglobulin heavy-chain was juxtaposed to the IRF4 locus, and as a result, IRF4 became overexpressed. Furthermore, overexpression of IRF4 led to transformation in Rat-1 fibroblasts.¹⁴ In human T cells, a high level of IRF4 expression was preferentially observed in HTLV-I-positive T cell lines, but not other T cell lines, and in ATL cells in lymph nodes.^{16–18} Based on these observations, we intended to investigate the expression of IRF4 in ATL cells freshly isolated from patients of different clinical subtypes. Here, we firstly demonstrate that the expression of IRF4 was constitutively high in HTLV-I positive cell lines, and that this expression was not dependent on the expression level of Tax. Importantly, constitutive expression of IRF4 was observed in primary ATL cells, and high-level expression was specifically associated with the acute type of ATL. We also present evidence that IRF4 was induced prior to cell cycle progression in mitogen-activated T cells.

MATERIALS AND METHODS

Cell culture Jurkat and MOLT4 are human T cell lines. HUT102 and MT-2 are human T cell lines transformed by HTLV-I.^{19–21} ST-1 and KK-1 are human T cell lines derived from ATL patients.²² These cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (50 U/ml), and streptomycin (50 µg/ml) in a humidified incubator containing 5% CO₂ in air. Rat-1,²³ a rat fibroblast cell line kindly provided by Dr. K. Matsumoto (Osaka Red Cross Blood Center, Osaka), was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS.

Patient samples Peripheral-blood samples were drawn from ATL patients before chemotherapy or healthy volunteers, after informed consent had been obtained. The diagnosis was based on clinical features, hematological findings, and serum anti-HTLV-I antibodies. Monoclonal HTLV-I provirus integration into the chromosomal DNA of leukemic cells was confirmed by Southern blot hybridization (data not shown). Subclassification of ATL was made according to previously described criteria.³ Mononuclear cells were obtained by density gradient separation from the peripheral blood of ATL patients. Peripheral blood mononuclear cells (PBMC) from 15 ATL patients were analyzed.

Plasmids The IRF4 cDNA was cloned into the expression vector, pcDNA3 (Invitrogen, San Diego, CA), via the *Bam*HI and *Eco*RI sites (pcDNA3/IRF4).

Detection of IRF4 and Tax expression by reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA was extracted using the RNeasy Mini kit (QIAGEN, Hilden, Germany), according to the protocol provided by the manufacturer. The first-strand cDNA was synthesized

using the RT-PCR kit (STRATAGENE) with oligo dT primers. Thereafter, the cDNA was amplified for 32, 30, and 23 cycles for IRF4, Tax, and β-actin, respectively. The oligonucleotide primers used for RT-PCR were as follows: for IRF4, sense 5'-GCA GAT GTC CAT GAG CCA CC-3', antisense 5'-CAG GTG GCT CAG CAG CTT CT-3',¹⁶ for Tax, sense 5'-ATC CCG TGG AGA CTC CTC AA-3', antisense 5'-AAC ACG TAG ACT GGG TAT CC-3',¹⁰ for β-actin, sense 5'-AAG AGA GGC ATC CTC ACC CT-3', antisense 5'-TAC ATC GCT GGG GTG TTG AA-3'. Product sizes were 499 bp, 145 bp, and 218 bp, respectively. Cycling conditions were as follows: denaturing at 94°C for 60 s, annealing at 62°C for 60 s, and extension at 72°C for 60 s. The PCR products were resolved in a 2% agarose gel, visualized by ethidium bromide staining, and quantitated by NIH image analyzer.

IRF4 and Tax protein detection by immunoblot analysis Approximately 5×10⁶ cells were solubilized at 4°C in lysis buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) for 30 min at 4°C. Cellular lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp, Bedford, MA). The blots were incubated with the mouse anti-human IRF4 monoclonal antibody (MAB) (a kind gift from Dr. Tak W. Mak, Ontario Cancer Institute, Amgen Institute), rinsed, and incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Life Science, Inc., Arlington Heights, IL). Proteins recognized by the antibodies were visualized using the enhanced chemiluminescence detection system (Amersham Life Science, Inc.). The same blots were reprobbed with anti-Tax MAB (Lt-4)²⁴ or anti-Actin MAB (CHEMICON International, Inc., Temecula, CA).

Flow cytometric analysis of IRF4-positive cells and cell cycle The intracellular expression of IRF4 protein was analyzed by flow cytometry in fixed and permeabilized cells.²⁵ Briefly, 3–5×10⁵ cells were fixed with ice-cold 1% paraformaldehyde for 4 min, permeabilized with 0.2% Tween 20 at 37°C for 15 min, and then washed twice with phosphate-buffered saline (PBS) containing 2% FBS (FBS/PBS). Cells were incubated at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-human IRF4 MAB or isotype control Ab (PharMingen, San Diego, CA) for 60 min, washed and suspended in FBS/PBS, and analyzed by FACScan using the CELLQuest software (Becton Dickinson, San Jose, CA). For double-staining with anti-human IRF4 and anti-CD3, cells were pre-stained with phycoerythrin (PE)-conjugated anti-CD3 MAB (PharMingen) before fixation. RNase and propidium iodide (PI) were added to stain DNA for a two color analysis of IRF4 (FITC) and DNA (PI). For a single color analysis of DNA, cells were treated with the lysis-DNA staining solution

(PBS with 0.1% Triton X, 25 μ g/ml PI, and 20 μ g/ml RNase). The percentage of cells in the S, G0/G1, and G2/M phase was determined using the ModFIT software (Verity Software House, Topsham, ME).

