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# Transcriptional Control of Spliced and Unspliced Human T-Cell Leukemia Virus Type 1 bZIP Factor (*HBZ*) Gene[▽]

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**The human T-cell leukemia virus type 1 (HTLV-1) basic leucine zipper factor (*HBZ*) gene is encoded by the minus strand of the HTLV-1 provirus and transcribed from the 3′ long terminal repeat (LTR). *HBZ* gene expression not only inhibits the Tax-mediated activation of viral gene transcription through the 5′ LTR but also promotes the proliferation of infected cells. However, the *HBZ* promoter region and the transcriptional regulation of the gene have not been studied. In this study, we characterize the promoters of the spliced version of the *HBZ* gene (s*HBZ*) and the unspliced version of the *HBZ* gene (us*HBZ*) by luciferase assay. Both promoters were TATA-less and contained initiators and downstream promoter elements. Detailed studies of the promoter for the s*HBZ* gene showed that Sp1 sites were critical for its activity. The activities of the s*HBZ* and us*HBZ* gene promoters were upregulated by Tax through Tax-responsible elements in the 3′ LTR. We compared the functions of the proteins derived from the s*HBZ* and us*HBZ* transcripts. sHBZ showed a stronger suppression of Tax-mediated transcriptional activation through the 5′ LTR than did usHBZ; the level of suppression correlated with the level of protein produced. The expression of s*HBZ* had a growth-promoting function in a T-cell line, while us*HBZ* expression did not. This study demonstrates that Sp1 is critical for s*HBZ* transcription, which accounts for the constitutive expression of the s*HBZ* gene. Functional differences between s*HBZ* and us*HBZ* suggest that the s*HBZ* gene plays a significant role in the proliferation of infected cells.**

Human T-cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL) (9, 33). Since HTLV-1 is transmitted in a cell-to-cell fashion (13), HTLV-1 facilitates its transmission by increasing the number of infected cells via the action of regulatory and accessory genes encoded in the pX region (11, 22). The plus strand of HTLV-1 encodes the regulatory (*tax* and *rex*) and accessory (*p12*, *p13*, and *p30*) genes. Among them, the *tax* gene is thought to play a critical role in the proliferation of infected cells and in oncogenesis by its pleiotropic actions (11, 22). In addition to the genes encoded by the plus strand, a gene encoded by the minus strand is also recognized (17). The gene is designated the HTLV-1 basic leucine zipper factor (*HBZ*) gene (10), and the encoded protein can bind to cyclic AMP response element (CRE) binding protein 2 (CREB2). The HBZ protein, which has a bZIP domain in the C-terminal region, can interact with c-Jun, JunB, JunD, and CREB (2, 18, 35). Through interactions with HBZ and c-Jun, HBZ inhibits transcription from the HTLV-1 long terminal repeat (LTR) and from activator protein 1 (AP-1)-dependent promoters (2). Recently, we reported that a spliced form of *HBZ* (s*HBZ*) is also transcribed from the 3′ LTR in all ATL cases and supports the proliferation of ATL cells (28). Two other groups also reported this spliced form of the *HBZ* gene transcript (4, 24). In addition, another alternative splice form of the *HBZ* gene transcript has been reported (4). However, the transcriptional regulation of the *HBZ* gene remains unelucidated.

Bidirectional transcription through viral LTRs has been recognized (6, 29); most such LTRs belong to endogenous retroviruses. However, only a few coding genes encoded by the minus strands of proviruses have been found. The *HBZ* gene is the first one proven to have important functions in viral replication and in the proliferation of infected cells (1, 2, 10, 28). A similar gene encoded by the minus strand of the provirus has been identified in simian T-cell leukemia virus type 1 (STLV-1) but not in HTLV-2 and STLV-2 (32). It is noteworthy that both HTLV-1 and STLV-1 can induce cancers, while neither HTLV-2 nor STLV-2 is associated with oncogenesis. Transcription from the 5′ LTR of HTLV-1 has been extensively characterized, and this transcription is highly inducible by Tax cooperating with CREB and CREB-binding protein and p300 (CBP/p300) (11, 16). On the other hand, the ubiquitous expression of the *HBZ* gene in infected cells and ATL cells suggests that its transcriptional control differs from that of the plus-strand genes.

In this study, we characterize the promoter regions of the spliced and unspliced versions of the *HBZ* gene. We report that in contrast to the highly inducible 5′ LTR, the s*HBZ* promoter is activated by the constitutively expressed transcription factor Sp1. However, in the unspliced *HBZ* (us*HBZ*) promoter, Tax has an effect on transcription activity through Tax-responsible elements (TREs), as observed in the 5′ LTR. Analysis of the ability of HBZ to inhibit transcription showed that sHBZ had a stronger function than usHBZ. In addition, s*HBZ* RNA could promote T-cell growth, whereas us*HBZ* RNA did not have growth-promoting activity.

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## MATERIALS AND METHODS

**Cell lines.** Four HTLV-1-transformed cell lines and two HTLV-1-uninfected T-cell lines were used in this study: ATL-55T, ATL-43T, and MT-1 were derived from leukemic cells (34). Jurkat and Kit225 were not infected with HTLV-1. These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The 293FT cell line is a subline derived from transformed HEK293T embryonal kidney cells. 293FT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 500 μg/ml G418.

**5′-RACE.** 5′ rapid amplification of cDNA ends (RACE) for us*HBZ* was performed using the Smart RACE cDNA amplification kit (BD Biosciences Clontech) according to the manufacturer's instructions. The cDNAs were synthesized from 1 μg total RNA of ATL-43T or MT-1 cells using reverse transcriptase (RT). The first-strand cDNAs were used in 5′-RACE PCR. For nested amplifications, primers specific for the us*HBZ* gene (5′-CGTCACGCCCTACT GGCCACCTGTCCAG-3′ and 5′-CGGCCCGCCTACATCGTCACGCCCTA CT-3′) were used. After nested PCR, bands were cloned, and the nucleotide sequences were determined.

