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成人T細胞性白血病(ATL)をモデルとしたウイルス感染関連がんに対する革新的治療法の開発に関する研究（臨床研究実施チームの整備）

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主任研究者　　松岡 雅雄

（京都大学ウイルス研究所・教授）

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主任研究者:松岡 雅雄　京都大学ウイルス研究所 教授

研究要旨
ヒトT細胞白血病ウイルスI型(HTLV-I)の感染により発症する成人T細胞白血病(ATL)は極めて難治性血液悪性腫瘍であり化学療法では平均生存期間は1年に過ぎない。ATLに対する移植療法の有効性が明らかになりつつあり新たな展開がもたらされている。本研究ではATLに対する骨髄非破壊的移植療法(ミニ移植)の有効性とATL細胞の分子生物学的解析を行い、ミニ移植有効性の分子基盤を明らかにすることを目的とする。本研究はミニ移植が何故ATLに有効かを明らかにするだけでなく、ATLの予後予測も可能にすることが期待される。本年度の解析から移植例ではHTLV-Iプロウイルスにおいてtax遺伝子の変異、5'側long terminal repeat(LTR)の高メチル化がなくtax遺伝子の発現が保たれていることが示唆された。この知見はミニ移植のATLに対する有効性を説明しうるものと考えられる。

A. 採択された研究事業での研究概要

本研究では難治性血液悪性腫瘍である成人T細胞白血病(adult T-cell leukemia: ATL)に対して骨髄非破壊的移植療法(ミニ移植)の有効性を明らかにすると共にヒトT細胞白血病ウイルスI型(human T-cell leukemia virus type I: HTLV-I)の分子生物学的解析、ウイルスに対する宿主の免疫反応の解析を行うことによって移植療法有効性の分子基盤を解明し、この研究をウイルスによる悪性腫瘍に対する治療法開発のモデルとすることを目的とする。京都大学ウイルス研究所ではATL細胞におけるHTLV-Iプロウイルスの分子生物学的解析を行うと共に残存腫瘍細胞検出系を開発し、ATLの治療法の確立に向けた臨床研究を研究班内の研究グループと共に展開する。本研究班は、ATLの多発地域である鹿児島、長崎、沖縄など九州の主要施設に、国立がんセンター、東京医科歯科大学、京都大学の研究者などを加えた全国的かつ特徴的な組織であり、エビデンスに基づくATL治療法の確立と標準化を図る。ATLの分子生物学的解析を行い、ミニ移植の有効性の分子基盤を明らかにする臨床研究である。本年度の解析から移植例ではHTLV-Iプロウイルスにおいてtax遺伝子の変異、5'側long terminal repeat(LTR)の高メチル化がなくtax遺伝子の発現が保たれていることが示唆された。この知見はミニ移植のATLに対する有効性を説明しうるものと考えられる。

B. 採択された研究事業での研究実績

1)移植症例におけるHTLV-Iプロウイルスの解析:我々の研究からATLでは約半数の症例でTaxの発現が認められないことが明らかとなっている。その機序として1)tax遺伝子の変異・欠失、2)5'LTRの高メチル化、3)5'LTRの欠失が存在する。移植症例のtax遺伝子発現と移植成績の関連を明らかにすることによって移植療法有効性の基盤を解明するために5'LTRのメチル化を解析した。9例の移植症例において5'LTRにおけるDNAメチル化をCOBRA法により解析したところ5例でメチル化を認めず4例で部分的なメチル化(35.1, 35.0, 17.4, 16.4%)を確認した。5'LTRは高度にメチル化された時にtax遺伝子の転写が抑制されることが明らかになっており(Taniguchi et al., Retrovirology, 2005)、解析した移植症例ではtax遺伝子の配列も保たれ転写可能であることが示された。

2)移植症例のtax遺伝子配列:同種造血細胞移植後に完全寛解を維持している4症例においてパラフィン標本からDNAを回収しtax遺伝子配列を解析した。2例でmis-sense

mutation を検出したが non-sense mutation, deletion 等は認めなかった。
これまでの解析結果より移植症例では全例、tax 遺伝子配列が保たれており、また発現可能な構造であった。tax を発現できない構造の ATL は予後不良であり、移植可能な状態になっていない可能性があり、今後の重要な検討課題である。

(倫理面への配慮)

本研究は各施設の倫理委員会、京都大学「医の倫理委員会」の承認の下に行われている。

C. 考察

ATL に対する移植療法の有効性が明らかにされつつあるが(Okamura J et al. Blood 2005)、特記すべきはプロウイルス量が検出感度以下にまで下がる症例が認められることである。この知見は HTLV-I に対する宿主免疫機構が抗腫瘍効果を発揮していることを示唆している。移植症例においてTaxを認識する細胞傷害性 T リンパ球の増加も報告されている(Harashima N et al, Cancer Res 2004; J Virol 2005)ことも、この考えを支持する。しかし、HTLV-I に対する免疫が抗腫瘍効果を発揮しているという直接の証拠は得られていない。

ATL は極めて予後が悪い血液腫瘍であり、しばしば治療抵抗性となる。移植ではドナーの選択等で時間がかかる場合が多く、治療によって病勢がコントロールされた症例で移植が施行されている可能性が考えられる。これまでの我々の解析から移植症例では tax 遺伝子の配列が保たれており、5'側 LTR の DNA メチル化も高度ではないことが示された。このことから tax 発現が保たれている症例が比較的、病勢のコントロールが付き移植可能となる可能性が示唆される。この可能性を検討するために HTLV-I プロウイルスの配列、存在様式と予後の関連を解析する予定である。今後、移植に症例も蓄積し HTLV-I プロウイルスと予後の関連について解析を進める。

D. 健康危険情報

特になし

E. その他実施した臨床研究・治験の概要及び実績

なし

研究成果の刊行に関する一覧表

主任研究者：京都大学ウイルス研究所　松岡　雅雄

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
	なし						

雑誌

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HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells

Yorifumi Satou, Jun-ichirou Yasunaga, Mika Yoshida, and Masao Matsuoka*

Laboratory of Virus Immunology, Institute for Virus Research, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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Human T cell leukemia virus type I (HTLV-I) causes adult T cell leukemia (ATL) in 2–5% of carriers after a long latent period. An HTLV-I encoded protein, Tax, induces proliferation and inhibits apoptosis, resulting in clonal proliferation of infected cells. However, *tax* gene expression in ATL cells is disrupted by several mechanisms, including genetic changes in the *tax* gene and DNA methylation/deletion of the 5′ long terminal repeat (LTR). Because Tax is the major target of cytotoxic T-lymphocytes *in vivo*, loss of Tax expression should enable ATL cells to escape the host immune system. The 5′ LTR of HTLV-I is frequently hypermethylated or deleted in ATL cells, whereas the 3′ LTR remains unmethylated and intact, suggesting the involvement of the 3′ LTR in leukemogenesis. Here we show that a gene encoded by the minus strand of the HTLV-I proviral genome, *HTLV-I basic leucine zipper factor* (*HBZ*), is transcribed from 3′-LTR in all ATL cells. Suppression of *HBZ* gene transcription by short interfering RNA inhibits proliferation of ATL cells. In addition, *HBZ* gene expression promotes proliferation of a human T cell line. Analyses of T cell lines transfected with mutated *HBZ* genes showed that *HBZ* promotes T cell proliferation in its RNA form, whereas HBZ protein suppresses Tax-mediated viral transcription through the 5′ LTR. Thus, the single *HBZ* gene has bimodal functions in two different molecular forms. The growth-promoting activity of *HBZ* RNA likely plays an important role in oncogenesis by HTLV-I.

oncogenesis | retrovirus | bimodal function

Human retroviruses use their genomes very efficiently because of their limited genome size. Their accessory genes elaborately control replication of genome copy (1). The HIV vigorously replicates to yield progeny virus, whereas human T cell leukemia virus type I (HTLV-I) increases the number of infected cells by the activity of accessory genes (2, 3). HTLV-I was the first human retrovirus associated with human disease (4, 5). After transmission of HTLV-I, 2–5% of carriers are likely to develop adult T cell leukemia (ATL) after a long latent period (6). HTLV-I belongs to the δ-retrovirus group, which includes bovine leukemia virus and simian T-cell leukemia virus. In contrast to HIV, HTLV-I is transmitted in a cell-to-cell fashion requiring transfer of infected cells (7). To facilitate transmission, HTLV-I increases the number of infected cells through the activity of accessory genes, which are encoded by the pX region located between *env* and the 3′ long terminal repeat (LTR). These genes include *tax*, *rex*, *p30*, *p12*, *p13*, and *HTLV-I basic leucine zipper factor* (*HBZ*) (3, 8). Among them, *tax* is thought to play a central role in increasing the number of infected cells. Tax activates transcriptional pathways, including nuclear factor κ-B, cAMP response element-binding protein, activator protein-1, and serum responsive factor (2, 3). In addition, Tax can functionally inactivate p53 (9), resulting in inhibition of apoptosis. Thus, Tax promotes proliferation and suppresses apoptosis of infected cells, leading to clonal proliferation (10–12). As a consequence, HTLV-I causes ATL, a fatal neoplastic disease of CD4-positive T-lymphocytes.

Despite its critical role in proliferation of infected cells, Tax expression in ATL cells is disrupted by several mechanisms, including genetic changes in the *tax* gene (13), DNA methylation (14, 15), or deletion of the 5′ LTR (16). Because Tax is the major target of cytotoxic T-lymphocytes (17), Tax-expressing cells are rapidly eliminated *in vivo*. Therefore, loss of Tax expression could enable ATL cells to evade the host immune system. On the other hand, the role of HTLV-I-encoded viral genes has not yet been determined in ATL cells that lack Tax expression. In ATL cells, the HTLV-I 3′ LTR remains unmethylated and intact (18), whereas the 5′ LTR is frequently hypermethylated or deleted. Based on these observations, we hypothesized that promoter/enhancer activity of the HTLV-I 3′ LTR was essential for proliferation and survival of ATL cells. Transcription from the minus strand of HTLV-I has been reported (19), and the HBZ was subsequently found to inhibit Tax-mediated transactivation of viral gene transcription from the 5′ LTR by heterodimerizing with either cAMP response element-binding protein 2, c-Jun or JunB (20, 21).

In this study, we found that the *HBZ* gene was expressed in all ATL cells and that suppression of *HBZ* transcription by short interfering RNA (siRNA) decreased ATL cell proliferation. Mutant analyses showed that the *HBZ* gene promoted proliferation of T cells in its RNA form, whereas HBZ protein inhibited Tax-mediated transactivation through the HTLV-I LTR. These findings suggest that *HBZ* plays an important role in oncogenesis by HTLV-I.

Results

The Spliced Form of *HBZ* Is Expressed in All ATL Cells. We first determined the transcription start site of *HBZ* by using 5′ RACE (Fig. 1*A*). Contrary to a previous report (20), the *HBZ* gene was spliced and transcriptional start sites were identified in the R and U5 region of the 3′ LTR. The first 4 amino acids of the predicted HBZ protein differed from the previously reported sequence (Fig. 1*B*). The 3′ end of the transcript was also determined by 3′ RACE (Fig. 1*A*). A polyadenylation signal was found in 3′ untranslated region of *HBZ*. The spliced *HBZ* gene does not overlap with the *tax*-encoding region, indicating that an antisense RNA to *tax* is not generated. We next analyzed *HBZ* transcription in ATL cell lines, fresh ATL cells, and peripheral blood mononuclear cells from HTLV-I carriers by RT-PCR. In three ATL cell lines (ED, ATL-43T, and TL-Om1), *tax* gene transcription was silenced, whereas *HBZ* transcription was detected in all cell lines (Fig. 1*C*). *HBZ* was transcribed in all seven fresh ATL cell samples, whereas *tax* gene transcription was observed in two cases (Fig. 1*D*). Furthermore, *HBZ* gene transcription was detected in two of three carriers. Although genetic changes (nonsense mutations, insertions, and deletions) in *tax* have been reported in refs. 13 and 15, *HBZ* sequences did not contain

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ATL, adult T cell leukemia; HBZ, HTLV-I basic leucine zipper factor; HTLV-I, human T cell leukemia virus type I; siRNA, short interfering RNA; SM HBZ, *HBZ* gene with silent mutations.