Effect of transient IRF4 expression on the cell cycle
The IRF4 expression vector, pcDNA3/IRF4, or the control vector, pcDNA3, was co-transfected with pMACSK^k (Miltenyi Biotec, Bergisch Gladbach, Germany) into Rat-1 fibroblasts using the FuGENE6 transfection reagent (Roche, Indianapolis, IN). Six hours after transfection, the serum concentration of the culture medium was reduced from 10% to 0.1% for serum starvation. Forty-eight hours after transfection, cells were harvested and double-stained with the FITC-conjugated anti-H-2K^k MAb (Miltenyi Biotec) and PI, and cell cycle analyses were performed for the H-2K^k-positive cells, as described above.

Statistical analysis Statistical significance was determined by Fisher's exact probability test and Student's *t* test. Differences were considered to be statistically significant at *P*<0.05.

RESULTS

Constitutive expression of IRF4 in HTLV-I-positive T cell lines Expression of IRF4 mRNA in several T cell lines was analyzed by RT-PCR using IRF4-specific primers (Fig. 1). All four of the HTLV-I-positive T cell lines examined (HUT102, MT-2, ST-1, and KK-1; lanes 3 to 6) strongly expressed IRF4 mRNA, whereas the two HTLV-I-negative T cell lines (Jurkat and MOLT4, lanes 1 and 2) did not. In the HTLV-I-positive cell lines, no significant difference was observed in the expression level of IRF4. However, the levels of Tax mRNA were quite different; it

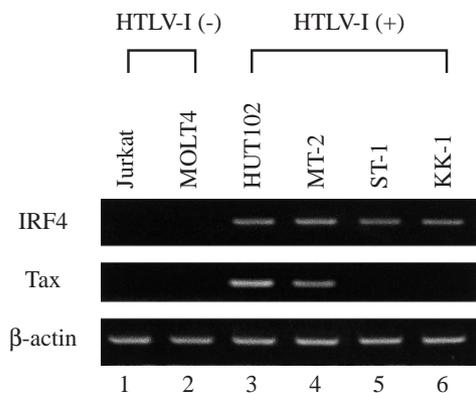


Fig. 1. Constitutive expression of IRF4 mRNA in HTLV-I-positive T cell lines. Expression of IRF4 and Tax mRNA in human T cell lines was analyzed by RT-PCR. Jurkat and MOLT4, HTLV-I-negative T cell lines. HUT102 and MT-2, HTLV-I-infected T cell lines. ST-1 and KK-1, T cell lines derived from ATL patients. β -Actin mRNA expression serves as a control.

was high in the HTLV-I-transformed cell lines (HUT102 and MT-2), whereas it was extremely low in the T cell lines derived from ATL patients (ST-1 and KK-1). Thus, the expression level of IRF4 mRNA did not correlate with that of Tax mRNA, although it has been reported that Tax induced IRF4 expression.¹⁶⁾ To investigate the level of IRF4 protein, immunoblot analysis was performed using the IRF4-specific MAb, which can detect a single 51 kDa signal of human IRF4 only in Rat-1 fibroblasts transfected with the human IRF4-expression vector (Fig. 2A, lane 2).

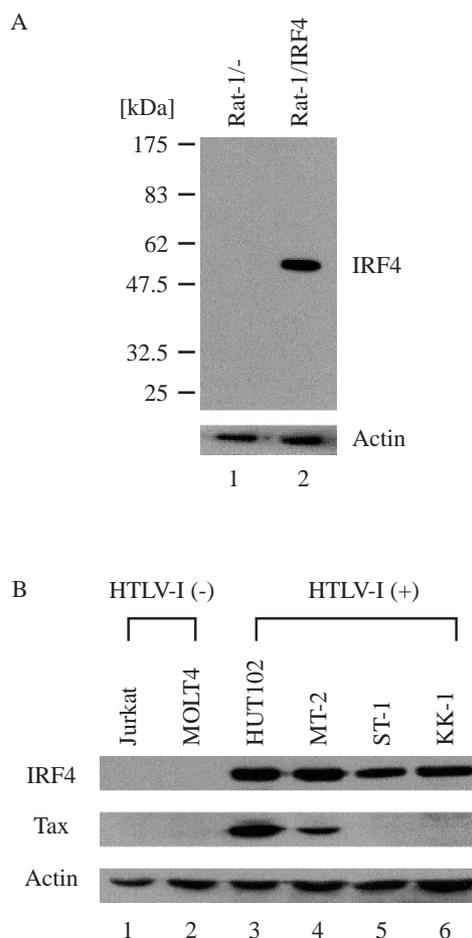


Fig. 2. Constitutive expression of IRF4 protein in HTLV-I-positive T cell lines. A, the expression of IRF4 protein was analyzed by immunoblotting. Cell extracts from Rat-1 fibroblasts transfected with the IRF4 expression vector, pcDNA3/IRF4 (Rat-1/IRF4), or the empty vector, pcDNA3 (Rat-1/-), were analyzed by immunoblotting using an anti-human IRF4 monoclonal antibody (MAb) or an anti-Tax MAb. The same blots were reprobbed with the anti-Actin antibody as a loading standard. B, the expression of the IRF4 and Tax proteins in human T cell lines was analyzed by immunoblotting. The same blots were reprobbed with an anti-Actin antibody as a loading standard.

