Human T-cell leukemia virus type I U5 repressive element binding protein 1 (HTLV-I U5RE Binding protein 1; HUB1 and renamed ZNF282) — a member of the Krüppel type zinc finger family — represses HTLV-I long terminal repeat (LTR)-mediated transcription by binding to the TCCACCC motif of the U5RE. Sp1 and other Sp1 family proteins also recognize the CACCC box of the U5RE, as well as the GC box (GGGCGG). We would therefore expect HUB1 to compete with Sp1 for binding to the former. In the present study, we demonstrated that Sp1 activates transcription via the U5RE, and that HUB1 represses these Sp1-mediated effects. Electrophoretic mobility shift assays (EMSA) confirmed that HUB1 was bound to the CACCC box, but not the GC box. Consistent with that finding, overexpression of HUB1 repressed Sp1-mediated transcription reporter genes controlled via the CACCC box, but not via the GC box. These results suggest that, by binding to the CACCC box, HUB1 represses the Sp1-dependent transcriptional activation.

**Key words** —– transcriptional repression, gene expression, repressor, HTLV-I, Sp1

**INTRODUCTION**

The zinc finger transcription factor Sp1 regulates a large number of cellular and viral gene promoters through binding to the GC or CACCC box. Three other Sp1-related genes, Sp2, Sp3 and Sp4, have also been identified. Sp1 and Sp3 are ubiquitously expressed in most tissues and mammalian cell lines, whereas expression of Sp4 is restricted to cell types found in brain. While Sp3 often exerts an inhibitory effect, repressing gene expression mediated by Sp1, it can also activate transcription driven by some promoters, including the sis/PDGF-B and p21Cip/WAF1 promoters.

HTLV-I is the etiologic agent for adult T-cell leukemia (ATL) and HTLV-I-associated myelopathy. HTLV-I is transmitted sexually or by blood, blood products, and breast milk and induces a lifelong chronic infection. ATL occurs in 1–2% of infected carriers generally 20–30 years after infection. This long latency period of ATL suggests that the accumulation of genetic mutations and other events, in addition to HTLV-I infection, may be required for the induction of ATL. In vivo, HTLV-I is low level in infected T cells upon viral gene expression in HTLV-I carriers. We have identified a 27-bp transcriptional repressive element in the U5 region of 5'-long terminal repeat (LTR) (U5RE) to which several nuclear proteins bind — a process involving Sp1 and Sp3. We also found that HUB1, a Krüppel type zinc finger protein, binds to U5RE by recognizing the TCCACCC sequence. HUB1 is ubiquitously expressed and can repress the transcription of HTLV-I LTR. Like most other Krüppel type zinc finger proteins, it contains a KRAB domain; however in this case, KRAB acts as a transcriptional activating domain, unlike others whose function is repressive. Indeed, we identified a novel HUB1 repression domain (HUR) comprised of amino acids 96-184 of HUB1, which suggests the CACCC box of the U5RE probably plays an important role in virus gene repression. HUB1 was reported to be an autoantigen in 70% of ATL patients, in 41.7% of HTLV-I carriers and in 37.5% of healthy donors.
suggesting development of ATL might be associated to production of antibodies against HUB1.

Although the physiological function of HUB1 has not yet been clarified, the fact that its binding motif contains an Sp1 binding sequence led us to hypothesize that HUB1 is involved in regulation of Sp1 function. In the present study, cotransfection of Sp1 and HUB1 enabled us to demonstrate that HUB1 inhibits Sp1-mediated activation of transcription by binding to the U5RE CACCC box, but not to the GC box. We therefore propose that a key function of HUB1 is as a sequence-specific repressor of Sp1.

**MATERIALS AND METHODS**

**Construction of Plasmids** —— thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT) contains a CAT gene under the control of a minimal promoter of herpes simplex virus TK. While 2 U5REs or four GC boxes were situated upstream of the TK promoter, yielding 2×U5RE-TK-CAT and TKCAT9, respectively. The bars depict the means ± S.D. (n = 3) of the CAT activities in HeLa cells transfected with 0.5 µg of 2×U5RE-TK-CAT, TKCAT9 or TK-CAT relative to the basal activity observed with the TK-CAT.

**Cell Culture and Transfection** —— Human HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Before transfection, the cells were seeded to a density of 2×10⁵ cells/well in a 6-well plate. They were then transfected by incubation for 16 hr with the appropriate plasmids and lipofectin reagent (Invitrogen) in OPTI-MEM medium, after which they were cultured for 48 hr and harvested for use in CAT assays.

**Electrophoretic Mobility Shift Assay (EMSA)** —— Bacterially synthesized His-tagged HUB1, which was constructed in pRSET vector (Invitrogen), was purified by using Ni-coated beads column. Binding reactions were carried out using 50 ng of His-tagged HUB1 protein in binding buffer [20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 µg poly (dl-dC) and 5% glycerol]; after addition of 50 fmol of ³²P-labeled (2×10⁷ cpm/fmol) U5RE, U5RE M17 or GC box, which served as probes, the reactants were incubated for 30 min at 25°C. Thereafter, the reaction mixtures were loaded onto 5% polyacrylamide gels in Tris-acetate EDTA.
The upper strand sequences of the probes were as follows: GC box type Sp1 consensus probe, 5′-ATTCGATCGGGGCGGGGCGAGC-3′; wild type U5RE probe, 5′-AAGTTCCACCCCTTTCCCTTTCATTC-3′; and U5RE M17 mutant probe, 5′-AAGTTCCACCaCTTTCCCTTTCATTC-3′. Probes were 32P end-labeled using a Klenow fragment or T4 kinase.