Data deposition: The spliced *HBZ* sequence reported in this paper has been deposited in the GenBank database (accession no. DQ273132).

*To whom correspondence should be addressed. E-mail: mmatsuok@virus.kyoto-u.ac.jp.

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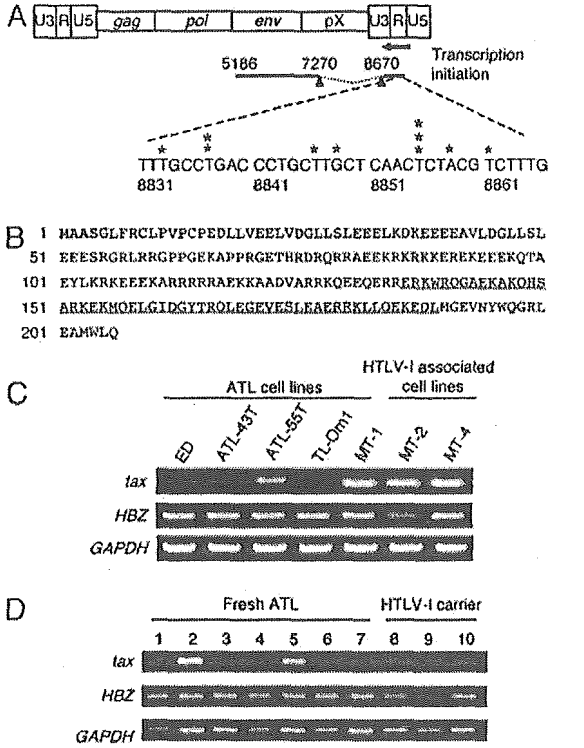


Fig. 1. *HBZ* gene expression in ATL cells. (*A*) 5′ RACE was performed by using total RNA from the ATL cell line ATL-55T. The schema represents the HTLV-I provirus and spliced *HBZ* mRNA. Asterisks show transcription initiation sites identified by 5′ RACE. The 3′ end of the transcript (5186) was identified by 3′ RACE, and polyadenylation signal was found upstream (5206–5211) of this transcript. Nucleic acids are numbered with reference to ATK-1 according to Seiki *et al.* (22). (*B*) Hypothetical amino acid sequence derived from spliced *HBZ*. Amino acids different from the previously reported HBZ are shown in bold type. The basic leucine zipper domain is underlined. (*C*) Expression of *tax* and *HBZ* genes in ATL and HTLV-I-immortalized cell lines analyzed by RT-PCR. (*D*) Expression of *tax* and *HBZ* genes in fresh ATL cells and peripheral blood mononuclear cells from HTLV-I carriers. Lanes: 1–7, fresh ATL cases; 8–10, peripheral blood mononuclear cells from HTLV-I carriers.

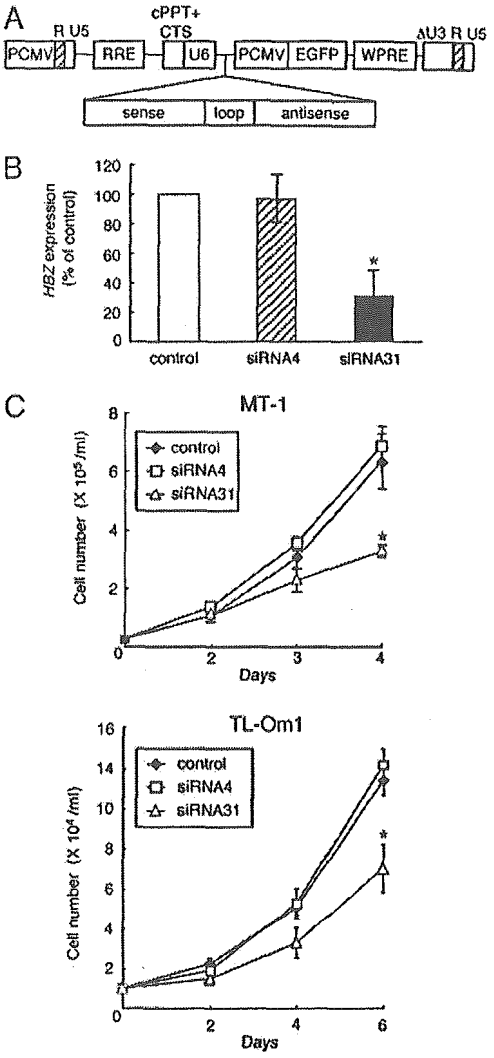


Fig. 2. Knockdown of *HBZ* gene expression inhibits cell growth of ATL cells. (*A*) Schematic diagram of the short hairpin RNA-expressing lentiviral vector. PCMV, immediate early cytomegalovirus promoter; RRE, Rev-responsive element; cPPT, central polypurine tract; CTS, central termination sequence; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. (*B*) siRNA31 decreases *HBZ* gene transcription, whereas siRNA4 does not (*Supporting Methods*, which is published as supporting information on the PNAS web site). Efficiencies of lentivirus vector transfection, which were determined by EGFP expression, were 99.3% and 93.0% for MT-1 and TL-Om1, respectively. *HBZ* transcripts in siRNA transfectants of MT-1 are quantified by a densitometer and shown as percentages compared with a mock transfectant. Values are means ± SD from three independent experiments. (*C*) Transfection of lentivirus vector expressing siRNA31 suppresses the growth of MT-1 and TL-Om1. The transfectants of siRNAs were harvested at day 7 after transfection, and seeded into a 96-well plate at 5×10^3 cells per well. Cell numbers of each transfectants were counted in triplicate by Trypan blue dye exclusion method. Values are means ± SD. *, $P < 0.05$.

disruptive genetic changes in any cell line or in fresh ATL samples (17 cases), except for polymorphisms (Table 1, which is published as supporting information on the PNAS web site).

Inhibition of *HBZ* by siRNA Suppresses Growth of ATL Cells. Although inhibition of Tax-mediated viral gene transcription has been reported as a function of HBZ (20), *HBZ* was expressed in ATL cells lacking *tax* gene transcription, suggesting that *HBZ* has other roles. To clarify the role of *HBZ* in ATL cells, we suppressed *HBZ* gene expression by using siRNAs. Recombinant lentiviruses transcribing short hairpin RNAs against *HBZ* under control of the U6 promoter (Fig. 2*A*) were generated and transduced to *tax*-expressing MT-1 and *tax*-nonexpressing TL-Om1 cells. In MT-1 cells, siRNA31 suppressed *HBZ* gene mRNA expression, whereas siRNA4 did not (Fig. 2*B*). Growth of both cell lines was suppressed by siRNA31 (Fig. 2*C*) along with *HBZ* transcription (Fig. 6, which is published as supporting information on the PNAS web site). Apoptosis was not induced by siRNA31. These findings indicate that *HBZ* gene expression is required for ATL cell proliferation, regardless of *tax* gene expression.

To analyze *HBZ* function, a vector expressing *HBZ* was transfected into Kit 225 cells, an IL-2-dependent human T cell line derived from T cell chronic lymphocytic leukemia cells (23), and stable transformants were selected in the presence of G418. *HBZ*-transfected Kit 225 cells showed prolonged survival after removal of IL-2 (Fig. 3*A*) compared with control cells, and *HBZ*-transduced Kit 225 cells showed an increase in the number of cells in S phase after depletion of IL-2 (Fig. 3*B*). In addition, *HBZ*-transfected Kit 225 cells showed enhanced responsiveness to IL-2 (Fig. 3*C*, WT). To identify *HBZ* target genes in Kit 225 cells, transcriptional profiles of *HBZ*-transfected and control cells after withdrawal of IL-2 (48 h) were analyzed by an

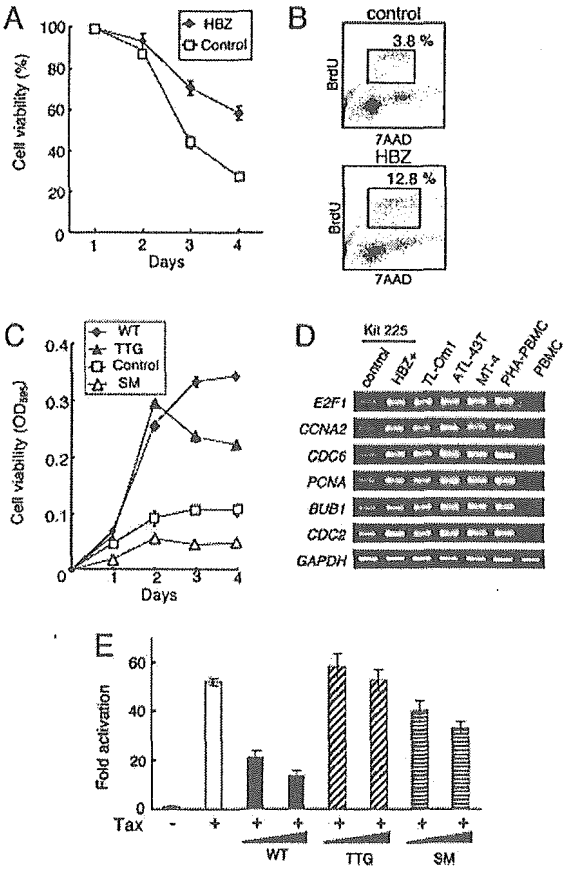


Fig. 3. Functional analyses of the *HBZ* gene. (*A*) *HBZ* gene expression prolongs survival of transfected Kit 225 cells after withdrawal of IL-2. Cell viabilities are measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Values are means ± SD. (*B*) Cell cycle analyses of *HBZ*-expressing Kit 225 cells, and Kit 225 cells transfected with a control vector. Cells in S phase were identified by BrdUrd incorporation and staining with 7AAD after withdrawal of IL-2. (*C*) Effects of wild-type (WT) and mutant forms of *HBZ* on proliferation of IL-2-stimulated Kit 225 cells. A suboptimal level of IL-2 (2.5 units/ml) was added after removal of IL-2 (48 h), and cell viability was measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The first ATG of *HBZ* is mutated to TTG, which blocks synthesis of HBZ protein. All codons in the *HBZ* gene are replaced with silent mutations (SM). The sequence of SM *HBZ* has been shown in Fig. 7. (*D*) Semiquantitative RT-PCR analysis for differentially expressed genes in *HBZ*-expressing Kit 225 cells compared with control cells. (*E*) Effects of *HBZ* and its mutants on Tax-mediated transactivation through the HTLV-I LTR. Lusiferase reporter (WT-Luc) and Tax expressing vector were transfected into Jurkat cells with or without vectors expressing wild-type (WT) or mutated *HBZ* (TTG or SM) (0.3 or 1 μg). Fold activation were calculated compared with basal luciferase activity of WT-Luc. Results are means ± SD in triplicate.