**Plasmids.** The transcriptional start sites of s*HBZ* were reported previously (28). The putative promoter regions of s*HBZ* or us*HBZ* were obtained by PCR from genomic DNA of ATL-43T cells and then cloned into the luciferase reporter vector pGL4.22[*luc2CP*/Puro] (Promega, Madison, WI). pGL4-3′LTR300(1-300) contains the promoter region of the s*HBZ* gene, which spans positions −354 to −54 relative to the translation initiation site (position +1). pGL4-3′LTR240(61-300), spanning positions −299 to −54; pGL4-3′LTR180(121-300), spanning positions −234 to −54; pGL4-3′ LTR120(181-300), spanning positions −174 to −54; and pGL4-3′LTR60(241-300), spanning positions −114 to −54, are 5′ deletion mutants of pGL4-3′ LTR300. pGL4-TRE+300 was made from pGL4-3′LTR300; this construct contained a $TRE^S$ region (positions 481 to 960) upstream of the 3′LTR300 sequence. Base substitution mutants [pGL4-3′LTRGATA-2mut, pGL4-3′LTRT axCREBmut, pGL4-3′LTRAP1mut, pGL4-3′LTRNF1mut, pGL4-3′LTRSp1. 2mut, pGL4-3′LTRSp(1.2.3)mut, and pGL4-3′LTRSp1(3)mut] were generated in pGL4-3′LTR300 by site-directed mutagenesis. Factor binding sites were analyzed by TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Oligonucleotides designed to mutate each element are as follows (mutated bases are shown in boldface type): AGGATGGG (positions 34 to 41)→AG**TCG**GGG for the GATA-binding protein 2 (GATA-2) site, GCTGTCGCT (positions 41 to 49)→ GC**GCTAC**CT for the Tax and CREB (TaxCREB) sites, GCTGGCTC (positions 47 to 54)→GC**ACCG**TC for the AP-1 site, CGCTGGCTCCGAGCCAA (positions 46 to 62)→CGC**ATT**CTCCGAG**GA**AA for nuclear factor 1 (NF1) site A, CTTGGCCGTGGGCCAAG (positions 77 to 93)→CT**ATT**CCGTGGG**G A**AAG for NF1 site B, GATGGGCTGT (positions 36 to 45)→GATG**TT A**TGT for Sp1 site A, CGCTGGCTCCGA (positions 46 to 57)→CGCT**TTA** TCCGA for Sp1 site B, and GGGGTGGAAC (positions 123 to 132)→GG**T TA**GGAAC for Sp1 site C. pGL4-3′LTRNF1mut contains mutations in two NF1 regions (NF1 sites A and B). pGL4-3′LTRSp(1.2)mut contains mutations in two Sp1 regions (Sp1 sites A and B). pGL4-3′LTRSp(1.2.3)mut contains mutations in all three Sp1 regions (Sp1 sites A, B, and C). pGL4-3′LTRSp1(3) mut contains mutations in only one Sp1 region (Sp1 site C). pGL4-uHBZup1, pGL4-uHBZup2, pGL4-uHBZup3, and pGL4-3′LTRwhole contain the estimated promoter sequences of us*HBZ*, which are from positions 1 to 1461 (up1), 247 to 1461 (up2), 725 to 1461 (up3), and 1 to 757 (3′LTRwhole), respectively, in the HTLV-1 sequence. Base substitution mutants (pGL4-uHBZup2TRE1. 3mut and pGL4-uHBZup2TRE1.3+NF-κBmut) were generated in pGL4-uHBZup2 by site-directed mutagenesis. Oligonucleotides designed to mutate each element are as follows (mutated bases are shown in boldface type): GGT TGTCGTCA→**ACTGACACATC** (positions 490 to 500) for TRE site 1, GGGA CACGTCA→**ACGGACACATC** (positions 590 to 600) for TRE site 2, and GG GAGACGTCA→**ACGGACACATC** (positions 639 to 649) for TRE site 3 in the pGL4-uHBZup2 construct, and GTGGCTTCCC→**AAACGCAGGA** (positions 550 to 559) for the nuclear factor κB (NF-κB) site in the pGL4-uHBZup2 construct. pME18SneoHBZ is the *HBZ* expression vector. pCG-Tax is the vector wild-type *tax* gene. pWT-Luc, which contains five copies of the 21-bp TRE sequences derived from the 5′ LTR of HTLV-1, was derived from 21WT-CAT (8). pME18Sneo/sHBZ, pME18Sneo/usHBZ, pcDNA3.1(−)sHBZ-MycHis, and pcDNA3.1(−)usHBZ-MycHis are *HBZ* expression vectors. pcDNA3.1(−)sHBZ-MycHis and pcDNA3.1(−)usHBZ-MycHis express HBZ with a C-terminal Myc-His tag.

**Luciferase assay.** Jurkat cells were plated onto six-well plates ($2 \times 10^5$ cells/well). After 24 h, cells were transfected with 1 μg/well of luciferase reporter plasmid, 10 ng/well of *Renilla* luciferase control vector (phRL-TK) (Promega), and/or approximately 0.5 to 1 μg/well of the *tax* expression vector and/or the *HBZ* expression vector. Plasmids were transfected using TransFectin lipid reagent (Bio-Rad, Hercules, CA). After incubation for 48 h, cells were collected, and the luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega) and measured by a luminometer (Lumat LB 9507 luminometer; EG&G Berthold, Australia). Relative luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity, and the luciferase values were expressed as increases relative to that of each negative control. Values represent means ± standard deviations (SD) (error bars) ($n = 3$). Three independent experiments, each with triplicate transfections, were performed, and typical results are shown.

**Transcription factor/oligonucleotide binding study.** Sp1 binding to the 5′ *HBZ* promoter Sp1 binding sequence was studied using a competitive biotinylated oligonucleotide binding assay (NoShift transcription factor assay kit; Novagen, Germany), which is a colorimetric assay similar to an electrophoretic mobility shift assay. Jurkat cell nuclear extracts were prepared by using the NucBuster protein extraction kit (Novagen). Double-stranded probes comprised of two 3′-biotinylated oligonucleotides corresponding to the Sp1 site (positions 118 to 137) were used. First, probes were incubated with nuclear extract in NoShift bind buffer, poly(dI-dC) · poly(dI-dC), and salmon sperm DNA in a 96-well streptavidin-coated plate. After washing, anti-Sp1 antibodies were added to the samples and incubated for 1 h. Wells were then washed again and incubated with secondary antibody conjugated with horseradish peroxidase (HRP). Finally, after washing, TMB substrate (3.3′,5.5′-tetramethylbenzidine) was added to each well, and the absorbance (450 nm) was measured by a plate reader. To investigate specific binding between the wild-type probe and Sp1 factors, we performed competition studies using a 10-fold molar excess of either a nonbiotinylated oligonucleotide or a nonbiotinylated oligonucleotide containing point mutations within the transcription factor binding sequence. The Sp1 oligonucleotides used are shown in Fig. 3A. Values represent means ± SD ($n = 3$). Three independent experiments, each with triplicate transfections, were performed, and typical results are shown.

**Cell proliferation assay.** For the construction of vectors expressing the s*HBZ*, us*HBZ*, and TTG-*HBZ* genes, those cDNAs were subcloned into the pME18Sneo vector. Vectors were transfected into an interleukin-2 (IL-2)-dependent human T-cell line, Kit225 (12), using Nucleofector (Amaxa Biosystems, Cologne, Germany). Cells were suspended in 100 μl of Cell Line Nucleofector solution T and then nucleofected with vectors (5 μg) using program T-16 of the Nucleofector device (Amaxa Biosystems). Stable transfectants were selected in G418 (600 μg/ml). After IL-2 depletion, proliferation was studied in the presence of a small amount of IL-2 (2.5 U/ml). Cell viabilities were assessed by measuring 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye absorbance. Values represent means ± SD (error bars) ($n = 3$). Three independent experiments, each with triplicate transfections, were performed, and typical results are shown.