RESULTS

Sp1 Activates Transcription through U5RE

We previously showed that the sequence recognized by both Sp1 and HUB1 includes a CACCC motif within the U5RE, and that whereas Sp1 mainly functions as a transcriptional activator of many genes, HUB1 is a repressor. Basal activity of 2×U5RE-TK-CAT and TK-CAT9, which respectively contain two U5RE and four GC box motifs (Fig. 1, left), were about 10-fold higher than TK-CAT in HeLa cells (Fig. 1, right).

Although we previously showed that Sp1, Sp3 and other proteins bind to the U5RE, it is not clear whether these factors are able to activate transcription via the U5RE. We verified the effects of Sp1 using Drosophila SL2 cells, which are known to be devoid of endogenous Sp1 activity. The luciferase activities of both 4×U5RE-TK-luc and p4×GC-luc were strongly stimulated in the presence of Sp1 (Figs. 2A and 2B). By contrast, Sp1 did not activate 4×M17-TK-luc, which contains four U5RE M17 mutant sequences (CACCC→CACCΔ), and TK-luc under the same conditions (Fig. 2B). Sp1 thus appears to activate gene transcription through both the U5RE and the GC box.

HUB1 Represses Sp1-Mediated Transcription of Reporter Genes Containing U5RE

We previously used competition assays to show that HUB1 specifically binds to the CACCC box, thereby repressing transcription, but does not bind to the GC box. HUB1 repressed basal 2×U5RE-TK-CAT activity, as described elsewhere. We therefore carried out electrophoretic mobility shift assays (EMSAs) using recombinant HUB1 and confirmed that HUB1 binds to the U5RE probe, but not to the U5RE M17 mutant and GC box (Fig. 3). Based on these observations, to further elucidate the function of HUB1 with respect to the CACCC box, we carried out the cotransfection assay both Sp1 and HUB1 in SL2 cells (Fig. 4). When Sp1 and HUB1 were cotransfected with 4×U5RE-TK-luc, activation of the promoter by Sp1 was dose-dependently repressed by HUB1. ∆HUB1 also decreased the activity of Sp1 via the U5RE in cotransfection assays, although the repression was less marked than the wild type protein. On the other hand, neither HUB1 nor ∆HUB1 inhibited activation by Sp1 via the GC box, confirming that HUB1 selectively represses Sp1-induced transcriptional activation mediated via the CACCC box. HUB1 may thus act as a sequence-specific inhibitor of Sp1.
DISCUSSION

Our findings indicate that HUB1 selectively represses Sp1-stimulated gene expression mediated via the CACCC box within the U5RE, which overlaps the Sp1 binding site. This conclusion is based on the observations that Sp1 clearly enhances transcription in SL2 cells, which lack endogenous Sp1 activity; that Sp1-dependent activation is repressed by HUB1 in SL2 cells cotransfected with Sp1 and HUB1 expression plasmids; and that ΔHUB1 — an N-terminal HUB1 deletion mutant — also diminished the Sp1-induced transcription via the U5RE.

Since the repressive effect of HUB1 was more efficient than the ΔHUB1, HUB1 might further repress transcription through the action of its HUR domain — an intrinsic transcriptional repression domain. We previously showed the presence of the HUR domain using a GAL4 DNA binding domain-HUB1 chimeric protein and a promoter containing GAL4 binding sites.11) In fact, several transcription factors possessing repressive domains also competitively inhibit Sp1-mediated transcription by binding to promoter sequences that overlap or are adjacent to the Sp1 site; these include Sp3,4) T3R,18) G10BP,19) Zif26820) and ZBP89 (a Krüppel type zinc finger).21) These factors are known to compete with Sp1 for binding to the GC rich element and to re-

![Fig. 3. HUB1 Protein Binds to CACCC Sequence](image1)

EMSAs were carried out as described in MATERIALS AND METHODS. 50 ng of purified HUB1 protein was used in each reaction. 3P-labeled (2 x 10³ cpm/fmol) U5RE (lane 1), U5RE M17 (lane 2), and Sp1 GC box (lane 3) served as probes.

![Fig. 4. Functional Analysis of the Effect of HUB1 on Sp1-Mediated Activation of Transcription via the CACCC and GC Boxes in SL2 Cells](image2)

One µg of 4 x U5RE-TK-luc (solid bars) and p4 x GC-luc (hatched bars) were cotransfected with the indicated amounts of pPacSp1 and either pPacHUB1 or pPacΔHUB1. Luciferase assays were carried out, and the resultant activities were normalized to the β-galactosidase activity of pRSVβgal. Total amounts of plasmids were adjusted to 1 µg by addition of pPacU. The bars depict the means ± SEM (n = 3) of the luciferase activity relative to the basal activity seen with pPacU.
press both basal and inducible expression of their respective target genes.\textsuperscript{5,18–21} Nonetheless, the present results show the effect of HUB1 to be critical to the loss of Sp1 function on the CACCC box, but not the GC box.

Although the precise physiological function of HUB1 is not yet known, we suggest that it regulates the expression of target genes by modulating Sp1 activity. As Sp1 is an essential promoter-specific factor, in vivo, its repression is critical for regulation of gene expression mediated via the CACCC box. Therefore, determination of the specific cellular target genes of HUB1 was important for understanding its function, which will likely have crucial implications for the regulation of Sp1-mediated gene expression.

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REFERENCES

17) Courey, A. J., Holtzman, D. A., Jackson, S. P. and...