These results confirmed the specificity of the MAb. When the expression of IRF4 protein in the T cell lines was analyzed, the level of IRF4 protein correlated with that of IRF4 mRNA (Fig. 2B). The Tax protein was detected in the HTLV-I transformed T cell lines (lanes 3 and 4), but not in the ATL-derived T cell lines (lanes 5 and 6), in accordance with the mRNA profile described above and with results reported previously.²⁶⁾ These data indicate that IRF4 is constitutively expressed in HTLV-I-positive T cell lines, either with or without Tax expression.

Expression of IRF4 in primary ATL cells Next, we examined the expression of IRF4 in PBMC derived from ATL patients. We analyzed the samples from 15 ATL patients, 8 with acute-type ATL and 7 with chronic-type ATL. The clinical features of the patients are summarized in Table I. There were no statistically significant differences between the acute and chronic patients in white blood cell (WBC) count and the percentage of abnormal lymphocytes in WBC. No signal or only faint expression of IRF4 was detected in PBMC from healthy donors under our RT-PCR conditions (Fig. 3, lanes 1 and 2). However, in 8 of 15 ATL cases, we could observe definite expression of IRF4 (Fig. 3, lanes 3–13, and data not shown). Interestingly, 7 of 8 IRF4-positive samples originated from acute ATL patients (Fig. 3, lanes 8–13, which correspond to patient No. 6–11, respectively, in Table I). On the other hand, only one of the samples from the chronic ATL patients showed detectable IRF4 expression (Fig. 3, lane 7, which corresponds to patient No. 5 in Table I). Thus,

IRF4 expression in PBMC correlated well with the clinical subtype of ATL (acute type versus chronic type, $P < 0.02$). In addition, the chronic ATL patient who was positive for IRF4 (lane 7) transformed to acute-type ATL and died within 6 months after the diagnosis. When the expression of Tax mRNA was examined, no definite band could be detected in the samples from ATL patients under these experimental conditions, indicating that the expression of IRF4 is independent of the Tax level (data not shown).

To evaluate further the expression of IRF4 in ATL cells, we managed to detect intracellular IRF4 by flow cytometry. The FITC-labeled anti-IRF4 MAb efficiently stained the IRF4-positive cell line, KK-1, but not the IRF4-negative cell line, Jurkat. Moreover, Rat-1 cells expressing exogenous IRF4 could be identified (Fig. 4A). Thus, the current flow cytometry protocol could actually distinguish IRF4-expressing cells from a mixed cell population. We then examined PBMC from ATL patients by this method. In acute ATL samples, CD3-positive T cells were strongly labeled with the anti-IRF4-FITC, whereas the extent of labeling in T cells from chronic ATL patients was weak, like that of healthy donors (Fig. 4B. Samples from patient No. 2, 13, 14, and 15 in Table I, were analyzed). In these samples from ATL patients, more than 90% of the lymphocytes were morphologically diagnosed as abnormal (data not shown). These results indicate that an anomaly in the expression of IRF4 may be a characteristic associated with acute-type ATL cells.

Mitogenic activation induces IRF4 expression prior to cell cycle progression in PBMC To evaluate the role of IRF4 in human peripheral T cells, we examined the relationship between IRF4 induction and cell cycle progression in mitogen-activated PBMC, by flow cytometric

Table I. Clinical Features of ATL Patients at the Time of Sample Collection

Patient No.	WBC count (/mm ³)	Ab-Ly (%) ^{a)}	Type ^{b)}
1	10 300	20	chronic
2	41 800	68	chronic
3	17 600	23	chronic
4	6 400	31	chronic
5	23 400	53	chronic
6	51 900	80	acute
7	98 900	82	acute
8	33 800	37	acute
9	35 200	66	acute
10	70 200	87	acute
11	36 700	55	acute
12	18 300	38	chronic
13	73 600	94	chronic
14	18 100	61	acute
15	66 500	71	acute

a) Ab-Ly indicates the percentage of abnormal lymphocytes in WBC.

b) Type indicates the clinical subtype of ATL.

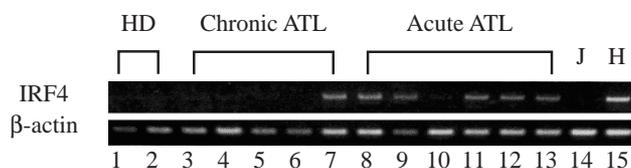


Fig. 3. Constitutive expression of IRF4 mRNA in peripheral blood mononuclear cells (PBMC) from ATL patients. Expression of IRF4 mRNA in freshly isolated PBMC from ATL patients and normal healthy donors was analyzed by RT-PCR. Lanes 1 and 2, PBMC RNA from healthy donors (HD). Lanes 3–7, PBMC RNA from chronic ATL patients. Lanes 8–13, PBMC RNA from acute ATL patients. Lane 14, Jurkat RNA as a negative control (J). Lane 15, HUT102 RNA as a positive control (H). Expression of β -actin mRNA is shown as a control. The relative expressions of IRF4 to β -actin quantitated by NIH image analyzer were as follows: lanes; 1–6 (ND), 7 (0.96), 8 (0.89), 9 (1.56), 10 (ND), 11 (0.73), 12 (0.62), 13 (0.53), 14 (ND), 15 (1.04). ND: not detectable.