**ChIP assay.** Chromatin shearing was performed using the Shearing chromatin immunoprecipitation (ChIP) kit as recommended by the manufacturer (Diagenode SA, Belgium). ATL-43T cells ($2 \times 10^6$ cells) were used for each ChIP assay. Formaldehyde was added to cells at a final concentration of 1.5%, and cells were incubated at room temperature for 7 min. Sonication was performed on ice using a Bioruptor apparatus (Cosmo Bio Co., Ltd., Japan) (power setting on "high") for 10 cycles of 30 s on and 30 s off, corresponding to a total time of 10 min. The size of the sheared chromatin was around 300 to 1,000 bp. Sheared chromatin was precleaned with 20 ml of preblocked *Staphylococcus* A cells at 4°C with rotation for 15 min and diluted in immunoprecipitation dilution buffer. A 25-ml aliquot (2.5% of the total) was removed to serve as an input sample. ChIP was performed with 2 mg of Sp1 antibody (PEP 2) (sc-59X; Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit immunoglobulin G (Santa Cruz Biotechnology) at 4°C overnight with rotation. To collect immune complexes, 20 ml of preblocked *Staphylococcus* A cells were added, and the sample was incubated first at room temperature for 15 min and then at 4°C with rotation for 30 min. After washing of the immune complexes, we isolated DNA from the immunoprecipitated chromatin. Finally, purified DNA was used in quantitative PCR. For the 3′ LTR/Sp1 site, PCR was performed under the following conditions: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 10 min at 72°C. For an Sp1-ChIP positive control, we used PCR amplification of the dihydrofolate reductase (*DHFR*) gene, which has Sp1-binding sites within its promoter; this PCR was performed under the following conditions: 2 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 57.4°C, and 30 s at 72°C; and 10 min at 72°C. Primers used were 5′-GCCGTGGGCCAAGCCGGCAGTCAGTCGTG A-3′ (positions 81 to 110) (sense) and 5′-CCTTGGAGCCTACCTAGACTCA GCCGGCTC-3′ (position 211 to 240) (antisense) for the 3′ LTR/Sp1 site and 5′-TCGCCTGCACAAATAGGGAC-3′ (sense) and 5′-AGAACGCGCGGTC

AAGTTT-3′ (antisense) for *DHFR*. DNA from input lysates was used as the input control.

**Western blot analysis.** 293FT cells were transiently transfected with pcDNA3.1(−)sHBZ-MycHis or pcDNA3.1(−)usHBZ-MycHis. The cells were then treated with 20 μM proteasome inhibitor, MG132 (Calbiochem, Darmstadt, Germany), for 12 h and then lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X, 1 mM $MgCl_2$, and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After 30 min on ice, lysates were cleared by centrifugation at 13,000 × *g* for 15 min at 4°C. Extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto a polyvinylidene difluoride membrane (Atto, Tokyo, Japan). The blots were blocked in blocking buffer for 1 h at room temperature and incubated in 2,000-fold-diluted anti-His tag polyclonal antibody (MBL, Nagoya, Japan) for 1 h at room temperature. After three washes in 5% Tween–phosphate-buffered saline (PBS), the blots were incubated in 4,000-fold-diluted anti-rabbit immunoglobulin-HRP (Amersham Biosciences) for 1 h at room temperature. After three washes in Tween-PBS, bound antibodies were detected using the Western Blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology). The blots were then exposed on Hyperfilm ECL film (Amersham Biosciences). Next, we made Tax (wild-type or mutated Tax)-expressed cells. 293FT cells were plated onto six-well plates ($2 \times 10^5$ cells/well). After 24 h, cells were transfected with 1 μg/well of the empty or *tax* expression vector (pCG-BL, pCG-Tax, pCG-M22, or pCG-M47) and 1 μg/well of pGL4-uHBZup1. Plasmids were transfected using FuGENE 6 transfection reagent (Roche, Mannheim, Germany). After incubation for 48 h, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 125 mM NaCl, 0.5% NP-40, and protease inhibitor cocktail. Sonication was performed on ice using the Bioruptor (power setting on "high") for 10 cycles of 30 s on and 30 s off, corresponding to a total time of 10 min. Lysates were cleared by centrifugation at 14,500 × *g* for 15 min at 4°C. Proteins (20 μg) were separated by SDS-PAGE followed by electrotransfer onto a polyvinylidene difluoride membrane (Atto). The blots were blocked in blocking buffer overnight at 4°C and incubated in 2,000-fold-diluted mouse serum anti-Tax antibody (MI-73) for 1 h at room temperature. After five washes in 0.5% Tween-PBS, the blots were incubated in 4,000-fold-diluted anti-mouse HRP-conjugated secondary antibody (Amersham Biosciences) for 1 h at room temperature. After two washes in Tween-PBS, the protein bands were visualized with the Western Blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology) and quantified by LAS 3000 (Fujifilm, Tokyo, Japan).

**cDNA synthesis and RT-PCR.** Total RNA was extracted from transfectants using Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 1 μg of total RNA with the Superscript preamplification system (Invitrogen) according to the manufacturer's protocol. PCR was performed using 1 μl of RT reaction sample mixed with 50 μl of PCR buffer containing 0.2 μM of each deoxynucleotide triphosphate, 2 μM $MgCl_2$, 1.25 units of ExTaq polymerase (TaKaRa, Shiga, Japan), and 0.5 μM of each primer using the hot start technique with AmpliWax PCR Gem 100 (Perkin-Elmer Applied Biosystems, Boston, MA). Primers for *HBZ* were 5′-GCTGAGGAGAAGAGGAAGCG-3′ (sense) and 5′-TTATTGCAACCACATCGCCT-3′ (antisense). PCR was performed in a T-Gradient thermocycler (Biometra, Gottingen, Germany) under the following conditions: 2 min at 94°C and 28 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C.

**Cycloheximide pulse-chase.** 293FT cells were transiently transfected with pcDNA3.1(−)sHBZ-MycHis or pcDNA3.1(−)usHBZ-MycHis. Forty-eight hours later, cells were treated with 100 μg/ml cycloheximide (Nacalai Tesque, Kyoto, Japan) and then harvested at different time points (0, 12, 24, 48, and 72 h). Extracted proteins were separated by SDS-PAGE and blotted with the appropriate antibodies as previously mentioned in Western blot analysis section. Densitometric data was calculated by a ATTO CS Analyzer 2.1 software (Atto).

**Nucleotide sequence accession number.** The 3′ LTR and partial coding sequence of *HBZ* gene was submitted to the DDBJ (DNA Data Bank of Japan) database. The assigned accession number is AB373952.

## RESULTS

**Identification of the promoter regions of the *HBZ* gene.** As reported previously (28), the start sites of the s*HBZ* transcript are scattered in the U5 and R regions (at nucleotides 173, 176, 179, 186, 188, 197, and 201) (Fig. 1). There is no TATA box sequence surrounding the transcriptional start sites, indicating that this promoter is TATA-less. Instead of a TATA box, putative initiator and downstream promoter element consensus sequences were identified downstream of the transcriptional start sites. This 300-bp DNA region (comprised of 175 bp from the U5 region and 125 bp from the R region) was used for further analysis.