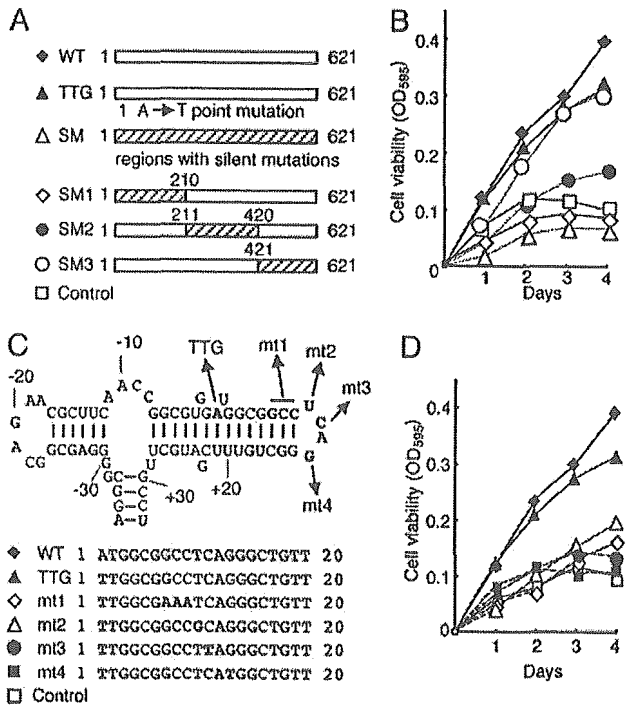


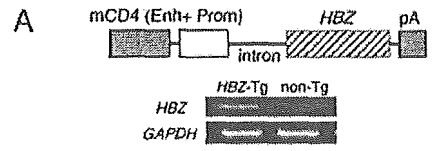
Fig. 4. Functional analyses of mutated *HBZ* genes on proliferation of T cells. (*A*) Schemas of mutant *HBZ* genes. (*B*) The effects of mutated *HBZ* genes on cell growth were measured by assays. The hatched area represents the region containing silent mutations. Numbers indicate the nucleotide positions in the *HBZ* coding sequence. (*C*) Predicted stem-loop structure in *HBZ* mRNA of the native *HBZ* gene (from −35 to +33). Nucleotides are numbered from the first ATG (A: +1) of the *HBZ* gene. Structural predictions were analyzed by using MFOLD (24). Mutated sequences of each vector are shown in bold type. (*D*) The results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays with these vectors are shown.

oligonucleotide microarray. We identified 687 genes up-regulated (by >2-fold) and 719 genes down-regulated (by >2-fold) in *HBZ*-transfected cells (Tables 2 and 3, which are published as supporting information on the PNAS web site). Expression of selected candidate genes was confirmed by RT-PCR (Fig. 3*D*), which indicated that transcription of the *E2F1* gene and its targets, *CCNA2*, *CDC6*, *PCNA*, *CDC2*, and *BUB1*, were up-regulated in *HBZ*-transfected Kit 225 cells. We speculated that up-regulation of *E2F1* is involved in enhanced proliferation mediated by *HBZ*.

To confirm whether HBZ protein promotes cell proliferation, we generated vectors expressing two *HBZ* gene mutants. Mutation that the first ATG of *HBZ* is replaced by TTG blocks synthesis of HBZ protein (TTG *HBZ*). Surprisingly, expression of TTG *HBZ* still promoted proliferation (Fig. 3*C*), suggesting that *HBZ* functions to promote cell proliferation as an RNA. To confirm this hypothesis, we replaced all coding regions in the *HBZ* gene with silent mutations (SM *HBZ*) (Fig. 7, which is published as supporting information on the PNAS web site). Such mutations completely altered the secondary structure of the RNA, but the SM *HBZ* gene still produced intact HBZ protein. SM *HBZ* gene expression did not promote cell proliferation (Fig. 3*C*), indicating that the growth-promoting effect of *HBZ* depended on RNA structure.

Because an inhibitory effect of HBZ on Tax-mediated transactivation through the 5′ LTR has been reported (20), we analyzed this function by using mutant forms of *HBZ*. As shown in Fig. 3*E*, wild-type *HBZ* suppressed Tax-mediated transactivation of transcription from the HTLV-I 5′ LTR. Although the SM *HBZ* gene also suppressed transactivation, TTG *HBZ* completely lacked this activity. Taken together, these findings suggest that *HBZ* promotes T cell proliferation in the form of RNA, whereas HBZ protein inhibits Tax-mediated transactivation through the 5′ LTR.

To further analyze the growth-promoting activity of *HBZ* RNA, we constructed vectors expressing a series of mutated *HBZ* genes (Fig. 4 *A* and *C*). When *HBZ* genes with silent mutations in different regions were analyzed, we found that mutations in the first 210 bp of *HBZ* (SM1) abolished growth-promoting activity (Fig. 4*B*). In addition, silent mutations in SM2 partially decreased growth-promoting activity of *HBZ*. When we compared the predicted secondary RNA structures of the native *HBZ* gene and the form of *HBZ* expressed in the pME18Sneo



B

Thymus	CD4⁺CD8⁺ (%)	CD4⁺ (%)	CD8⁺ (%)
Non-Tg (n = 5)	80.2 ± 6.2	9.5 ± 2.7	3.1 ± 1.6
HBZ-Tg (n = 5)	81.2 ± 6.5	12.3 ± 5.5	2.8 ± 1.1

Spleen	CD4⁺ (%)	CD8⁺ (%)
Non-Tg (n = 5)	26.0 ± 1.9	11.0 ± 1.3
HBZ-Tg (n = 5)	30.3 ± 2.1**	11.2 ± 0.8

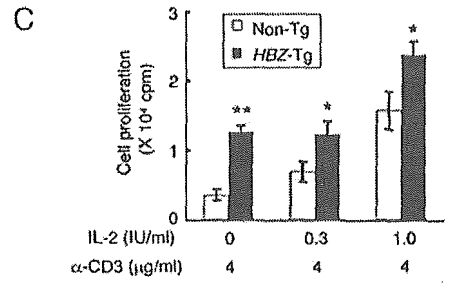


Fig. 5. Generation and analyses of *HBZ*-transgenic (Tg) mice. (*A*) Schematic representation of the *HBZ* transgene. The promoter (Prom) and enhancer (Enh) of the mouse CD4 (mCD4) gene were ligated to *HBZ* cDNA (including the 5′ UTR) plus the polyadenylation signal sequence of SV40. Expression of *HBZ* transcripts was detected by RT-PCR in purified $CD4^+$ splenocytes from *HBZ*-Tg mice. (*B*) T cell subsets in *HBZ*-Tg mice. Values are means ± SD from five transgenic mice. **, $P < 0.01$. (*C*) Proliferative responses of thymocytes from *HBZ*-Tg mice to IL-2 and/or stimulation with an anti-CD3 antibody. Proliferative responses were measured by ^{3}H-thymidine incorporation. ^{3}H-thymidine uptake of thymocytes without anti-CD3 antibody and IL-2 was 5.8 ± 4.6 cpm for control mice and 21.0 ± 16.0 cpm for *HBZ* transgenic mice. Values are means ± SD in triplicate. *, $P < 0.05$; **, $P < 0.01$.

vector, the first stem-loop of *HBZ* RNA was present in both cases (Fig. 4*C*). Although the TTG *HBZ* gene retained strong growth-promoting activity, TTG *HBZ* genes with mutations in the first stem-loop structure showed reduced or no activity (Fig. 4 *C* and *D*, mutants 1–4), indicating that it is essential for growth-promoting activity.

Microarray analyses of Kit 225 cells expressing wild-type and TTG *HBZ* genes identified transcriptional changes mediated by *HBZ* RNA, showing that RNA was responsible for up-regulation of *E2F1* (Table 2). Taken together, we conclude that the first stem-loop structure is important for growth-promoting activity of *HBZ*. To study the possibility that microRNA is responsible for growth-promoting activity, we performed Northern blot analysis by using oligonucleotides from the region containing the first stem-loop structure as probes. However, microRNA was not detected by Northern blot analysis (data not shown).

***In Vivo* Effect of *HBZ* Gene Expression in Transgenic Mice.** To analyze the role of *HBZ in vivo*, we generated transgenic mice expressing *HBZ* under the control of the mouse CD4 promoter/enhancer, which induces specific transgene expression in CD4-positive cells (Fig. 5*A*) (25). *HBZ* expression in transgenic mice was confirmed in CD4-positive splenocytes by RT-PCR (Fig. 5*A*). Thymocyte subpopulations in transgenic mice did not differ from those observed in nontransgenic littermates. However, the percentage of CD4-positive T lymphocytes increased in splenocytes of transgenic mice ($P < 0.01$) (Fig. 5*B*). In addition, proliferation induced by cross-linking with an immobilized anti-CD3 antibody was augmented in thymocytes of transgenic mice (Fig. 5*C*). These data indicate that the *HBZ* gene promotes proliferation of CD4-positive T lymphocytes *in vivo*.

Discussion

HTLV-I infection causes a fatal neoplastic disease, ATL, after a long latent period. Because transfection of retroviral vectors expressing *tax* can immortalize T lymphocytes *in vitro* (26), it has been suggested that *tax* plays a central role in oncogenesis among HTLV-I accessory genes. Tax has pleiotropic actions by interacting with cellular proteins, which promotes clonal proliferation of infected cells (2, 3). However, the role of the *tax* gene remains an enigma in ATL cells, because *tax* gene transcripts cannot be detected in ≈60% of ATL cases (12). However, the 3′ LTR and *HBZ* sequences are conserved in all ATL cells regardless of deletion or hypermethylation of the 5′ LTR or of genetic changes in the *tax* gene, suggesting that an intact 3′ LTR and *HBZ* gene are critical for oncogenesis. In this study, we showed that the *HBZ* gene encoded by the minus strand of the HTLV-I provirus is transcribed in all ATL cells. HBZ has been reported to suppress Tax-mediated transactivation of viral gene transcription through the 5′ LTR. In addition to this function, we showed that *HBZ* promotes proliferation of T lymphocytes *in vitro* and *in vivo*. These findings indicate that *HBZ* plays a critical role in oncogenesis mediated by HTLV-I, even in late stages of oncogenesis when *tax* is not expressed.

In oncogenic DNA viruses, oncoproteins, such as the SV40 T antigen and human papillomavirus E7 protein, target retinoblastoma (Rb) protein and inactivate its function (27). Loss of Rb function is associated with deregulation of E2F transcription factors (28). E2F1, a critical regulator of cell cycle progression, plays a pivotal role in the G_1-to-S-phase transition by transactivating specific target genes (29). Furthermore, overexpression of E2F1 has been reported to be associated with oncogenesis (30). As shown in this study, *HBZ* RNA increased *E2F1* gene transcription, which is likely associated with *HBZ*-induced proliferation. Although Tax was also reported to induce *E2F1* expression (31), *E2F1* is overexpressed in ATL cell lines lacking Tax expression, indicating that *HBZ* may be responsible for up-regulating *E2F1* expression.

Tax promotes cell growth and inhibits apoptosis of infected cells. However, because Tax is the major target of cytotoxic T lymphocytes, its expression is also disadvantageous to the survival of infected cells (17, 32). Therefore, HTLV-I encodes several other viral genes, such as *rex*, *p30*, and *HBZ*, to suppress Tax production by different mechanisms (20, 21, 33, 34). Because such suppression has a negative effect on growth of infected cells, the *HBZ* gene is likely to support proliferation of infected cells in addition to its activity in suppressing *tax* gene transcription. Because growth-promoting activity of *HBZ* could be a critical factor in maintaining a leukemic state, *HBZ* should be a therapeutic target.