analysis. When PBMC were treated with a T cell mitogen, concanavalin A (ConA), expression of IRF4 was induced by 1 day after stimulation. At the time, most of the cells,

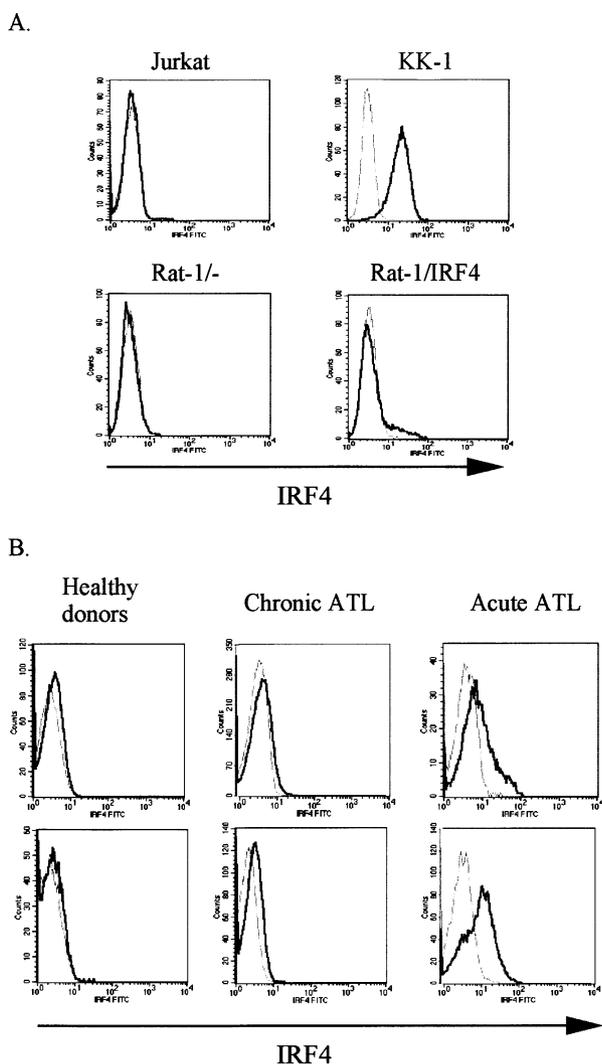


Fig. 4. Constitutive expression of IRF4 protein in ATL cells from acute ATL patients. A, intracellular expression of IRF4 in fixed and permeabilized cells was analyzed by flow cytometry using the FITC-conjugated anti-IRF4 MAb. Jurkat, KK-1, Rat-1/- (Rat-1 cells transiently transfected with empty pcDNA3) and Rat-1/IRF4 (Rat-1 cells transiently transfected with IRF4 expression vector, pcDNA3/IRF4) were used for the assay. B, intracellular expression of IRF4 in ATL cells. PBMC from healthy donors, and chronic and acute-type ATL patients (upper middle, patient No. 2; lower middle, patient No. 13; upper right, patient No. 14; lower right, patient No. 15 in Table I), were double-stained with PE-conjugated anti-CD3 MAb and FITC-conjugated anti-IRF4 MAb. Histograms of CD3-positive cells are shown for staining with the anti-IRF4 MAb (black lines) and the isotype control antibody (gray lines).

including the IRF4-positive cells, remained in the G0/G1 phase (Fig. 5B). On day 2, the S-G2/M transition could be detected in ConA-stimulated cells. Interestingly, most of the cells in the S-G2/M phase were positive for IRF4 staining (Fig. 5C). These data clearly showed that upon ConA-stimulation, IRF4 was induced in PBMC prior to cell proliferation, and that these IRF4-positive cells predominantly cycled into the S-G2/M phase.

Ectopic expression of IRF4 increases the S and G2/M phase populations in Rat-1 fibroblasts To examine the correlation of IRF4 expression with cell cycle progression, cell cycle analyses were performed in cells transiently expressing IRF4. We used Rat-1 fibroblasts in this assay, because they can be transformed by IRF4 *in vitro*.¹⁴ The S and G2-M phase populations in Rat-1 cells transfected with the IRF4-expression vector, as compared with those transfected with the empty control vector (Table II). These data indicate that IRF4 may have a positive effect on cell cycle progression and suggest that this

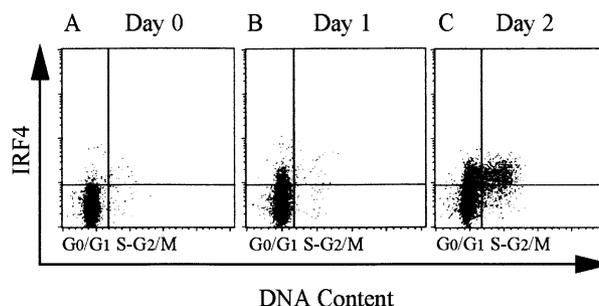


Fig. 5. Correlation of IRF4 induction with cell cycle progression. PBMC from healthy donors were treated with the T cell mitogen, concanavalin A (ConA), and were harvested at the indicated time points after stimulation. They were then double-stained with FITC-conjugated anti-IRF4 MAb and propidium iodide, and were analyzed by flow cytometry for IRF4 staining and DNA content. The vertical marker discriminates cells in G0/G1 from those in the S-G2/M phases of the cell cycle. In each dot plot, the right panel contains cells in the S-G2/M phases, and the upper panel contains IRF4-positive cells.