In addition to the s*HBZ* transcript, a us*HBZ* transcript was reported previously (4, 24). To identify the us*HBZ* promoter region, we performed 5′-RACE using us*HBZ*-specific primers. We also identified scattered transcriptional initiation sites at the pX region, as shown by asterisks in Fig. 1A. Like the promoter of the s*HBZ* gene, this putative promoter was also TATA-less and contained putative initiator and downstream promoter element consensus sequences (Fig. 1A). These scattered distributions of transcriptional initiation sites have been confirmed in the reporter plasmid DNA after transfection into a cell line (data not shown).

Both promoter regions were cloned into reporter plasmids as shown in Fig. 1A (bp 1 to 300 for the s*HBZ* promoter; bp 1 to 1461 for up1, bp 247 to 1461 for up2, and bp 725 to 1461 for up3 for the us*HBZ* promoter; and bp 1 to 757 for the whole 3′ LTR). Their promoter activities were measured by luciferase assay. We found that the s*HBZ* promoter had stronger activity than did the us*HBZ* promoter (Fig. 1B). This difference in promoter activities likely accounts for the observation that the transcript of the s*HBZ* gene was about fourfold higher than that of the us*HBZ* gene (36). Therefore, we focused first on the s*HBZ* promoter region.

**The Sp1 binding sites are critical for s*HBZ* promoter activity.** We generated deletion mutants of this promoter region as shown in Fig. 1C. Deletion of the first 60 bp (pGL4-3′LTR240) decreased the promoter activity up to 60%, suggesting that the presence of positive regulatory elements. The deletion of the first 120 bp (pGL4-3′LTR180) resulted in an 83% decrease in promoter activity, and the deletion of the first 180 bp (pGL4-3′LTR120) or 240 bp (pGL4-3′LTR60) produced plasmids with no more promoter activity than the empty vector (Fig. 1C). This observation suggests that positive regulatory elements are confined to the first 180 bp, which corresponded to the U5 region. Next, we searched for possible transcription factor binding sites in the first 180 bp using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). GATA-2, TaxCREB, AP-1, two NF1, and two Sp1 binding sites were found in this region. We made mutations in these binding sites and examined the promoter activity of each mutant (Fig. 2). Mutations of GATA-2, TaxCREB, AP-1, two NF1, and two Sp1 binding sites within the 5′-120 bp impaired the promoter activity only slightly (from −16% to −20%). Previous studies reported that some GC-rich TATA-less promoters contain several transcription initiation sites spread over a fairly large region and have several potential binding sites for Sp1 (7). Therefore, we mutated three Sp1 sites (within the 5′-180 bp) in pGL4-3′LTR300. Although mutating the first two Sp1 sites [Sp1(1.2)mut] did not significantly impair promoter activity, the mutating of all three Sp1 sites [Sp1(1.2.3)mut] decreased promoter activity strikingly (Fig. 2). In addition, mutation of the third Sp1 site alone also suppressed promoter activity [Sp1(3)mut], indicating that the third Sp1 site was the most important Sp1 binding site.

We next tested the binding of Sp1 to this site by a competitive biotinylated oligonucleotide binding assay, which was a

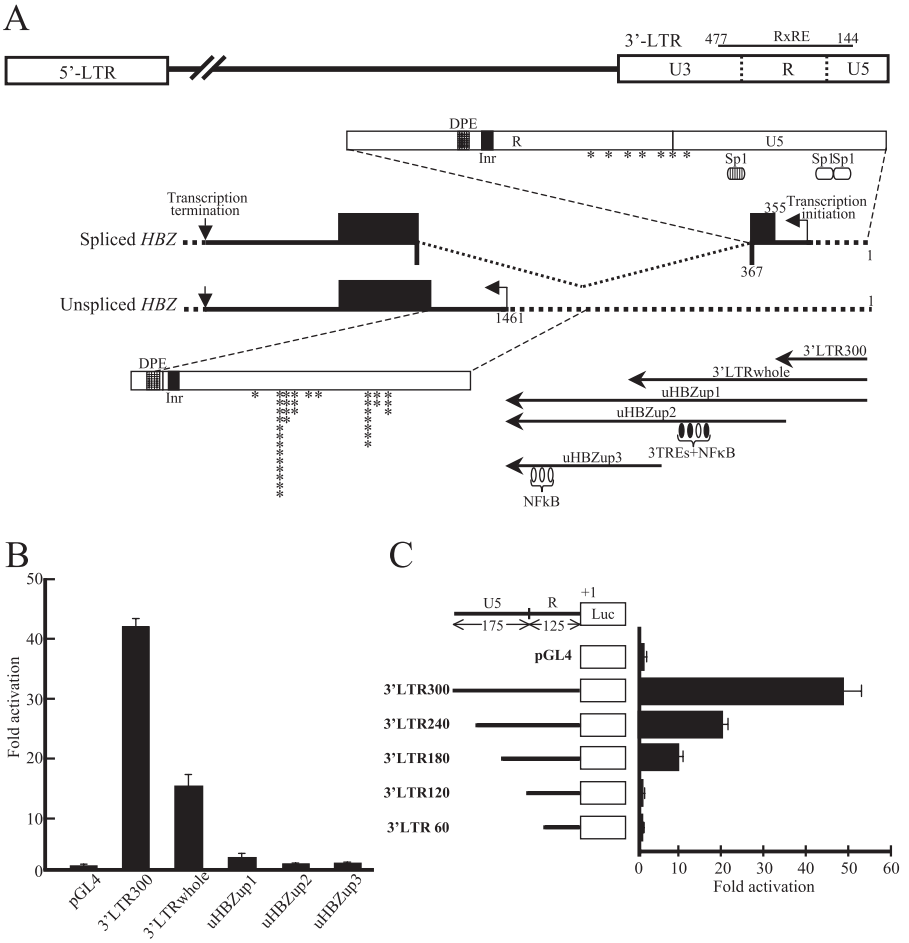


FIG. 1. Characterization of the *HBZ* promoter. (A) Identification of transcriptional start sites of us*HBZ* by 5′-RACE. Schematic diagrams show the HTLV-1 provirus, the s*HBZ* and us*HBZ* transcripts, and the expected promoter regions that we subcloned for analysis. The asterisks show identified transcriptional initiation sites of s*HBZ* and us*HBZ* transcripts. The expected promoter region of s*HBZ* (3′LTR300) consists of a 300-bp segment containing 175 bp from the U5 region and 125 bp from the R region, while the promoter for us*HBZ* is believed to lie somewhere upstream of the us*HBZ* coding sequence (uHBZup1, uHBZup2, and uHBZup3). 3′LTRwhole contains the whole LTR sequence. (B) Comparison of s*HBZ* and us*HBZ* promoter activities. Reporter constructs containing the promoter segments shown in A were transiently transfected into Jurkat cells ($2 \times 10^5$ cells) and assayed for luciferase activity. The luciferase activity relative to that in the cell lysate transfected by empty vector (pGL4) is shown. Values represent means ± SD ($n = 3$). (C) Luciferase activities of deletion mutants of the s*HBZ* promoter. A reporter plasmid containing a DNA segment upstream of the transcription initiation sites (full length [300 bp] or a deletion construct [1 μg each]) was transfected into Jurkat cells ($2 \times 10^5$ cells). After 2 days, luciferase activity was measured. The luciferase activity relative to that of cells transfected with empty vector (pGL4.22) is shown. Values represent means ± SD ($n = 3$).

colorimetric assay similar to an electrophoretic mobility shift assay. As shown in Fig. 3A and B, nuclear extracts from Jurkat cells specifically bound the tested Sp1 sequence, and this binding was inhibited by excess cold (nonbiotinylated) oligonucleotide but not by excess mutant oligonucleotide. In addition, we detected the binding of Sp1 to the promoter region (positions 81 to 240, which includes the third Sp1 site) by ChIP assay in an ATL cell line, ATL-43T (Fig. 3C).