Recently, microRNAs have been demonstrated to play important roles in regulating gene expression (35). Virus-encoded microRNAs have been also identified (36, 37), and some function in viral replication. In HIV-1, microRNAs have been demonstrated to function in viral transcription (38, 39). In HTLV-I, when *env* RNA is transcribed with the *HBZ* gene, such a long double-stranded RNA could be precursor for Dicer processing, resulting in formation of viral siRNAs. Such siRNA might function to suppress viral gene expression in HTLV-I. It is noteworthy that *HBZ* transcription is suppressed in MT-2 cells (Fig. 1*C*). In MT-2 cells, *env-tax* fusion gene is abundantly transcribed (15), which might generate siRNA and suppress *HBZ* expression.

Epstein–Barr virus (EBV)-encoded nonpolyadenylated RNA (also known as EBV-encoded small RNA or EBER) is known to

function in oncogenesis by activating transcription of genes such as insulin-like growth factor 1 (40) or interleukin 9 (41). Although these functional RNAs do not encode polypeptides, *HBZ* can function as both RNA and protein. Because of their limited genome size (8,506 bp for HTLV-I), complex retroviruses have evolved to use RNA splicing to express required genes. In addition to such a mechanism, HTLV-I not only expresses the *HBZ* gene on the minus strand but it also utilizes this gene as protein and RNA. Bimodal functions of viral gene may represent a previously uncharacterized strategy to regulate viral replication and proliferation of infected cells.

In conclusion, we showed that spliced *HBZ* gene was transcribed in all ATL cells. The *HBZ* gene promotes proliferation of ATL cells in the RNA form, whereas HBZ protein suppresses Tax-mediated viral transcription through the 5′ LTR. Although the role of *tax* gene remains undetermined in ATL cells, this study sheds light on the role of *HBZ* gene in oncogenesis.

Materials and Methods

Cells. Two HTLV-I immortalized lines (MT-2, and MT-4) and five ATL cell lines (ED, ATL-43T, ATL-55T, TL-Oml, and MT-1) were used in this study (15). The IL-2-dependent human T cell line Kit 225 was maintained in RPMI medium 1640 supplemented with 10% FBS and recombinant IL-2 (85 units/ml). Approval for this study was obtained from the institutional review board of Kyoto University. Informed consent was obtained from blood donors and patients according to the Declaration of Helsinki. To construct vectors encoding wild-type (WT) and mutant forms of *HBZ*, the coding sequence (621 bp) was amplified from cDNA of TL-Oml cells and subcloned into the pME18Sneo vector (42). Vectors were transfected into Kit 225 cells by using Nucleofector (Amaxa Biosystems, Cologne, Germany). Briefly, cells were suspended in 100 μl of Cell Line Nucleofector Solution T and then nucleofected with vectors (5 μg) by using program T-16 of the Nucleofector device (Amaxa Biosystems). Stable transfectants were selected in G418 (600 μg/ml).

Rapid Amplification of cDNA 5′and 3′ Ends (RACE). To determine the 5′ and 3′ ends of transcripts, RACE was performed by using the SMART RACE cDNA amplification kit (BD Biosciences Clontech) according to manufacturer's instructions (42). First-strand cDNAs were synthesized from 1 μg of total RNA of ATL-55T, ATL-43T, or MT-1 cells by reverse transcriptase and used for 5′ RACE PCR. For nested amplifications, primers specific for the *HBZ* gene (5′-CCTCTTTCTC-CCGCTCTTTTTTTCGC-3′ and 5′-CATGACACAG-GCAAGCATCGAAACA-3′) were used. For nested 3′ RACE amplifications, primers specific for the *HBZ* gene (5′-CTAGGTTAGGGCAGGGGGGCTGTAGGGC-3′ and 5′-GGGTCCACGAACAAACTGGCTGGGCAGG-3′) were used. PCR products were cloned into vectors, and the nucleotide sequences were determined.

Synthesis of cDNA and Semiquantitative RT-PCR. Spliced *HBZ*, *tax*, and *GAPDH* transcripts were quantified by using RT-PCR. The primers used were as follows: *HBZ* gene: 5′-TAAACTTACCTA-GACGGCGG-3′ (sense), 5′-CTGCCGATCACGATGCGTTT-3′ (antisense); *tax* gene: 5′-CCGGCGCTGCTCTCATCCCGGT-3′ (sense) and 5′-GGCCGAACATAGTCCCCCAGAG-3′ (antisense). PCR was performed in a PC-808 (Astec) under the following conditions: *HBZ*, 2 min at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 57.5°C, and 30 seconds at 72°C; *tax*, 2 min at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 61°C, and 30 seconds at 72°C. The intensity of PCR-amplified band was quantified by using ATTO densitograph 4.0 (Atto Instruments, Tokyo). Semiquantitative RT-PCR was performed to confirm microarray results by using primers (Table 4, which is published as supporting information on the PNAS web site).

Lentiviral Vector Construction and Transfection of Recombinant Lentivirus. We modified pCSII-EF-MCS (43) for delivery of anti-*HBZ* short hairpin RNAs, and recombinant lentivirus was produced as described in the *Supporting Methods*. The titer of concentrated virus stocks was measured on 293 T cells based on their EGFP expression. Cells were then transfected with concentrated vector stocks at a multiplicity of infection of 10–25 in the presence of polybrene (4 μg/ml, Sigma). Cells were harvested 7 days later, and EGFP expression of transfected cells was analyzed with an EPICS XL Flow Cytometer (Beckman Coulter). When >90% of transfected cells expressed EGFP, cell growth and *HBZ* gene expression were analyzed.

Microarray Analysis. Total RNAs were isolated from Kit 225 cells, which were transfected with a vector expressing the *HBZ* gene, the TTG *HBZ* gene (the first ATG of *HBZ* gene was replaced by TTG), or with a control vector by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNAs were then purified by RNeasy (Qiagen). Oligonucleotide microarray analyses (CodeLink, Human 20K I bioarrays, Amersham Pharmacia Biosciences) were performed by Kurabo Industries (Osaka) with the authorization of Amersham Pharmacia Biosciences. A 2.0-fold increase or decrease was considered significant, based on the manufacturer's recommendation.

Luciferase Assay. Jurkat cells were grown in RPMI medium 1640 containing 10% FBS. On day 1, cells were seeded into 6-well plates at 4×10^5 cells per well. After 24 h, cells were transfected with 1 μg per well of luciferase reporter plasmid (WT-Luc) (44), 40 ng per well of pRL-TK (Promega), and pCG-Tax (1 μg) (45), and/or an *HBZ* expression plasmid (0.3 or 1 μg), and/or blank expression vector (to normalize the DNA dose) mixed with Transfectin (Bio-Rad). After 48 h, cells were collected and luciferase activities were measured by using a Dual Luciferase Reporter Assay Kit (Promega).

Generation of Transgenic Mice. *HBZ* cDNA was cloned into the SalI site of the H/M/T-CD4 vector, which was designed for restricted expression of a transgene in $CD4^+$ cells (25). The purified fragment containing the *HBZ* transgene was microinjected into C57BL/6J F1 fertilized eggs. Transgenic founders were screened for integration of transgenes by PCR and mated with C57BL/6J mice to generate transgenic progeny. All animals used in this study were maintained and handled according to protocols approved by Kyoto University.

Cell Proliferation Assay. Proliferation assays of murine cells were carried out in RPMI 1640 medium supplemented with 10% FBS and 2-mercaptoethanol (50 μM). Thymocytes (1×10^6 cells per ml) were stimulated by an immobilized anti-CD3 antibody (4 μg/ml) with or without recombinant IL-2 in flat-bottomed 96-well plates. Thymocyte proliferation was measured by ^{3}H-thymidine uptake after 3 days of incubation. Cell viabilities were assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye absorbance (42).

Flow Cytometric Analysis. Cell cycles of *HBZ*-transfected and control Kit 225 cells were analyzed after removal of IL-2 (48 h) by using BrdU Flow Kits (Becton Dickinson Pharmingen) according to the manufacturer's instructions. To analyze CD4 and CD8 expression in *HBZ* transgenic mice, cells (5×10^5) were reacted with monoclonal antibodies against murine CD4 (FITC-labeled, Immunotech) and CD8 (phycoerythrin-labeled, Immu-

notech), according to the manufacturer's instructions, and analyzed with an EPICS XL Flow Cytometer (Beckman Coulter).

Statistical Analyses. Statistical analyses were performed by using the unpaired Student *t* test.

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ORIGINAL ARTICLE

Loss of interleukin-2-dependency in HTLV-I-infected T cells on gene silencing of thioredoxin-binding protein-2

MK Ahsan[1], H Masutani[1], Y Yamaguchi[1], Y-C Kim[1], K Nosaka[2], M Matsuoka[2], Y Nishinaka[1], M Maeda[1] and J Yodoi[1]

[1]*Department of Biological Responses, Institute for Virus Research, Kyoto University, Shogoin, Kawahara-cho, Sakyo-ku, Kyoto, Japan;* [2]*Research Center for Acquired Immunodeficiency Syndrome, Institute for Virus Research, Kyoto University, Shogoin, Kawahara-cho, Sakyo-ku, Kyoto, Japan*

The transition from interleukin-2 (IL-2)-dependent to IL-2-independent growth is considered one of the key steps in the transformation of human T-cell leukemia virus type-I (HTLV-I)-infected T cells. The expression of thioredoxin-binding protein-2 (TBP-2) is lost during the transition of HTLV-I-infected T-cell lines. Here, we analysed the mechanism of loss of TBP-2 expression and the role of TBP-2 in IL-2-dependent growth in the *in vitro* model to investigate multistep transformation of HTLV-I. CpGs in the *TBP-2* gene are methylated in IL-2-independent but not in IL-2-dependent cells. Sequential treatment with 5-aza-2′-deoxycytidine and a histone deacetylase inhibitor augmented histone acetylation and TBP-2 expression, suggesting that loss of TBP-2 expression is due to DNA methylation and histone deacetylation. In IL-2-dependent cells, a basal level of TBP-2 expression was maintained by IL-2 associated with cellular growth, whereas TBP-2 expression was upregulated on deprivation of IL-2 associated with growth suppression. Overexpression of TBP-2 in IL-2-independent cells suppressed the growth and partially restored responsiveness to IL-2. Knockdown of TBP-2 caused the IL-2-dependent cells to show partial growth without IL-2. These results suggested that epigenetic silencing of the *TBP-2* gene results in a loss of responsiveness to IL-2, contributing to uncontrolled IL-2-independent growth in HTLV-I-infected T-cell lines.
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Correspondence: Dr H Masutani, Department of Biological Responses, Institute for Virus Research, Kyoto University, 53 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: hmasutan@virus.kyoto-u.ac.jp
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Introduction

Adult T-cell leukemia (ATL) is a disease entity with characteristic clinical and immunological features (Yodoi *et al.*, 1974; Uchiyama *et al.*, 1977), caused by human T-cell leukemia virus type-I (HTLV-I; Yoshida *et al.*, 1982). The long latent period and the age-dependent accumulation of leukemogenic events within HTLV-I-infected T cells suggest that the development of ATL is a multistep process (Okamoto *et al.*, 1989; Matsuoka, 2003). HTLV-I-infected T cells generally grow only in the presence of interleukin-2 (IL-2) (IL-2-dependent phase/IL-2-dependent cells; Maeda, 1992). But when these cells are cultured with IL-2 for several months, some fractions become able to proliferate continuously in the absence of IL-2 (IL-2-independent phase/IL-2-independent cells; Morgan *et al.*, 1976). The transition from the IL-2-dependent phase to IL-2-independent phase is an *in vitro* model for investigating the multistep process of the malignant transformation of HTLV-I-infected T cells (Maeda *et al.*, 1985; Migone *et al.*, 1995). ATL-derived factor (ADF) was cloned from HTLV-I-infected T cells (Tagaya *et al.*, 1989) and revealed to be a human homologue of thioredoxin (TRX; Holmgren, 1985). TRX is an oxidoreductase with important regulatory roles in cell proliferation and signal transduction (Masutani and Yodoi, 2002). TRX expression is markedly enhanced in HTLV-I-infected T cells (Makino *et al.*, 1992). Using the yeast two-hybrid screening system, we identified thioredoxin-binding protein-2 (TBP-2; Nishiyama *et al.*, 1999), which is identical to vitamin D_3 upregulated protein 1 (VDUP1; Chen and DeLuca, 1994). We have reported that TBP-2 has growth suppressive activity (Nishinaka *et al.*, 2004a) and its expression is abrogated in HTLV-I-infected IL-2-independent T cells, but maintained in HTLV-I-infected IL-2-dependent T cells as well as in HTLV-I-negative T cells (Nishinaka *et al.*, 2004a). TBP-2 expression is also downregulated in human cancers (Butler *et al.*, 2002; Han *et al.*, 2003). However, the mechanisms for the downregulation of TBP-2 expression remain unaddressed. In this study, we have investigated the mechanism of loss of TBP-2 expression in IL-2-independent HTLV-I-infected T-cell lines. We have also analysed the role of TBP-2 in IL-2-dependent growth control in HTLV-I-infected T-cell lines.