Table II. Ectopic Expression of IRF4 Has a Positive Effect on Cell Cycle Progression in Rat-1 Fibroblasts

	Control	IRF4
G0/G1	79.86±2.64 ^{a)}	72.27±3.25 ^{b)}
S	11.39±1.17	17.21±2.60 ^{c)}
G2/M	7.78±1.21	10.38±1.16 ^{b)}

a) Data presented as means±SE from five experiments.

b) P<0.005 versus the control group.

c) P<0.05 versus the control group.

effect may be associated with the oncogenic potential of IRF4.

DISCUSSION

ATL is a fatal T-cell malignancy caused by HTLV-I. It is widely accepted that studies of HTLV-I-transformed cell lines have provided valuable information about the molecular mechanism of this malignant phenotype. However, HTLV-I-transformed cell lines have usually been established *in vitro* by cocultivation of target cells with irradiated cells producing HTLV-I, and consequently, these cell lines do not represent the original ATL cells. Indeed, Tax, a potent transactivator protein in many biological settings, is not expressed in clinically isolated ATL cells.^{9,10} MT-2, which distinctly expresses Tax (Figs. 1 and 2B), was established by *in vitro* transformation of normal T cells with HTLV-I.²⁰ HUT102, which strongly expresses Tax (Figs. 1 and 2B), was established from a patient diagnosed with mycosis fungoides, although its clonal origin was not examined.^{19,21} On the other hand, the ST-1 and KK-1 cell lines, whose origins were confirmed as ATL cells by a Southern blot analysis,²² express only low levels of Tax (Figs. 1 and 2B). Therefore it is possible that the gene expressions of leukemic cells of ATL patients are free from Tax transactivation, especially after malignant cell transformation, which emphasizes the importance of an oncogenic factor(s) whose expression is independent of Tax.

It has been reported that IRF4 is expressed in HTLV-I-positive T cell lines,¹⁶ and that the viral protein Tax might induce the expression of IRF4.^{16,27,28} When we studied the MT-2 and HUT102 cell lines, the IRF4 levels appeared to be proportional to the level of Tax expression. However, when we studied ATL-derived cell lines or clinically isolated samples, IRF4 was still strongly expressed, irrespective of the fact that Tax expression was below the detectable level (Figs. 1, 2B, and data not shown). A recent report suggested the existence of a Tax-responsive element in a 1.2 kb region of the human IRF4 promoter.²⁸ However, we could not detect any regions responding specifically in HUT102, as compared with Jurkat, an HTLV-I-negative cell line, in our preliminary study using the promoter region of human IRF4 up to about -4700 base pairs from the putative transcription start site (data not shown). Although we could not exclude the possibility that an undetectable amount of Tax is sufficient to induce IRF4 and/or a Tax-responsive element resides outside the region we studied, it seems reasonable to speculate that Tax is not directly involved in the constitutive expression of IRF4 in HTLV-I-infected cells. It was reported that c-Rel, a component of nuclear factor (NF)- κ B, regulates IRF4 expression in mice.²⁷ However, two putative NF- κ B binding sites, between -1733 and -1724 or -686 and -677,

reported in the mouse IRF4 promoter, are not conserved in the human *IRF4* gene promoter (data not shown). In addition, p50 and p65, but not c-Rel, were shown to be activated components of NF- κ B in primary ATL cells.²⁹ Furthermore, it was reported that NF- κ B subunits, including combinations of p50, p65, and c-Rel, failed to stimulate the human IRF4 promoter,²⁸ suggesting that a factor(s) other than NF- κ B is involved in IRF4 induction.

In the present study, we demonstrated preferential IRF4 expression in acute-type ATL cells, rather than in the chronic type. IRF4 was reported to cause transformation of Rat-1 fibroblasts *in vitro*.¹⁴ On the other hand, IRF4-transgenic mice, in which the expression of IRF4 was restricted to lymphoid tissue, did not develop T cell leukemia/lymphoma,³⁰ indicating that IRF4 alone is not sufficient for developing T cell leukemia/lymphoma. In view of these observations, we speculate that IRF4 is required for the progression, rather than the initiation, of ATL. Alternatively, it is also possible that ATL cells simply represent activated T lymphocytes in which IRF4 is concurrently expressed. Indeed, the constitutive expression of IRF4 was observed in anaplastic large cell lymphoma, another T cell malignancy derived from activated T cells.¹⁷ To clarify this issue, we examined the proliferative role of IRF4 in human PBMC. We firstly established a flow cytometric protocol to analyze the intracellular expression of IRF4 together with other parameters, such as cell surface markers or DNA content. With this flow cytometric analysis, we could clearly demonstrate that IRF4 was induced prior to cell cycle progression, and that the IRF4-positive cells predominantly entered the cell cycle through the S-G2/M phase, when PBMC were treated with a T cell mitogen (Fig. 5). The ectopic expression of IRF4 increased the S-G2/M phase population in Rat-1 fibroblasts, further supporting the notion that IRF4 functions as a positive regulator of cell cycle progression (Table II). In addition, our preliminary study revealed that the inhibition of IRF4 by the transduction of a truncated form of IRF4 protein, with a possible dominant negative activity, reduced the S phase population significantly. This cell cycle inhibition was remarkable but incomplete, probably because the amount of transduced mutant IRF4 protein was not enough to compete with the endogenous IRF4 and/or some subset of T cells could proceed through the cell cycle without IRF4, as seen in IRF4-deficient mice.¹³