**Influence of Tax or HBZ on the 5′ LTR and the *HBZ* promoter.** The HBZ protein has been reported to suppress Tax-mediated transactivation through the 5′ LTR. Thus, Tax and HBZ may form a complex network in which they regulate their own and one another's transcription. We analyzed the effects of HBZ and Tax on the promoter of the s*HBZ* gene. We performed a luciferase assay in the presence of Tax or HBZ using a reporter plasmid containing the s*HBZ* promoter (pGL4-3′LTR300). We found that Tax enhanced promoter activity by 28.8-fold for a reporter plasmid containing known Tax-inducible elements (WT-Luc) (see Materials and Methods) in a Jurkat cell line (data not shown). On the other hand, Tax and HBZ had only modest effects on the s*HBZ* promoter (Fig. 4A and B). On the other hand, Tax remarkably enhanced transcription from the us*HBZ* promoters (pGL4up1 and pGL4up2) (Fig. 5A). pGL4up3 showed no such transcriptional activation by Tax, indicating that the region spanning positions 247 to 724 is responsible for this Tax-mediated transcriptional activation.

The elements, which impart Tax responsiveness to the 5′ LTR, consist of three imperfect 21-bp repeats (TREs) and an NF-κB site between the promoter-central and promoter-proximal 21-bp repeats (5). Each TRE contains a near-consensus CRE, which might act as a enhancer for the transcription of

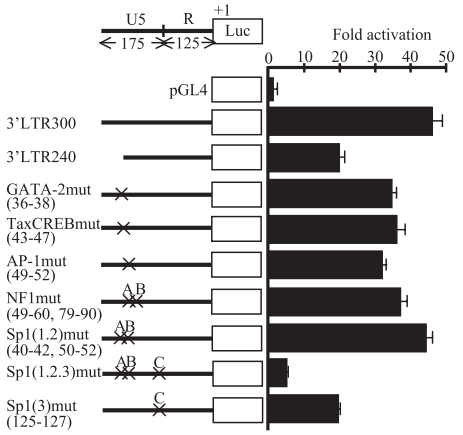


FIG. 2. Mutation analysis of the s*HBZ* promoter. A reporter construct (1 μg) containing the full-length s*HBZ* promoter (pGL4-3′LTR300), a 60-bp deletion mutant (pGL4-3′LTR240), or one of seven other mutated constructs was transfected into Jurkat cells ($2 \times 10^5$ cells), and luciferase activity (relative to that of an empty vector control) was measured after 2 days. Each × represents the position of a site-directed mutation in a transcriptional factor recognition site. Each mutation resulted in a loss of consensus sequence. Values represent means ± SD ($n = 3$).

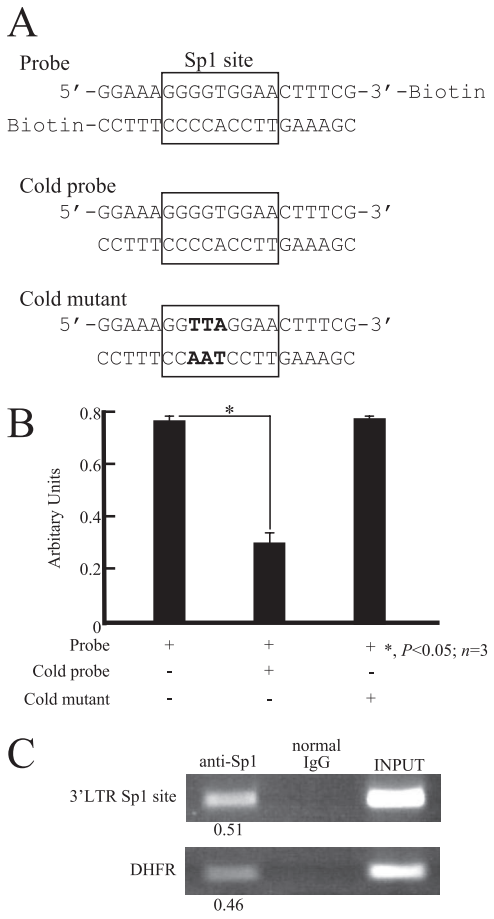


FIG. 3. Binding of Sp1 to the 3′ LTR. (A) Oligonucleotides used to study the binding activities of Sp1. Double-stranded biotinylated or unbiotinylated oligonucleotides corresponding to the Sp1 site in the s*HBZ* promoter were synthesized. The biotinylated wild-type probe (Probe), the competing nonbiotinylated oligonucleotide (Cold probe), and an oligonucleotide modified to contain a mutation in the Sp1 recognition sequence (Cold mutant) (mutated bases are in boldface type) are shown. (B) NoShift assay to detect Sp1 binding activity. The activity of Sp1 binding to the recognition site was abolished by excess cold (nonbiotinylated) oligonucleotide but not by excess mutant oligonucleotide (*, $P = 0.026$). (C) ChIP assay with anti-Sp1 antibody. Formaldehyde-cross-linked chromatin was isolated from ATL-43T cells. Chromatin was immunoprecipitated with anti-Sp1 or normal rabbit immunoglobulin G (IgG) (as a negative control). Immunoprecipitated DNA was purified and analyzed by PCR using primers specific for the 3′ LTR Sp1 site or the *DHFR* gene (positive control for anti-Sp1 ChIP). Relative values (each input band density equals 1) are shown below anti-Sp1 bands.

the us*HBZ* gene. To investigate this possibility, we used mutant Tax proteins. Tax mutant M22 is defective in NF-κB activation and has only half the CREB/ATF activity of wild-type Tax. In contrast, Tax mutant M47 has NF-κB activity similar to that of wild-type Tax, while its CREB/ATF-activating activity is almost completely lost (31). The transcriptional activation by Tax mutants M22 and M47 was severely impaired in pGL4uHBZup1 or pGL4uHBZup2 (Fig. 5B and C). Similarly, the promoter of the s*HBZ* gene might be influenced by Tax expression in the presence of this enhancer element. We inserted this enhancer element into pGL4-3′LTR300 and found that Tax expression enhanced the transcription from the promoter of the s*HBZ* gene (Fig. 5D). Furthermore, this enhanced transcription was impaired by Tax mutants M22 and M47 as observed in the promoter of the us*HBZ* gene (Fig. 5E). Next, we studied the effect of Tax expression on up2 promoters containing mutated TREs and/or a mutated NF-κB site. We found that mutated TREs abolished transcriptional activation by Tax, indicating that TREs were essential for the transactivation of up2 by Tax (Fig. 5F). There was no statistical difference between mutated TREs and mutated TREs plus the mutated NF-κB site.