Results

Treatment with a demethylating reagent 5-aza-2′-deoxycytidine (5-aza-CdR) restored TBP-2 mRNA expression in HTLV-I-infected IL-2-independent cells

In the early stages of culture, HTLV-I-infected T cells generally grow only in the presence of IL-2 (IL-2-dependent cells). When these cells are cultured in the presence of IL-2 for several months; however, some fractions become able to proliferate continuously in the absence of IL-2 (IL-2-independent cells) (Morgan *et al.*, 1976; Maeda, 1992). Each set of IL-2-dependent and IL-2-independent cells has the same clonal origin as confirmed by the T-cell receptor-β gene rearrangement and HTLV-I proviral integration sites (Maeda, 1992). We analysed the expression of TBP-2 mRNA in sets of IL-2-dependent and IL-2-independent cells. The expression was lost in IL-2-independent cells (ATL-2, ED-40515, ATL-35T and ATL-43T) but not in IL-2-dependent cells (Figure 1a). However, we did not find genetic alterations in the promoter or coding region of the *TBP-2* gene (data not shown). The *TBP-2* promoter region nearest the TATA-box contained more than 49% GC and the observed CpG (number of CpG multiplied by total number of nucleotides of the region)/expected CpG (number of C multiplied by total number of nucleotides of the region) ratio was 0.64. The CpG/GpC ratio was 1. These values are considered sufficient to meet the criteria for CpG-dinucleotides susceptible to methylation (Gardiner-Garden and Frommer, 1987). Since hyper-methylation of promoter CpG-dinucleotides is often associated with inactivation of gene transcription, we first examined whether the *TBP-2* gene is methylated by testing the effect of a demethylating reagent, 5-aza-CdR, on TBP-2 expression in IL-2-independent ATL-43T and ATL-2 cells. Consistent with a previous report (Nosaka *et al.*, 2000), treatment with 5-aza-CdR restored CDKN2A gene expression (Figure 1b, mid gel). The treatment also restored the mRNA expression of TBP-2 in the cells (Figure 1b, upper gel, and 1c). To further examine the methylation status of the *TBP-2* promoter region, we directly sequenced the genomic DNA after treatment with sodium bisulfite. Sodium bisulfite converts C to T, except the C of methylated CpG. As shown in Figure 2, the CpGs in the promoter region and exon-1 of *TBP-2* were heavily methylated in IL-2-independent ATL cell lines, compared with those in IL-2-dependent cell lines. On treatment with 5-aza-CdR, about one-third of the methylated CpGs were demethylated in IL-2-independent ATL-43T and ATL-2 cells. These results strongly suggested that DNA methylation is involved in the mechanism of abrogation of TBP-2 mRNA expression in HTLV-I-infected IL-2-independent cells.

Involvement of histone deacetylation in TBP-2 gene silencing in HTLV-I-infected IL-2-independent cells

The restoration of TBP-2 expression by 5-aza-CdR in IL-2-independent cells seemed to be partial. Moreover, DNA methylation and histone deacetylation are

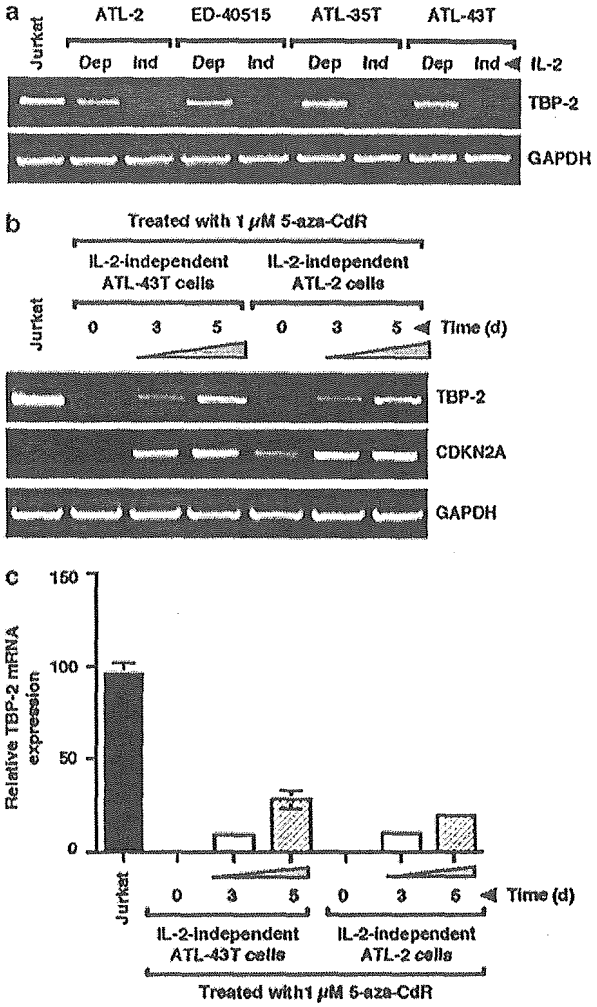


Figure 1 Restoration of thioredoxin-binding protein-2 (*TBP-2*) expression by 5-aza-2′-deoxycytidine (*5-aza-CdR*) in interleukin-2 (IL-2)-independent HTLV-I-infected T cells. An equivalent amount of total RNA was analysed by semiquantitative RT–PCR. GAPDH was amplified as an internal control. (a) Loss of TBP-2 expression in the HTLV-I-infected IL-2-independent T cells. *Dep*: IL-2-dependent cells and *Ind*: IL-2-independent cells. (b) Restoration of TBP-2 expression by 5-aza-CdR. IL-2-independent ATL-43T and ATL-2 cells were treated with 1 μM 5-aza-CdR for 0, 3 or 5 days and expression levels of TBP-2 and CDKN2A were analysed by semiquantitative RT–PCR. (c) Quantitative real-time RT–PCR analysis of TBP-2 mRNA expression. Data are representative of three separate experiments and the mean$\pm$s.d. from three replicates. 18S ribosomal mRNA expression was used as a cDNA loading control.

dynamically linked and synergistically silenced tumor suppressor genes (Cameron *et al.*, 1999). Therefore, we next analysed whether histone deacetylation is involved in *TBP-2* gene silencing. Treatment with suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor, alone was unable to restore TBP-2 expression in IL-2-independent ATL-43T and ATL-2 cells (Figure 3a). Then, the cells were sequentially treated with 5-aza-CdR and SAHA. TBP-2 gene expression was restored by treatment with 5-aza-CdR

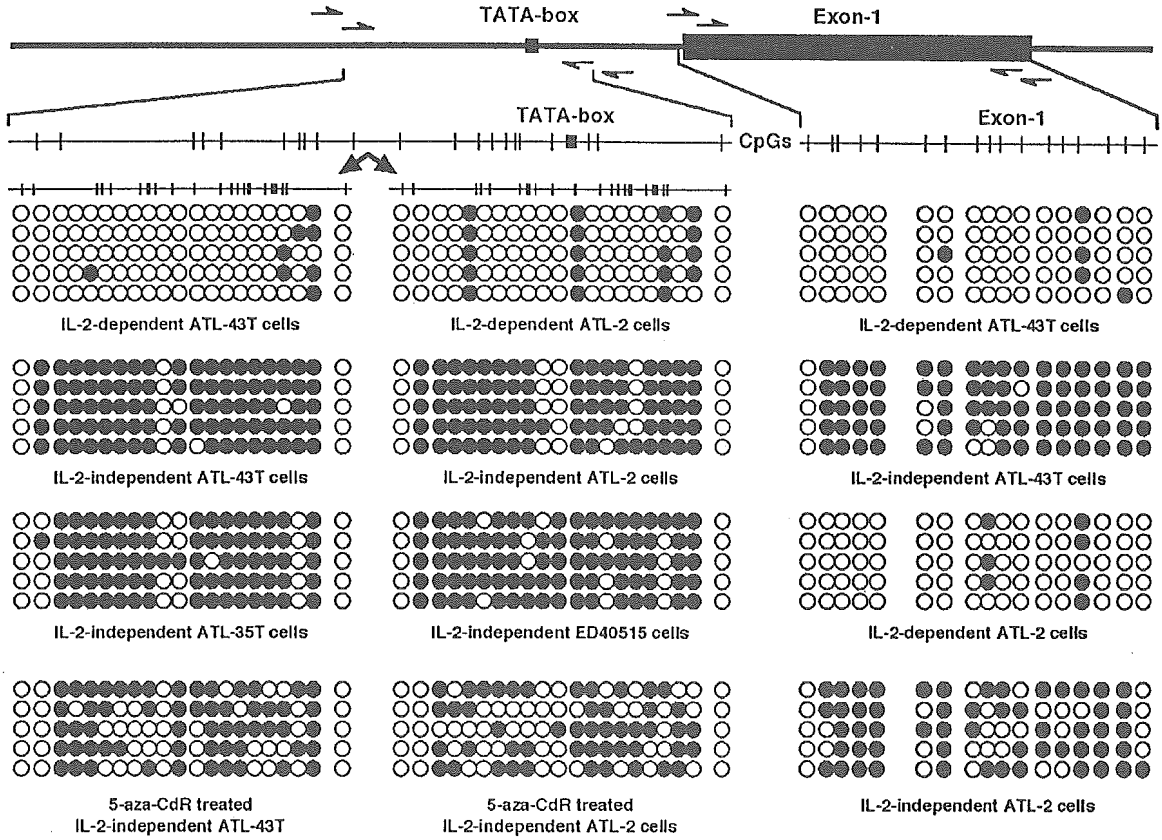


Figure 2 Methylation status of the thioredoxin-binding protein-2 (*TBP-2*) gene in HTLV-I-infected T-cell lines. Genomic DNA from HTLV-I-transformed IL-2-dependent and IL-2-independent cells before and after treatment with 5-aza-CdR were subjected to sodium bisulfite sequencing analyses as described in 'Materials and methods'. O, unmethylated CpG sites; •, methylated CpG sites.

for 3 days (Figure 3b, upper gel: lanes 3–5 and lanes 8–10), and further enhanced by additional treatment with SAHA for 1 day (Figure 3b, upper gel: lanes 6 and 11) in IL-2-independent ATL-43T and ATL-2 cells. The expression of TRX was not significantly altered by this treatment (Figure 3b, mid gel). These results suggested that both DNA-methylation and histone deacetylation synergistically silence the *TBP-2* gene in HTLV-I-infected IL-2-independent cells. In an earlier report (Butler *et al.*, 2002), SAHA augmented TBP-2 expression through the CCAAT element of the *TBP-2* promoter. However, SAHA alone was unable to restore the TBP-2 expression in our cases. To clarify whether the effect of SAHA is a cancellation of histone deacetylation, we performed a chromatin immunoprecipitation (Chip) assay in IL-2-independent ATL-43T and ATL-2 cells. The PCR signal obtained using anti-acetylated-H3 or H4 antibody was very weak in IL-2-independent ATL-43T and ATL-2 cells, before treatment or on treatment with SAHA only (Figure 3c). After treatment with 5-aza-CdR, the PCR signal was markedly enhanced. Sequential treatment with 5-aza-CdR and SAHA further strengthened the PCR signal. These results strongly suggested that DNA methylation and histone deacetylation are synergistically involved in the *TBP-2* gene silencing in HTLV-I-infected IL-2-independent cell lines.