Regarding HTLV-I associated leukemogenesis, the involvement of several cell cycle-related genes has been suggested.³¹⁻³³ Deletion of the genes encoding the cyclin-dependent kinase inhibitors p15INK4B and/or p16INK4A, and repressed expression of the *p16INK4A* gene by methylation, were found in ATL and correlated with the ATL subtype.³⁴⁻³⁶ In addition, the deletion of p15INK4B and/or p16INK4A during disease progression was also reported in ATL.³⁴ Another tumor suppressor gene, *p53*, is

also known to be involved in cell cycle regulation.^{37, 38)} It was reported that mutations of p53 were detected in some ATL cells, and that the occurrence of these mutations was correlated with disease progression.^{39, 40)} Hence, the deregulation of cell cycle-related factors may be an important event in the late phase of ATL leukemogenesis. In our study, the constitutively high-level expression of IRF4 was specifically observed in most of the acute-type ATL patients, and flow cytometric analysis revealed that IRF4 was positive in a part of the leukemic cells. This implied the heterogeneous nature of ATL cells, and cells positive for IRF4 might represent an aggressive proliferative population of ATL cells, as suggested by Falini *et al.*¹⁷⁾ Intriguingly, a patient once diagnosed with chronic-type ATL, but expressing a high level of IRF4, proceeded to leukemic crisis months later, suggesting IRF4 involvement in the late stage of ATL. Although the relationship between the above-mentioned cell cycle regulators and IRF4 in leukemogenesis remains to be elucidated, some members of the IRF family were found to be involved in cell cycle regulation through the expression of the cognate down-stream genes.^{41–47)} A full understanding of the mechanism(s) by which IRF4 participates in the regulation of the cell cycle will help us to know the role of IRF4 in leukemogenesis and may allow us to identify new therapeutic targets.

mogesis remains to be elucidated, some members of the IRF family were found to be involved in cell cycle regulation through the expression of the cognate down-stream genes.^{41–47)} A full understanding of the mechanism(s) by which IRF4 participates in the regulation of the cell cycle will help us to know the role of IRF4 in leukemogenesis and may allow us to identify new therapeutic targets.

ACKNOWLEDGMENTS

We are grateful to Drs. H. Ichinose, A. Koda, and M. Miyazaki for encouragement. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and a grant by N.D.R. Gifu, Japan.

(Received May 14, 2001/Revised July 10, 2001/Accepted September 17, 2001)

REFERENCES

- 1) Yoshida, M., Miyoshi, I. and Hinuma, Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA*, **79**, 2031–2035 (1982).
- 2) Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. and Gallo, R. C. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA*, **77**, 7415–7419 (1980).
- 3) Shimoyama, M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). *Br. J. Haematol.*, **79**, 428–437 (1991).
- 4) Shimoyama, M. Peripheral T-cell lymphoma in Japan: recent progress. *Ann. Oncol.*, **2** (Suppl. 2), 157–162 (1991).
- 5) Tanaka, A., Takahashi, C., Yamaoka, S., Nosaka, T., Maki, M. and Hatanaka, M. Oncogenic transformation by the tax gene of human T-cell leukemia virus type I *in vitro*. *Proc. Natl. Acad. Sci. USA*, **87**, 1071–1075 (1990).
- 6) Grassmann, R., Dengler, C., Muller-Fleckenstein, I., Fleckenstein, B., McGuire, K., Dokhelar, M. C., Sodroski, J. G. and Haseltine, W. A. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. *Proc. Natl. Acad. Sci. USA*, **86**, 3351–3355 (1989).
- 7) Grassmann, R., Berchtold, S., Radant, I., Alt, M., Fleckenstein, B., Sodroski, J. G., Haseltine, W. A. and Ramstedt, U. Role of human T-cell leukemia virus type I X region proteins in immortalization of primary human lymphocytes in culture. *J. Virol.*, **66**, 4570–4575 (1992).
- 8) Akagi, T. and Shimotohno, K. Proliferative response of Tax1-transduced primary human T cells to anti-CD3 antibody stimulation by an interleukin-2-independent pathway. *J. Virol.*, **67**, 1211–1217 (1993).
- 9) Mori, N., Nunokawa, Y., Yamada, Y., Ikeda, S., Tomonaga, M. and Yamamoto, N. Expression of human inducible nitric oxide synthase gene in T-cell lines infected with human T-cell leukemia virus type-I and primary adult T-cell leukemia cells. *Blood*, **94**, 2862–2870 (1999).
- 10) Kinoshita, T., Shimoyama, M., Tobinai, K., Ito, M., Ito, S., Ikeda, S., Tajima, K., Shimotohno, K. and Sugimura, T. Detection of mRNA for the tax1/rex1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, **86**, 5620–5624 (1989).
- 11) Matsuyama, T., Grossman, A., Mittrucker, H. W., Siderovski, D. P., Kiefer, F., Kawakami, T., Richardson, C. D., Taniguchi, T., Yoshinaga, S. K. and Mak, T. W. Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). *Nucleic Acids Res.*, **23**, 2127–2136 (1995).
- 12) Eisenbeis, C. F., Singh, H. and Storb, U. Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. *Genes Dev.*, **9**, 1377–1387 (1995).
- 13) Mittrucker, H. W., Matsuyama, T., Grossman, A., Kundig, T. M., Potter, J., Shahinian, A., Wakeham, A., Patterson, B., Ohashi, P. S. and Mak, T. W. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science*, **275**, 540–543 (1997).
- 14) Iida, S., Rao, P. H., Butler, M., Corradini, P., Boccadoro, M., Klein, B., Chaganti, R. S. and Dalla-Favera, R. Deregulation of MUM1/IRF4 by chromosomal translocation in multiple myeloma. *Nat. Genet.*, **17**, 226–230 (1997).