**Functional differences between sHBZ and usHBZ.** The proteins derived from the s*HBZ* and us*HBZ* transcripts differ in their N-terminal regions (4 amino acids for sHBZ and 7 amino acids for usHBZ), as shown in Fig. 6A. We studied the functional differences between these HBZ proteins. Tax-mediated transcriptional activation through the 5′ LTR is suppressed much more strongly by s*HBZ* than by us*HBZ* (Fig. 6B). When both sHBZ and usHBZ were present, no synergistic or antagonistic effect was observed. To clarify the reason for this functional difference, we studied the expression levels of these proteins using tagged *HBZ* genes as shown in Fig. 6C. The amount of protein produced by us*HBZ* is much lower than that produced by s*HBZ*, as reported previously (4), indicating that the low level of the usHBZ protein accounts for the weak suppressive effect on transcription by us*HBZ*. To analyze mechanisms to confer higher protein levels of sHBZ, we analyzed the half-lives of the sHBZ and usHBZ proteins using a cycloheximide pulse-chase assay. As shown in Fig. 6D, the half-life of the sHBZ protein was longer than that of usHBZ.

In a previous report (28), we showed that the 5′ sequence of the RNA is critical for the growth-promoting activity of the *HBZ* gene. In this report, we investigate whether s*HBZ* and us*HBZ* RNAs have different effects on cell prolifera-

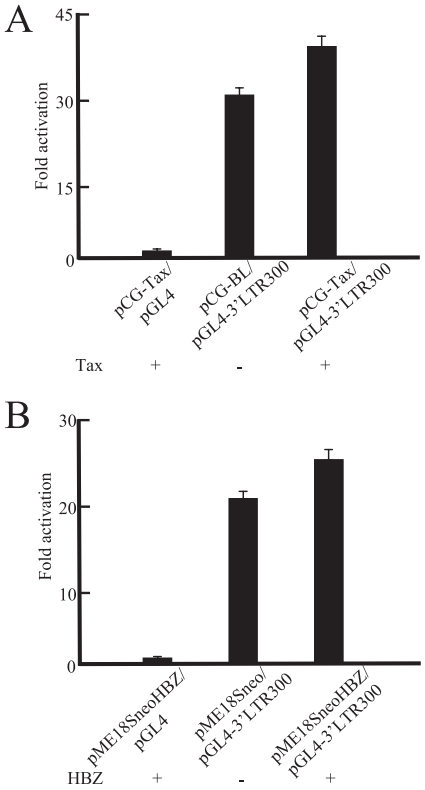


FIG. 4. Effects of Tax or HBZ on the s*HBZ* promoter. (A) Effect of Tax on the s*HBZ* promoter. Jurkat cells ($2 \times 10^5$ cells) were cotransfected with 1 μg each of pCG-Tax and pGL4-3′LTR300 and assayed for luciferase activity. The total amount of DNA to be introduced was equalized with empty vector (pCG-BL or pGL4.22). Activation relative to that of cells transfected with pCG-Tax and pGL4.22 is shown. (B) Effect of HBZ on the s*HBZ* promoter. Jurkat cells ($2 \times 10^5$ cells) were cotransfected with 1 μg each of pME18SneoHBZ and pGL4-3′LTR300 and assayed for luciferase activity. The total amount of DNA to be introduced was equalized with empty vector (pME18Sneo or pGL4.22). Activation relative to that of cells transfected with pME18sneoHBZ and pGL4.22 is shown. Values represent means ± SD ($n = 3$).

tion. As we reported previously, we found that the s*HBZ* gene and TTG-*HBZ* mutant had growth-promoting activity in a T-cell line, indicating that HBZ RNA is responsible for this growth-promoting activity (Fig. 6E). We confirmed that the TTG-HBZ mutant did not produce the protein (Fig. 6E). In the same experiment, the us*HBZ* gene lacked growth-promoting activity. The levels of mRNA were similar among these transfectants (Fig. 6E). Taken together, these observations suggest that the functions of both *HBZ* transcripts and HBZ proteins differ depending on the presence or absence of splicing.

## DISCUSSION

Bidirectional transcription from the LTR in the endogenous retrovirus has been reported. It was previously reported that the endogenous retrovirus 1 LTR transcribed two human genes, the human Down syndrome critical region gene 4 (*DSCR4*) and *DSCR8*, in the opposite direction, namely, sense and antisense (6). In that report, Sp1 was found to be a critical transcription factor for both genes. The transcription of both plus-strand and minus-strand viral genes has been reported for HTLV-1. Transcription of plus-strand DNA is highly inducible by Tax and is suppressed by HBZ and p30 (10, 37). In the minus strand, the transcription of s*HBZ* is dependent on Sp1. The promoter activity of the s*HBZ* gene was much higher than that of the us*HBZ* gene, although the transcription levels of both s*HBZ* and us*HBZ* are enhanced by Tax through the TREs of the 3′ LTR. These findings demonstrate that TREs function as enhancers for Tax-mediated transcriptional activation in both strands of the provirus. Thus, there are both similarities and differences between the transcriptional control of plus-strand and minus-strand viral genes in HTLV-1.

Promoters for both the s*HBZ* and us*HBZ* genes are TATA-less. In general, gene transcription is considered to begin at the +1 nucleotide in an initiator, while previous reports showed that RNA polymerase II is redirected to alternative start sites when a gene does not contain TATA (20, 27, 30). This fact might account for the scattered transcription start sites of s*HBZ*. Additionally, it was previously reported that Sp1 is critical for many TATA-less promoters (3, 19). In this study, the s*HBZ* gene promoter certainly fit this pattern. Since Sp1 is a well-known regulator of housekeeping genes, the transcription of the s*HBZ* gene may be relatively constant without Tax. Previous studies showed that the HTLV-1 5′ LTR enhancer contains multiple functional elements, including binding sites for at least CREB- and NF-κB-like factors, which synergistically cooperate in the activation of transcription in response to Tax (5, 26). Analysis of TRE and NF-κB mutations showed that TREs function as enhancers for the transcription of the s*HBZ* and us*HBZ* genes.

Since ATL cells frequently lose the expression of Tax due to genetic or epigenetic changes in the HTLV-1 provirus (34), the influence of Tax on the transcription of *HBZ* is likely limited in ATL cells. The finding of consistent transcription of the s*HBZ* gene in all ATL cells supports the idea that Sp1 plays a critical role in its transcription (28). Such constitutive transcription of the *HBZ* gene may be implicated in viral replication and the proliferation of infected cells. Since Tax is a major target of cytotoxic T lymphocytes in vivo (15), Tax-expressing cells are rapidly eliminated. The persistent expression of *HBZ* likely inhibits the excess expression of Tax in addition to growth-promoting activity.