Association between downregulation of TBP-2 expression in response to IL-2 and cellular growth

Augmented TBP-2 expression results in growth suppression (Nishinaka *et al.*, 2004a, b). We then analysed the relation between TBP-2 expression and growth in response to IL-2. We examined the level of TBP-2 expression and cell growth using [^{3}H]thymidine (Figure 4; ATL-43 T cells) and the MTT assay (Figure 5; ATL-2 cells). In IL-2-dependent ATL-43T (Figure 4a) and ATL-2 (Figure 5a) cells, TBP-2 expression was minimally maintained by the addition of IL-2, associated with cellular proliferation, whereas it was upregulated on deprivation of IL-2, associated with cell growth suppression. TBP-2 expression was recovered by sequential treatment with 5-aza-CdR and SAHA in IL-2-independent ATL-43T (Figure 4c) and ATL-2 (Figure 5c) cells. The expression of TBP-2 was also quantitated using real time RT–PCR (Figures 4d and 5d). These cells displayed a similar pattern of TBP-2 expression and growth in response to IL-2, mimicking that of IL-2-dependent cells. In contrast, IL-2-independent ATL-43T (Figure 4b) and ATL-2 (Figure 5b) cells

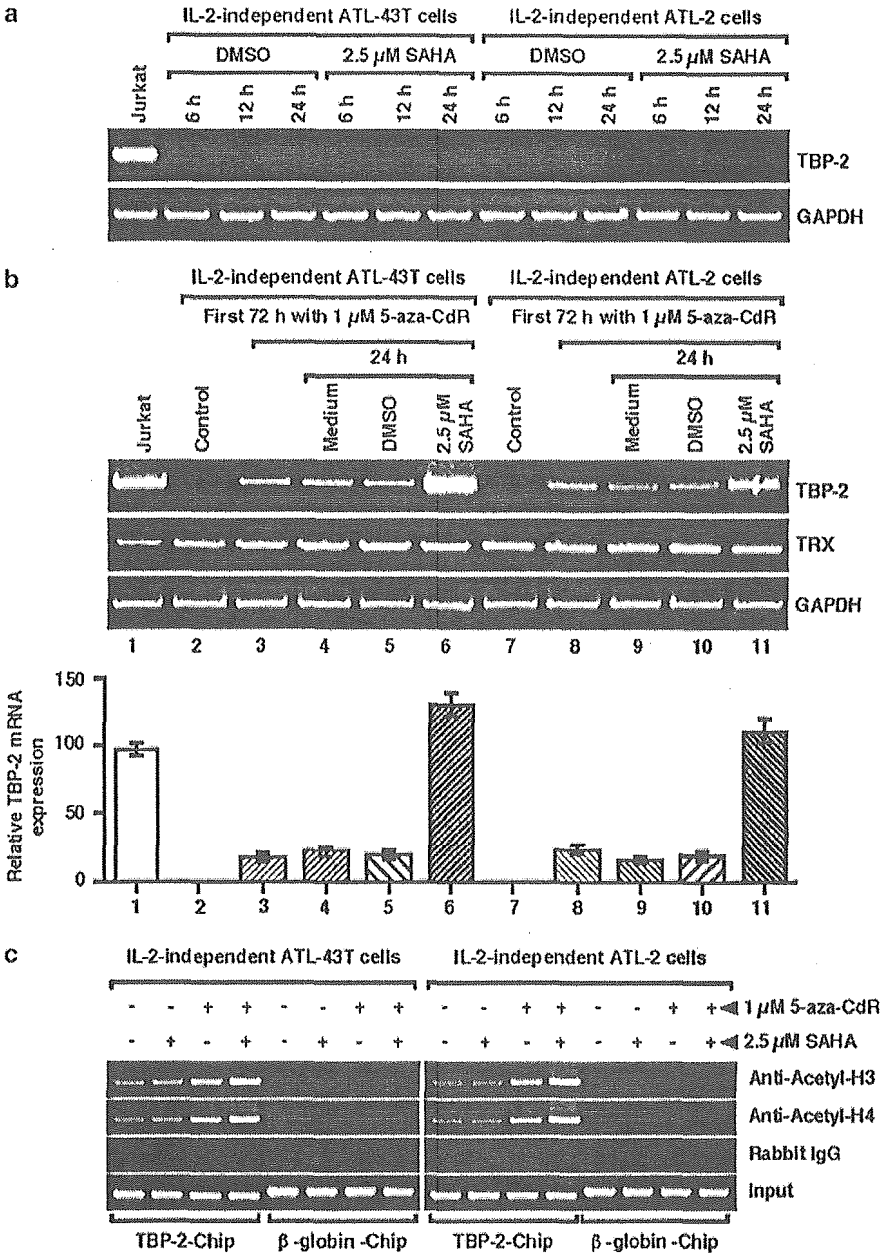


Figure 3 Restoration of thioredoxin-binding protein-2 (*TBP-2*) expression in interleukin-2 (IL-2)-independent HTLV-I-infected T cells by sequential treatment with 5-aza-2′-deoxycytidine (5-aza-CdR) and suberoylanilide hydroxamic acid (SAHA). (a) TBP-2 expression in SAHA-treated IL-2-independent ATL-43T and ATL-2 cells. The cells were treated with 2.5 μM SAHA at the indicated time points and analysed by semiquantitative RT–PCR. (b) TBP-2 and TRX expression in the cells sequentially treated with 5-aza-CdR and SAHA. The cells were treated with 1 μM 5-aza-CdR for the first 72 h, then with complete medium, DMSO, or 2.5 μM SAHA for 24 h. The bar graph shows mean values of real-time RT–PCR from three replicates. Similar results were obtained in three separate experiments. Error bars represent s.d. 18S ribosomal mRNA expression was used as a cDNA loading control. (c) The histone acetylation status of the *TBP-2* promoter region (TBP-2-Chip) and *β-globin* (β-globin-Chip) determined by chromatin immunoprecipitation (Chip) assay. Assays were performed three times with the indicated antibodies, followed by PCR analysis.

proliferated well regardless of IL-2. In these cells, TBP-2 expression was completely abrogated and the cell growth did not change in response to IL-2. In Figure 4e (ATL-43T) and Figure 5e (ATL-2), statistical analysis of the day-3 data showed a significant ($P<0.05$) difference in growth between the presence and absence of IL-2 in IL-2-dependent cells and sequentially treated IL-2-independent cells, but not in untreated IL-2-independent cells. These results suggested that restored TBP-2 expression is associated with the response to IL-2 and suppression of uncontrolled IL-2-independent growth.

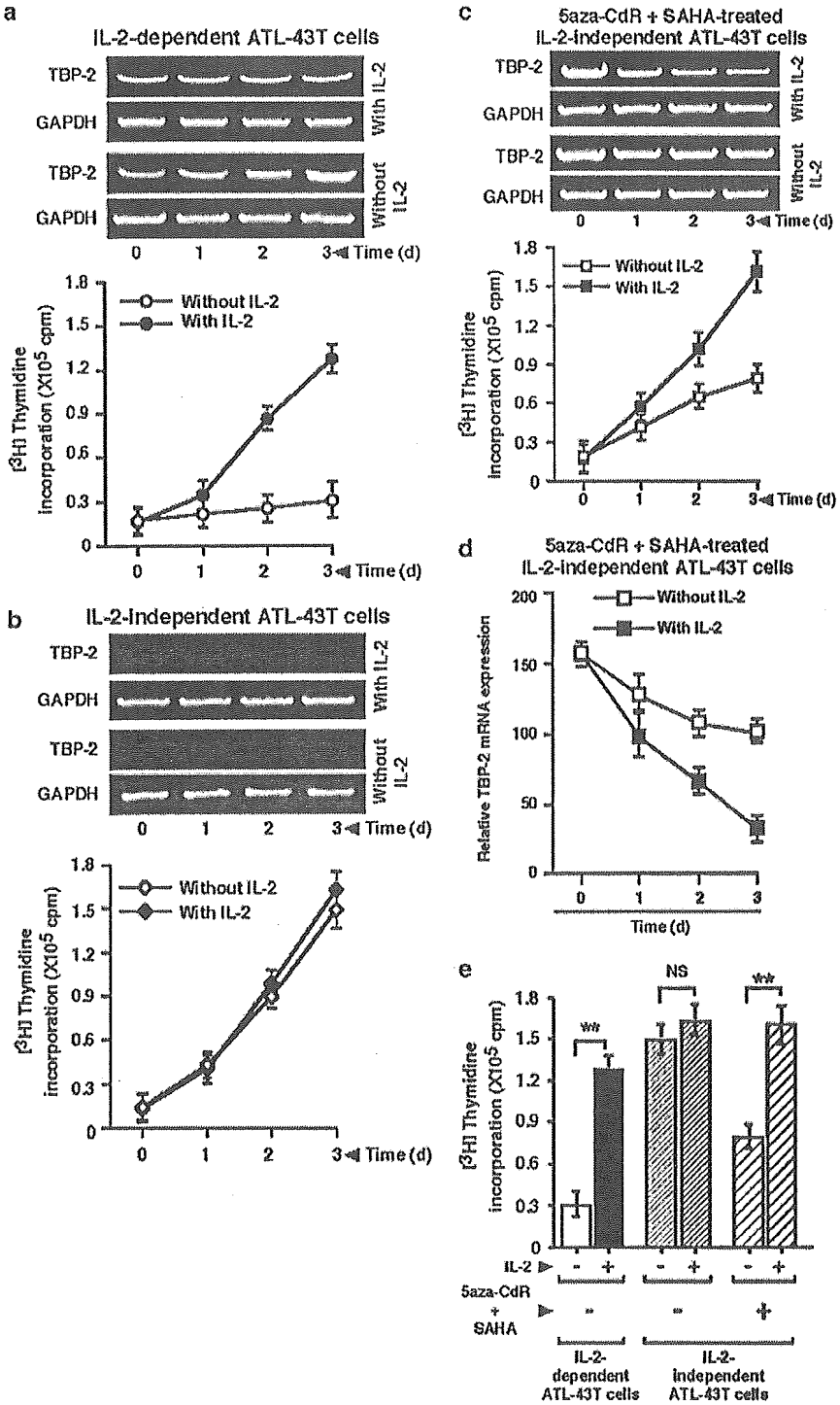
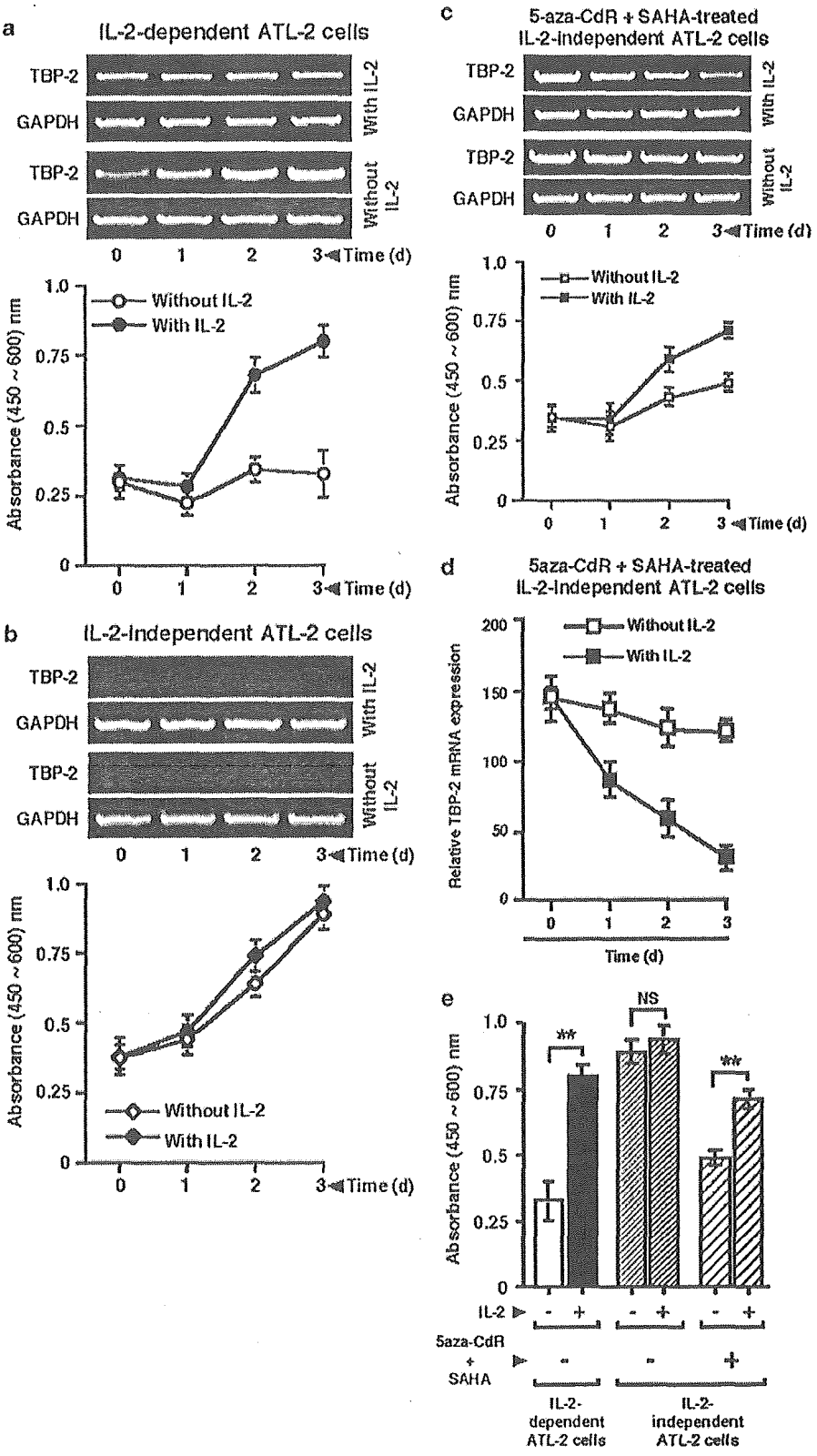