- 15) Yoshida, S., Nakazawa, N., Iida, S., Hayami, Y., Sato, S., Wakita, A., Shimizu, S., Taniwaki, M. and Ueda, R. Detection of MUM1/IRF4-IgH fusion in multiple myeloma. *Leukemia*, **13**, 1812–1816 (1999).
- 16) Yamagata, T., Nishida, J., Tanaka, S., Sakai, R., Mitani, K., Yoshida, M., Taniguchi, T., Yazaki, Y. and Hirai, H. A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon-regulated genes. *Mol. Cell. Biol.*, **16**, 1283–1294 (1996).
- 17) Falini, B., Fizzotti, M., Pucciarini, A., Bigerna, B., Marafioti, T., Gambacorta, M., Pacini, R., Alunni, C., Natali-Tanci, L., Ugolini, B., Sebastiani, C., Cattoretti, G., Pileri, S., Dalla-Favera, R. and Stein, H. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. *Blood*, **95**, 2084–2092 (2000).
- 18) Tsuboi, K., Iida, S., Inagaki, H., Kato, M., Hayami, Y., Hanamura, I., Miura, K., Harada, S., Kikuchi, M., Komatsu, H., Banno, S., Wakita, A., Nakamura, S., Eimoto, T. and Ueda, R. MUM1/IRF4 expression as a frequent event in mature lymphoid malignancies. *Leukemia*, **14**, 449–456 (2000).
- 19) Gazdar, A. F., Carney, D. N., Bunn, P. A., Russell, E. K., Jaffe, E. S., Schechter, G. P. and Guccion, J. G. Mitogen requirements for the *in vitro* propagation of cutaneous T-cell lymphomas. *Blood*, **55**, 409–417 (1980).
- 20) Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K. and Hinuma, Y. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature*, **294**, 770–771 (1981).
- 21) Bunn, P. A. and Foss, F. M. T-cell lymphoma cell lines (HUT102 and HUT78) established at the National Cancer Institute: history and importance to understanding the biology, clinical features, and therapy of cutaneous T-cell lymphomas (CTCL) and adult T-cell leukemia-lymphomas (ATLL). *J. Cell Biochem. Suppl.*, **24**, 12–23 (1996).
- 22) Yamada, Y., Ohmoto, Y., Hata, T., Yamamura, M., Murata, K., Tsukasaki, K., Kohno, T., Chen, Y., Kamihira, S. and Tomonaga, M. Features of the cytokines secreted by adult T cell leukemia (ATL) cells. *Leuk. Lymphoma*, **21**, 443–447 (1996).
- 23) Matsumoto, K., Shibata, H., Fujisawa, J. I., Inoue, H., Hakura, A., Tsukahara, T. and Fujii, M. Human T-cell leukemia virus type 1 Tax protein transforms rat fibroblasts via two distinct pathways. *J. Virol.*, **71**, 4445–4451 (1997).
- 24) Tanaka, Y., Yoshida, A., Takayama, Y., Tsujimoto, H., Tsujimoto, A., Hayami, M. and Tozawa, H. Heterogeneity of antigen molecules recognized by anti-tax₁ monoclonal antibody Lt-4 in cell lines bearing human T cell leukemia virus type I and related retroviruses. *Jpn. J. Cancer Res.*, **81**, 225–231 (1990).
- 25) Douglas, R. S., Tarshis, A. D., Pletcher, C. H., Jr., Nowell, P. C. and Moore, J. S. A simplified method for the coordinate examination of apoptosis and surface phenotype of murine lymphocytes. *J. Immunol. Methods*, **188**, 219–228 (1995).
- 26) Mori, N., Fujii, M., Iwai, K., Ikeda, S., Yamasaki, Y., Hata, T., Yamada, Y., Tanaka, Y., Tomonaga, M. and Yamamoto, N. Constitutive activation of transcription factor AP-1 in primary adult T-cell leukemia cells. *Blood*, **95**, 3915–3921 (2000).
- 27) Grumont, R. J. and Gerondakis, S. Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferon-regulated gene expression by rel/nuclear factor kappaB. *J. Exp. Med.*, **191**, 1281–1292 (2000).
- 28) Sharma, S., Mamane, Y., Grandvaux, N., Bartlett, J., Petropoulos, L., Lin, R. and Hiscott, J. Activation and regulation of interferon regulatory factor 4 in HTLV type 1-infected T lymphocytes. *AIDS Res. Hum. Retroviruses*, **16**, 1613–1622 (2000).
- 29) Mori, N., Fujii, M., Ikeda, S., Yamada, Y., Tomonaga, M., Ballard, D. W. and Yamamoto, N. Constitutive activation of NF-kappaB in primary adult T-cell leukemia cells. *Blood*, **93**, 2360–2368 (1999).
- 30) Saito, T., Yamagata, T., Takahashi, T., Honda, H. and Hirai, H. ICSAT overexpression is not sufficient to cause adult T-cell leukemia or multiple myeloma. *Biochem. Biophys. Res. Commun.*, **260**, 329–331 (1999).
- 31) Neuveut, C. and Jeang, K. T. HTLV-I Tax and cell cycle progression. *Prog. Cell Cycle Res.*, **4**, 157–162 (2000).
- 32) Tsukasaki, K., Koeffler, P. and Tomonaga, M. Human T-lymphotropic virus type 1 infection. *Baillieres Best Pract. Res. Clin. Haematol.*, **13**, 231–243 (2000).
- 33) Hollsberg, P. Mechanisms of T-cell activation by human T-cell lymphotropic virus type I. *Microbiol. Mol. Biol. Rev.*, **63**, 308–333 (1999).
- 34) Yamada, Y., Hata, Y., Murata, K., Sugawara, K., Ikeda, S., Mine, M., Maeda, T., Hirakata, Y., Kamihira, S., Tsukasaki, K., Ogawa, S., Hirai, H., Koeffler, H. P. and Tomonaga, M. Deletions of p15 and/or p16 genes as a poor-prognosis factor in adult T-cell leukemia. *J. Clin. Oncol.*, **15**, 1778–1785 (1997).
- 35) Hata, Y., Hiramata, T., Miller, C. W., Yamada, Y., Tomonaga, M. and Koeffler, H. P. Homozygous deletions of the p15 (MTS2) and p16 (CDKN2/MTS1) genes in adult T-cell leukemia. *Blood*, **85**, 2699–2704 (1995).
- 36) Nosaka, K., Maeda, M., Tamiya, S., Sakai, T., Mitsuya, H. and Matsuoka, M. Increasing methylation of the CDKN2A gene is associated with the progression of adult T-cell leukemia. *Cancer Res.*, **60**, 1043–1048 (2000).
- 37) Ko, L. J. and Prives, C. p53: puzzle and paradigm. *Genes Dev.*, **10**, 1054–1072 (1996).
- 38) Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331 (1997).
- 39) Sakashita, A., Hattori, T., Miller, C. W., Suzushima, H., Asou, N., Takatsuki, K. and Koeffler, H. P. Mutations of the p53 gene in adult T-cell leukemia. *Blood*, **79**, 477–480 (1992).
- 40) Nishimura, S., Asou, N., Suzushima, H., Okubo, T.,