Several mechanisms to suppress Tax expression by viral genes have been reported. Rex binds to a Rex-responsive element (RxRE) in the 3′ LTR of viral transcripts and enhances the transport of the unspliced *gag*/*pol* and the singly spliced *env* transcripts. By this mechanism, double-spliced *tax*/*rex* mRNA levels decrease, resulting in the suppressed expression of Tax (14). On the other hand, p30 binds to *tax*/*rex* transcripts and retains them in the nucleus, thus suppressing Tax protein production (25). In addition, p30 also repressed CRE-driven gene transcription in both the presence and absence of Tax (37, 38). HBZ directly interacts with c-Jun and suppresses viral gene transcription (10). Thus, viral gene products suppress Tax expression by several different mechanisms. Rex and p30 are encoded by the plus strand of the provirus and are transcribed from the 5′ LTR. Therefore, Rex expression and p30 expression are driven by Tax. On the other hand, s*HBZ* transcription is dependent on the 3′ LTR, in which Sp1 is a critical tran-

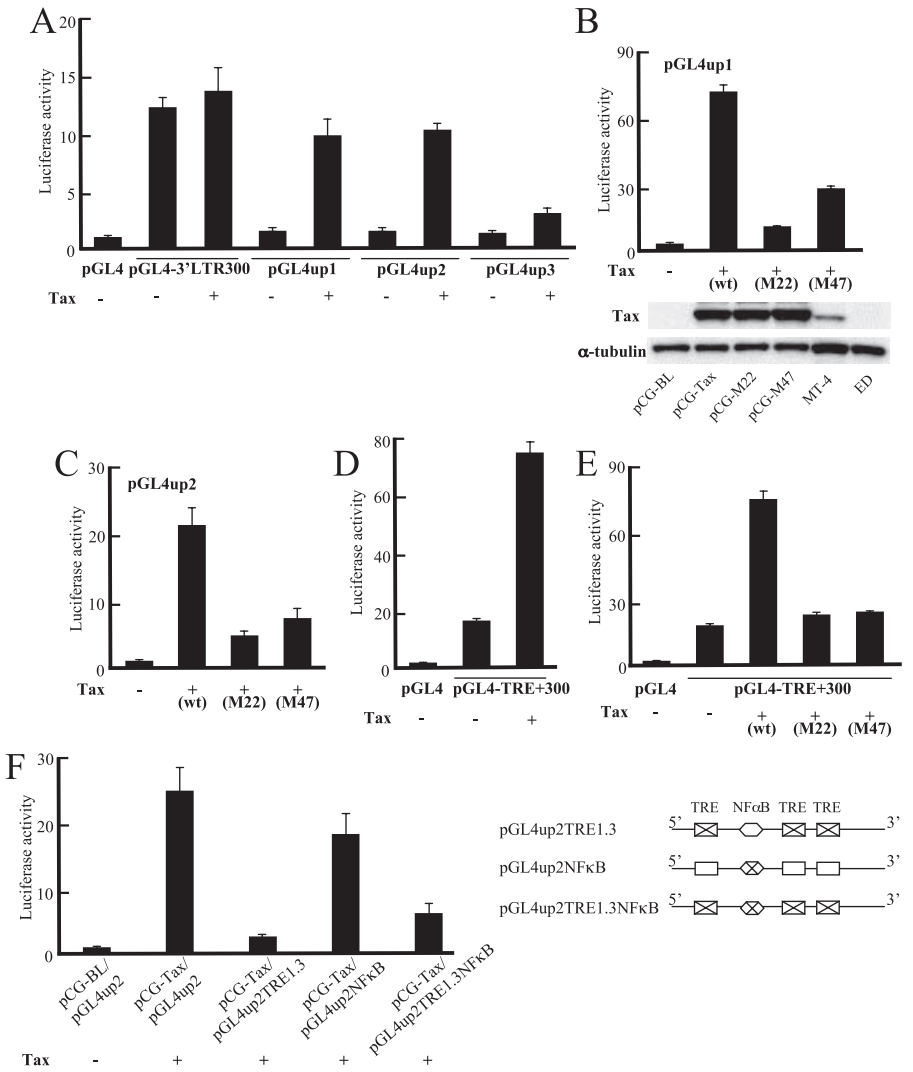


FIG. 5. Tax-mediated transactivation of the us*HBZ* and s*HBZ* gene promoters. Jurkat cells were cotransfected with 1 μg of wild-type (wt) or mutated Tax expression vector and 1 μg of s*HBZ* or us*HBZ* promoter luciferase reporter constructs. Luciferase activities were measured. Activation relative to that of cells transfected with pCG-BL and each fundamental vector (pGL4.22 [A, D, and E], pGL4up1 [B], and pGL4up2 [C and F]) is shown. Values represent means ± SD ($n = 3$). (A) Tax-mediated transactivation of the us*HBZ* promoter deletion mutants (up1 and up2). (B and C) Effects of Tax mutants (M22 and M47) on the promoter of us*HBZ* (B, up1; C, up2). (B, bottom) Western blot analysis of Tax in 293FT cells transfected pGL4up1 and each *tax* (wild type, M22, or M47) expression vector. MT-4 was used as the positive control. (D) Tax-mediated transactivation of the construct that contains both the s*HBZ* promoter and the TRE sequence. (E) Effects of Tax mutants (M22 and M47) on the TREs and s*HBZ* promoter. (F) Effects of mutations in the us*HBZ* promoter (up2). Constructs similar to up2, but with mutated TREs and/or a mutated NF-κB site, were made and tested as shown.

scription factor, although the transcription of the s*HBZ* and us*HBZ* genes is enhanced by Tax. These complementary mechanisms, which are Tax dependent and independent, are likely useful in maintaining Tax expression at the appropriate level in vivo.

As shown in this study (Fig. 6B), the s*HBZ* gene shows a more potent suppressive effect on the Tax-mediated activation of transcription than does the us*HBZ* gene. The different levels of protein produced by the s*HBZ* and us*HBZ* genes likely account for their different levels of transcriptional suppression. It was reported previously that HBZ binds to c-Jun and promotes the ubiquitination and degradation of c-Jun by proteasomes (21). Since the us*HBZ* gene was used in that report, it was possible that the protein derived from usHBZ was more susceptible to proteasomal degradation. However, we found that the HBZ protein derived from the unspliced transcript is not unduly influenced by proteasomal degradation. Different N-terminal modifications of proteins after translation affect their half-lives (23). The half-life of sHBZ is much lager than that of usHBZ. Thus, differences in the N-terminal amino acid sequences of sHBZ and usHBZ may be responsible for the different protein levels observed. In addition, the level of the s*HBZ* gene transcript is about fourfold higher than that for the us*HBZ* gene (36), which is possibly caused by the stronger promoter activity of the s*HBZ* gene, as shown in this study. Taken together, sHBZ plays a more important role in viral gene transcription and the proliferation of infected cells.