Figure 4 Thioredoxin-binding protein-2 (TBP-2) expression and cell growth in response to interleukin-2 (IL-2) in ATL-43T cells. The cells were cultured for 3 days in the absence or presence of IL-2 and cell growth was analysed using the thymidine incorporation assay. The expression of TBP-2 was analysed by semiquantitative RT–PCR. (a) IL-2-dependent ATL-43T cells, (b) IL-2-independent ATL-43T cells, and (c) IL-2-independent ATL-43T cells after sequential treatment with 5-aza-CdR and SAHA. (d) Quantitative real-time RT–PCR analysis of TBP-2 mRNA expression in the samples of Figure 4c. Data are representative of three separate experiments and the mean ± s.d. from three replicates. (e) Statistical analysis of the day-3 data. Data are representative of three separate experiments and the mean ± s.d. from three replicates. Nonsignificant and significant ($P<0.05$) differences are indicated by NS and (**), respectively, as determined with Student's *t* test.


a
IL-2-dependent ATL-2 cells
TBP-2
GAPDH
With IL-2
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0 1 2 3 Time (d)
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b
IL-2-Independent ATL-2 cells
c
5-aza-CdR + SAHA-treated
IL-2-Independent ATL-2 cells
d
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IL-2-Independent ATL-2 cells
Relative TBP-2 mRNA expression
Time (d)
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Ectopic expression of TBP-2 suppressed cell growth and partially restored IL-2 responsiveness

To further analyse the role of TBP-2 in the response to IL-2, we utilized HTLV-I-transformed MT-2 cells ectopically expressing TBP-2 (Nishinaka *et al.*, 2004a). TBP-2 protein expression in these transfectants was confirmed (Figure 6a). While the control clones C_1 and C_4 proliferated well regardless of IL-2, the TBP-2-overexpressing clones T_1 and T_2 proliferated less without IL-2 than with IL-2 ($P<0.05$; Figure 6b). Addition of IL-2 suppressed the expression of TBP-2 in TBP-2-overexpressing clones T_1 and T_2 (Figure 6b; Agarose gel snaps). These results suggested that the TBP-2 expression suppressed cell growth and partially restored responsiveness to IL-2. We further analysed the relationship between TBP-2 expression and IL-2-dependency in IL-2-dependent ATL-43T and ATL-2 cells by knocking down TBP-2 expression using RNA-interference (RNAi). The expression of TBP-2 was downregulated in these cells after the transfection with RNAi of TBP-2 but not with control RNAi (Figure 6c). The cells transfected with nonsilencing control RNAi proliferated in the presence of IL-2 but not in the absence of IL-2, while those cells transfected with TBP-2 RNAi showed partial proliferation in the absence of IL-2 (Figure 6d). These results suggest that loss of TBP-2 expression causes HTLV-I-infected IL-2-dependent cells to acquire a partial capability for growth without IL-2.

Discussion

We revealed that in IL-2-independent cells, the expression of TBP-2 is completely silenced due to DNA methylation and histone deacetylation (Figures 1–3). DNA methylation and histone deacetylation with the downregulation of gene expression for tumor suppressors such as CDKN2A, p53, E-cadherin and VHL tumor suppressor have been also reported (Ushijima and Okochi-Takada, 2005). TBP-2 is a regulator of the cell cycle and apoptosis (Butler *et al.*, 2002; Nishinaka *et al.*, 2004a). The *TBP-2* gene is downregulated during oncogenesis (Butler *et al.*, 2002; Han *et al.*, 2003), reported as a suppressor of melanoma metastasis (Goldberg *et al.*, 2003), and implicated in tumor progression in TBP-2 knockout mice (Oka *et al.*, in preparation). Therefore, the *TBP-2* gene seems to be an onco-suppressive gene. An HDAC inhibitor, SAHA, did not augment TBP-2 expression alone but induced the expression only after the reversal of DNA methylation (Figure 3a and b). Demethylation slightly induced histone acetylation (Figure 3c). These results indicate a link between DNA methylation and histone deacetylation, consistent with previous reports (Coombes *et al.*, 2003; Satoh *et al.*, 2003).

In HTLV-I-infected IL-2-dependent cells as well as murine IL-2-dependent CTLL-2 cells (data not shown), TBP-2 mRNA expression was upregulated on the deprivation of IL-2 and cells did not grow, whereas the expression was downregulated to within the normal range on the addition of IL-2 and cells started to grow, as shown by the current study and elsewhere (Nishinaka *et al.*, 2004a). TBP-2 gene expression is also induced by treatment with vitamin D_3 (Chen and DeLuca, 1994), HDAC inhibitors (Butler *et al.*, 2002), and serum deprivation (Han *et al.*, 2003). Therefore, expression of the *TBP-2* gene seems to be transcriptionally induced under conditions associated with cell growth arrest. TBP-2 mRNA expression paralleled the protein level (Nishinaka *et al.*, 2004a). The level of TBP-2 showed an inverse correlation with cell growth in response to IL-2 (Figure 4 and 5). TBP-2 may play a key role in the regulation of IL-2-dependent growth. TBP-2 gene regulation seems to be a sensor of deprivation of IL-2 signal. IL-2 deprivation upregulates TBP-2 expression transcriptionally, leading to cell growth arrest. Addition of IL-2 to the IL-2-deprived cells downregulates the expression of TBP-2 to the basal level, leading to proliferation. Therefore, loss of TBP-2 or loss of TBP-2 gene regulation causes loss of the growth control mechanism.

The ectopic expression of TBP-2 in MT-2 cells (Figure 6a) suppressed cell growth and restored the responsiveness to IL-2, although the response was partial (Figure 6b). Similarly, knockdown of TBP-2 by RNAi in IL-2-dependent cells (Figure 6c) caused a partial loss of IL-2-dependent growth control (Figure 6d). We consider the reason why TBP-2 overexpression only partially restored IL-2 dependence to be as follows. Since the transformation of HTLV-I seems to be a multistep process, TBP-2 alone may not be enough to restore the dependence on IL-2. In addition, the overexpressed *TBP-2* gene is less responsive to exogenous stimuli such as IL-2. The gene regulation of TBP-2 expression may be important for full IL-2-dependence. Overexpression of TBP-2 did not change the expression of the IL-2 receptor, or STAT binding activity (data not shown). Further study is needed to elucidate the mechanism of the regulation of TBP-2 expression in response to IL-2. The mechanism by which TBP-2 regulates growth suppression is under investigation. In our previous report, we observed a similar growth suppressive effect on overexpression of TBP-2 in other HTLV-I-transformed T cells (IL-2-independent

Figure 5 Thioredoxin-binding protein-2 (*TBP-2*) expression and cell growth in response to interleukin-2 (IL-2) in ATL-2 cells. The cells were cultured for 3 days in the absence or presence of IL-2 and cell growth was analysed by the MTT assay as described in 'Materials and methods'. The expression of TBP-2 was analysed by semiquantitative RT–PCR. (a) IL-2-dependent ATL-2 cells, (b) IL-2-independent ATL-2 cells, and (c) IL-2-independent ATL-2 cells after sequential treatment with 5-aza-CdR and SAHA. (d) Quantitative real-time RT–PCR analysis of TBP-2 mRNA expression in the samples of (c). Data are representative of three separate experiments and the mean ± s.d. from three replicates. (e) Statistical analysis of day-3 data. Data are representative of three separate experiments and the mean ± s.d. from three replicates. Nonsignificant and significant ($P<0.05$) differences are indicated by NS and (**), respectively as determined with Student's *t*-test.