- Fujimoto, T., Osato, M., Yamasaki, H., Lisha, L. and Takatsuki, K. p53 gene mutation and loss of heterozygosity are associated with increased risk of disease progression in adult T cell leukemia. *Leukemia*, **9**, 598–604 (1995).
- 41) Tanaka, N. and Taniguchi, T. The interferon regulatory factors and oncogenesis. *Semin. Cancer Biol.*, **10**, 73–81 (2000).
- 42) Tanaka, N., Ishihara, M., Lamphier, M. S., Nozawa, H., Matsuyama, T., Mak, T. W., Aizawa, S., Tokino, T., Oren, M. and Taniguchi, T. Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. *Nature*, **382**, 816–818 (1996).
- 43) Vaughan, P. S., Aziz, F., van Wijnen, A. J., Wu, S., Harada, H., Taniguchi, T., Soprano, K. J., Stein, J. L. and Stein, G. S. Activation of a cell-cycle-regulated histone gene by the oncogenic transcription factor IRF-2. *Nature*, **377**, 362–365 (1995).
- 44) Vaughan, P. S., van der Meijden, C. M., Aziz, F., Harada, H., Taniguchi, T., van Wijnen, A. J., Stein, J. L. and Stein, G. S. Cell cycle regulation of histone H4 gene transcription requires the oncogenic factor IRF-2. *J. Biol. Chem.*, **273**, 194–199 (1998).
- 45) Mamane, Y., Heylbroeck, C., Genin, P., Algarte, M., Servant, M. J., LePage, C., DeLuca, C., Kwon, H., Lin, R. and Hiscott, J. Interferon regulatory factors: the next generation. *Gene*, **237**, 1–14 (1999).
- 46) Pitha, P. M., Au, W. C., Lowther, W., Juang, Y. T., Schafer, S. L., Burysek, L., Hiscott, J. and Moore, P. A. Role of the interferon regulatory factors (IRFs) in virus-mediated signaling and regulation of cell growth. *Biochimie*, **80**, 651–658 (1998).
- 47) Harada, H., Taniguchi, T. and Tanaka, N. The role of interferon regulatory factors in the interferon system and cell growth control. *Biochimie*, **80**, 641–650 (1998).