As we reported previously, *HBZ* RNA has growth-promot-

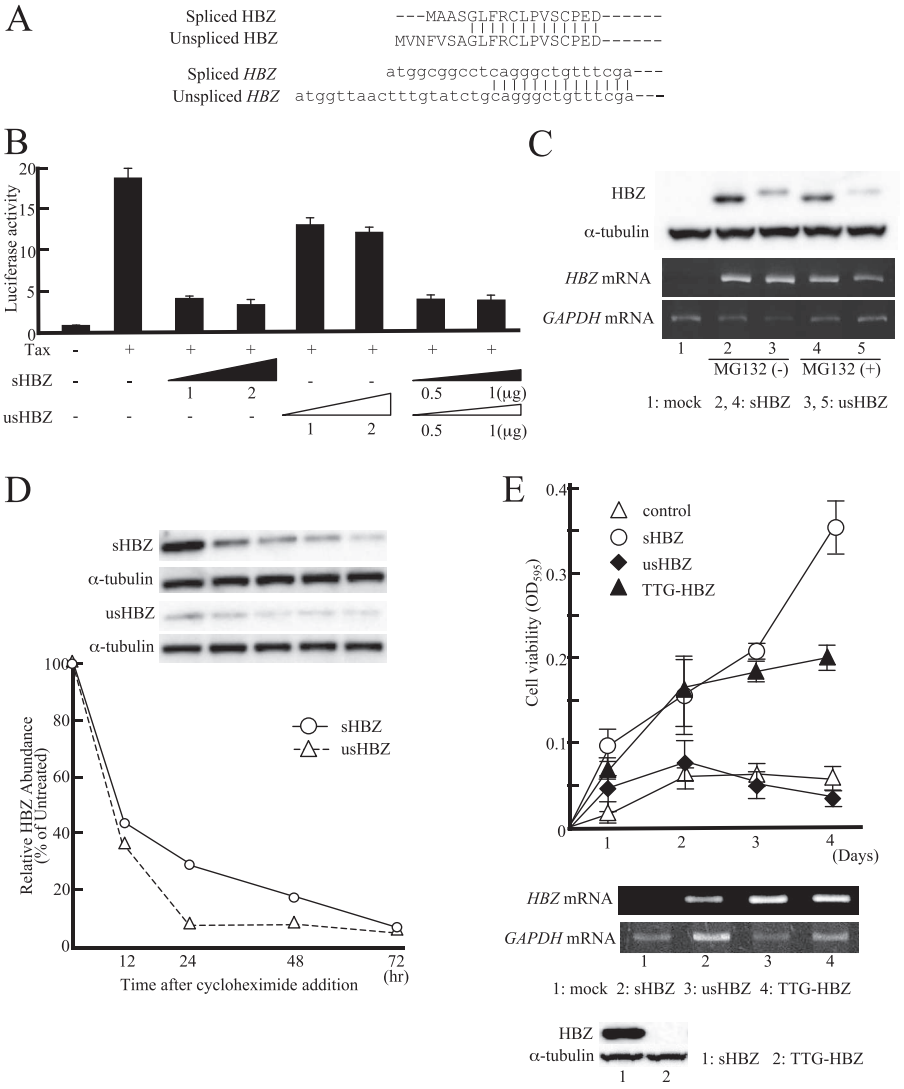


FIG. 6. Functional differences between sHBZ and usHBZ. (A) Nucleotide and peptide sequences at the N terminus of the two HBZ isoforms are shown. (B) Different functions of the sHBZ and usHBZ proteins in Tax-mediated viral gene transactivation. Jurkat cells were cotransfected with 1 μg of pCG-Tax and either pME18Sneo-splicedHBZ (lane 3, 1 μg; lane 4, 2 μg), pME18Sneo-unsplicedHBZ (lane 5, 1 μg; lane 6, 2 μg), or both (lane 7, 0.5 μg each; lane 8, 1 μg each) and assayed for luciferase activity. The total amount of DNA to be introduced was equalized with empty vector (pCG-BL and pME18Sneo). After 2 days, cells were lysed, and relative luciferase activities were determined. Activation relative to that of cells transfected with pCG-BL, pME18Sneo, and pGL4.22 is shown. Values represent means ± SD ($n = 3$). (C) Expression levels of protein and mRNAs of the two *HBZ* isoforms. Cell extracts were prepared from 293FT cells transfected with pcDNA3.1(−), pcDNA3.1(−)sHBZ-MycHis, or pcDNA3.1(−)usHBZ-MycHis. HBZ protein isoforms were detected by Western blotting using anti-His antibodies. At the same time, the transcription of the two *HBZ* isoforms was studied by semiquantitative RT-PCR. To evaluate whether differences in proteasome-dependent degradation contributed to the differences in HBZ expression levels, some samples were treated with MG132 before extraction of protein or mRNA. Lane 1, mock; lanes 2 and 4, sHBZ transfectants; lanes 3 and 5, usHBZ transfectants. (D) Stability of sHBZ and usHBZ proteins by cycloheximide pulse-chase assay. Forty-eight hours after transfection, 293FT cells were treated with cycloheximide (100 μg/ml) for the indicated times, followed by an immunoblotting assay with an anti-His antibody or an anti-tubulin antibody. Densitometric quantification of sHBZ (full line) and usHBZ (dashed line) is shown, with the quantification of each HBZ isoform at 0 h after cycloheximide treatment defined as 100%, upon which the change in expression levels was calculated. (E) Functional differences of s*HBZ*, us*HBZ*, and TTG-*HBZ*. The effects of the two *HBZ* isoform genes on Kit225 cell growth were measured as described in Materials and Methods. Values represent means ± standard deviations (error bars) ($n = 3$). Three independent experiments, each with triplicate transfections, were performed, and typical results are shown. (Bottom) Transcription of *HBZ* was analyzed by semiquantitative RT-PCR. Lane 1, mock; lanes 2, sHBZ transfectant; lane 3, usHBZ transfectant; lane 4, TTG-HBZ transfectant. (Bottom) The TTG-HBZ protein was analyzed by the Western blot method. Lane 1, sHBZ transfectant; lane 2, TTG-HBZ transfectant.

ing activity in the human T-cell line Kit225 (28). In this study, we report that only s*HBZ* has growth-promoting activity, confirming that the first exon of the *HBZ* RNA is critical for this growth-promoting activity. These results emphasize the significance of the 5′ portion of the s*HBZ* gene in the proliferation of infected cells. The 5′ portion of s*HBZ* RNA corresponds to the minus strand of the RxRE. Rex binds to a stem-loop structure formed by the RxRE, resulting in the enhanced

nuclear export of viral RNA. The 5′ portion of the s*HBZ* RNA is also predicted to form a stem-loop structure, which is quite different from that of RxRE (data not shown). It is possible that cellular factors bind to this region and enhance the growth of HTLV-1-infected cells. The genes encoded by the minus strand of the provirus were reported previously for HTLV-1, STLV-1, and HTLV-3 (32). However, HTLV-2 and STLV-2 do not have such genes. It is noteworthy that both HTLV-1 and STLV-1 can induce lymphomas, while HTLV-2 and STLV-2 cannot. This correlation suggests a possible connection between genes encoded by the minus strand and oncogenesis.

This study highlights the significance of the spliced form of *HBZ* over us*HBZ*. sHBZ may be more important for one or more of the three following reasons: (i) the sHBZ protein is present in greater amounts, (ii) s*HBZ* RNA contains the critical 5′ portion of the RNA that may form a stem-loop structure and interact with cellular proteins, and (iii) s*HBZ* is expressed ubiquitously, independent of Tax expression. The continuous expression of s*HBZ* is consistent with findings that *HBZ* supports the proliferation of HTLV-1-infected cells and ATL cells and inhibits the excess expression of Tax.

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