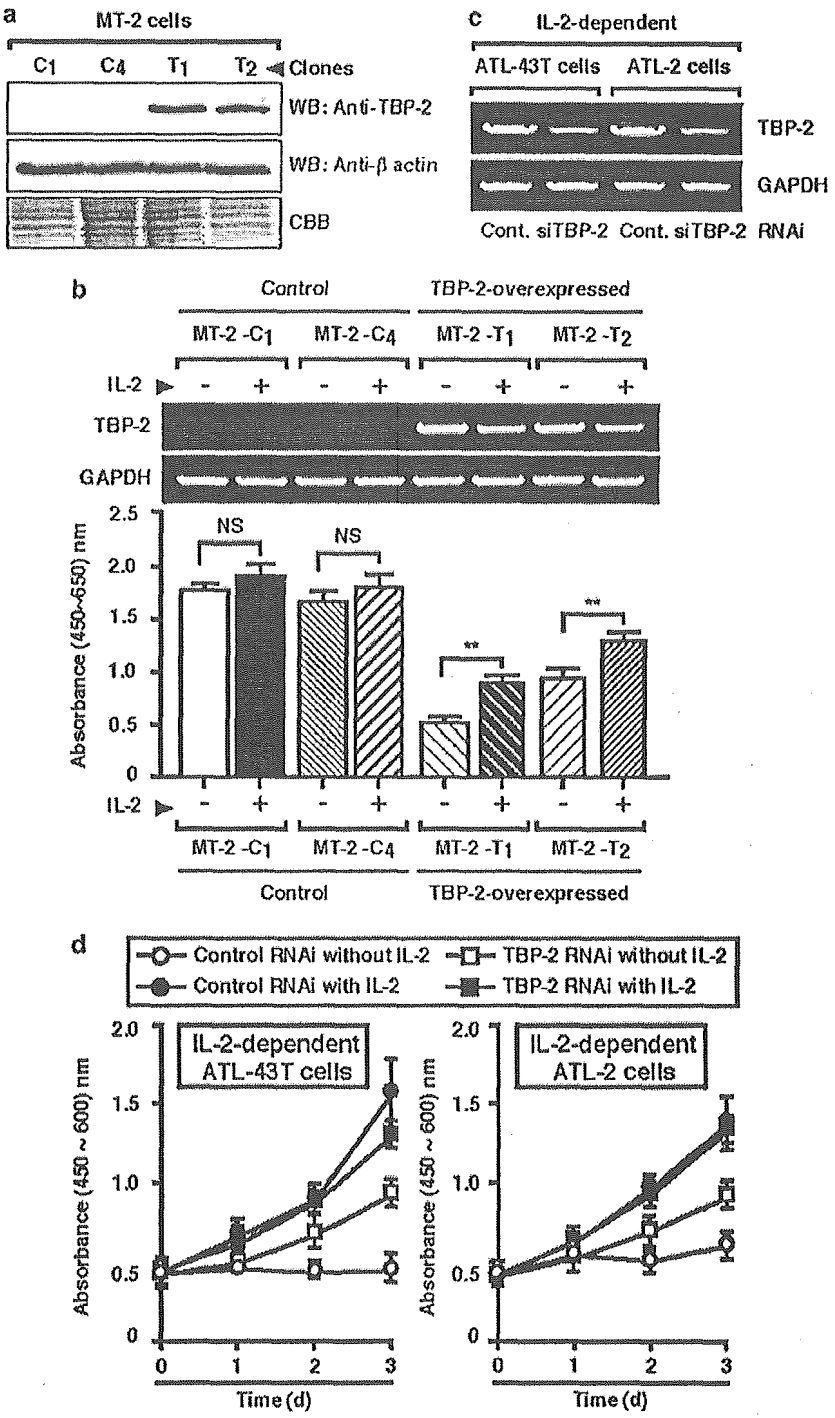

Figure 6 Relationship between TBP-2 expression and interleukin-2 (IL-2)-dependency in HTLV-I-infected T cells. (**a**) The MT-2 transfectants were stably transfected with *TBP-2* (T_1 and T_2) or control vector (C_1 and C_4) and the TBP-2 expression level was confirmed by Western blotting with monoclonal anti-TBP-2 antibody. Western blotting with monoclonal anti-β-actin antibody and staining with Coomassie Brilliant Blue R-250 (*CBB*) were used to monitor the amount of sample loaded. (**b**) Partial recovery of IL-2 dependency by overexpression of TBP-2. The TBP-2-overexpressing T_1 and T_2 or control C_1 and C_4 clones of MT-2 cells were cultured for 3 days in the absence or presence of IL-2 and cell proliferation was analysed by the MTT assay using WST-1 reagent. The upper panel shows the TBP-2 expression level analysed by semiquantitative RT–PCR. The cell proliferation data for day 3 in the absence or presence of IL-2 represent the mean ± s.d. from three replicates. Similar results were obtained in three separate experiments. Nonsignificant and significant ($P<0.05$) differences are indicated by NS and (**), respectively, as determined with Student's *t*-test. (**c**) The expression of TBP-2 in cells transfected with RNAi of TBP-2. The level of TBP-2 was analysed by semiquantitative RT–PCR after 48 h of transfection. (**d**) IL-2-independent growth by knockdown of TBP-2 in HTLV-I-infected IL-2-dependent cells. After transfection with RNAi of TBP-2 or control, the cells were cultured for 3 days in the absence or presence of IL-2 and cell growth was analysed by MTT assay as described in 'Materials and methods'. The same results were obtained from three separate experiments.

ATL-43T, ATL-2, and MT-2 cells). We also reported the disappearance of TBP-2 expression in Hut-102, ATL-2, MT-1 and MT-2 cells (Nishinaka *et al.*, 2004a), showing the generality of the finding in HTLV-I-infected cells. Loss of TBP-2 expression may augment the reducing activity of TRX (Nishiyama *et al.*, 1999), enhancing the growth of HTLV-I-infected T cells. In MT-2 cells ectopically expressing TBP-2, the expression of p16 was augmented (Nishinaka *et al.*, 2004a). Overexpression of TBP-2 also suppressed cell growth in MCF-7 cells, in which the p16 gene is deleted (Nishinaka *et al.*, 2004b), suggesting that there are other mechanisms to suppress cell growth. Analyses of gene expression in *TBP-2* knockout cells using Gene chips are underway. TBP-2 is localized to the nuclear compartment (Nishinaka *et al.*, 2004b). The interaction of TBP-2 with transcriptional repressors such as Fanconi anemia zinc-finger (FAZF), promyelocytic leukemia zinc-finger (PLZF) and HDAC1 has been suggested (Han *et al.*, 2003), and we have isolated several nuclear proteins that are important for the regulation of transcriptional events as partners of TBP-2 (Masutani *et al.*, in preparation). Therefore, it could be speculated that TBP-2, augmented by various stimuli, interacts with growth controlling molecules such as an HDAC complex to induce cell growth arrest.

In summary, we showed that the *TBP-2* gene is silenced due to DNA-methylation and histone deacetylation in IL-2-independent cell lines and that TBP-2 expression is closely associated with responsiveness to IL-2-dependent growth. The complete silencing of the *TBP-2* gene is seen in HTLV-I-infected IL-2-independent cells but not in other T-cell lines (Nishinaka *et al.*, 2004a), EBV-transformed cell lines, and other cell lines (unpublished observation). Therefore, the silencing may be specifically linked to the HTLV-I infection. Therefore, the role of the loss of TBP-2 expression seems more important in HTLV-I-infected leukemic cells. Although TBP-2 expression does not seem to be associated with Tax expression in the HTLV-I infected cell lines (data not shown), the role of viral proteins including Tax should be examined further. Clastogenetic changes (point mutations, deletions, substitutions, and translocations) are frequently found in HTLV-I-transformed cells. Disruption of base excision repair, nucleotide excision repair, DNA end stability, telomerase and cell cycle progression leads to an increase in the frequency of genomic mutation. ATL cells are convoluted and known to be aneuploid, suggesting an abnormality of the mitosis checkpoint in HTLV-I transformation (Jeang *et al.*, 2004). These aspects of HTLV-I transformation and ATL leukemogenesis are important. The role of loss of TBP-2 in these aspects of HTLV-I transformation should be also investigated further.

Materials and methods

Cell culture and transfections

Human lymphocytic leukemia T cells (Jurkat) and HTLV-I-infected T cells (ATL-2, MT-2, ED-40515, ATL-35T and ATL-43T) were cultured in RPMI-1640 medium containing 10% heat inactivated fetal calf serum (FCS) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) at 37°C with 5% CO_2 in air. For the maintenance of IL-2-dependent HTLV-I-infected T cells, IL-2 (7.5 ng/ml; Peprotech EC) was added to the culture medium. HTLV-I-infected IL-2-dependent T cells, such as ATL-2, ED-40515, ATL-35T and ATL-43T), were established from ATL patients by culture in the presence of IL-2, whereas IL-2-independent cells were established by the long-term culture of IL-2-dependent cells (Maeda, 1992). Each set of IL-2-dependent and IL-2-independent cells has the same clonal origin as confirmed by the T-cell receptor-β gene rearrangement and HTLV-I proviral integration sites (Maeda, 1992). Stable MT-2 transfectants overexpressing TBP-2 were generated as described previously (Nishinaka *et al.*, 2004a) and maintained in medium containing 2 mg/ml of G418 (Nacalai Tesque).

Treatment of cells with 5-aza-CdR or SAHA

For the treatment with a demethylating reagent, IL-2-independent ATL-43T and ATL-2 cells were cultured in medium supplemented with 1 μM 5-aza-CdR (Sigma) for 3 or 5 days. For the treatment with HDAC inhibitors, cells were cultured in medium supplemented with 2.5 μM SAHA (Alexis Biochemicals). For the sequential treatment with the demethylating reagent and HDAC inhibitors, the cells were cultured in medium supplemented with 1 μM 5-aza-CdR for the first 3 days, then with 2.5 μM SAHA for the next 24 h. After the sequential treatment, the cells were cultured in the presence or absence of IL-2 (1U) for an additional 3 days. SAHA was dissolved in dimethyl sulfoxide (DMSO). After each treatment, cells were placed in fresh medium.

Measurement of TBP-2 mRNA expression by RT–PCR

For the TBP-2 mRNA expression analyses, total RNA was isolated from the cells using Trizol reagent (Invitrogen). cDNA was synthesized using a SuperScript First-Strand Synthesis System (Invitrogen) with oligo dT_{12-18}. The cDNA was amplified by PCR using a KlenTaq-LA DNA polymerase mix (Sigma). The primers used for the amplification were as follows: *TBP-2*, 5′-CCATGGTGATGTTCAAGAAGAT CAAG-3′ (forward) and 5′-CTCAGGGGCATACATAAA GA-3′ (reverse); *TRX*, 5′-ATGGTGAAGCAGATCGAG-3′ (forward) and 5′-TTAGACTAATTCATTAATGGT-3′ (reverse); *CDKN2A*, 5′-TTCGGCTGACTGGCTGGCCA-3′ (forward; exon 1) and 5′-AGCTCCTCAGCCAGGTCCAC-3′ (reverse; exon 2); and *glyceraldehydes-3-phosphate dehydrogenase (GAPDH)*, 5′-ATGGGGAAGGTGAAGGTCGGAG TC-3 (forward) and 5′-CCATGCCAGTGAGCTTCCCGTT C-3′ (reverse). PCR was performed under the following conditions: 32 cycles for *TBP-2* (denaturing at 94°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 2 min), 20 cycles for *TRX* (denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min), 30 cycles for *CDKN2A* (denaturing at 94°C for 30 s, annealing at 61°C for 45 s, and extension at 72°C for 1 min), and 22 cycles for *GAPDH* (denaturing at 94°C for 30 s, annealing at 61°C for 1 min, and extension at 72°C for 90 s). The PCR products were visualized by electrophoresis in 3% NuSieve GTG agarose (Cambrex Bio Science) gel.

Quantitative real-time RT–PCR

For measurement of the quantity of *TBP-2* mRNA, real-time RT–PCR was conducted with a TaqMan Universal Master Mix (Applied Biosystems) using reverse transcribed cDNA as a template, in triplicate. The amplification was performed