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Transcription Factor Sp1 Plays an Important Role in the Regulation of Copper Homeostasis in Mammalian Cells

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Nonstandard abbreviations used: hCtr1, human copper transporter 1; DMT1, divalent metal transporter 1; CuRE, copper-responsive element; MRE, metal responsive element; MTF-1, metal-responsive transcription factor; BCS, bathocuproine disulfonic acid; CUP, bis-cyclohexanone oxaldihydrazone; BPS, bathophenanthroline disululfonic acid; DEF, deferoxamine mesylate; ZF, zinc finger domain

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ABSTRACT

Copper is an essential metal nutrient, yet Cu overload is toxic. Here, we report that human copper transporter 1 (hCtr1) plays an important role in the maintenance of Cu homeostasis by demonstrating that expression of hCtr1 mRNA was up-regulated under Cu-depleted conditions and down-regulated under Cu-replete conditions. Overexpression of full-length hCtr1 by transfection with a recombinant *hCtr1* cDNA clone reduced endogenous hCtr1 mRNA levels, whereas overexpression of N-terminus-deleted hCtr1 did not change endogenous hCtr1 mRNA levels, suggesting that increased functional hCtr1 transporter, which leads to increased intracellular Cu contents down-regulates the endogenous hCtr1 mRNA. A luciferase assay using reporter constructs containing the *hCtr1* promoter sequences revealed that three Sp1-binding sites are involved in the basal and Cu concentration-dependent regulation of *hCtr1* expression. Modulation of Sp1 levels affected the expression of hCtr1. We further demonstrated that zinc finger domain of Sp1 functions as a sensor of Cu that regulates hCtr1 up-and-down in response to Cu concentration variations. Our results demonstrate that mammalian Cu homeostasis is maintained at the hCtr1 mRNA level which is regulated by the Sp1 transcription factor.

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INTRDUCTION

Copper is an essential trace element required for a wide array of enzymatic activities in many important physiological processes (Harris, 2003; Puig and Thiele, 2002; Sharp, 2003). However, copper is toxic when its concentration is too high, due to the consequence of generating excess amounts of reactive oxygen species that damage proteins, lipids, and nucleic acids. In mammals, Cu concentration stress resulting from copper deficiency or Cu excess is associated with many pathophysiologic disorders (Cai et al., 2005; Shim and Harris, 2003; Uriu-Adams and Keen, 2005).

Eukaryotic cells have developed an evolutionarily conserved regulatory system to modulate Cu concentration stress. This system consists of transporters (Ctr) that facilitate Cu acquisition when intracellular Cu content is low, efflux pumps (ATP7A and ATP7B) that eliminate Cu when Cu content is high, and Cu chaperones for distribution of Cu to various intracellular compartments. Copper entry into cells is mainly carried out by copper transporter 1 (Ctr1) (Kuo et al., 2007; Puig and Thiele, 2002; Sharp, 2003) and to a lesser extent, by divalent metal transporter (DMT1) (Garrick et al., 2003). While DMT1 transports a broad range of divalent metal ions, including Cu(II); Ctr1 is Cu(I)-specific except recent studies demonstrated that it also transports platinum-based antitumor agents (Ishida et al., 2002; Song et al., 2004).

Ctr1 belongs to a group of evolutionarily conserved membrane proteins consisting of three transmembrane domains, with N-terminus extracellularly located and C-terminus inside the cytoplasm. Evidence that Ctr1 has an essential role includes the observation that intestinal epithelial cell-targeted *Ctr1* ablation resulted in striking neonatal defects in Cu accumulation in peripheral tissues resembling Cu deficiency (Nose et al., 2006).

The Ctr family plays an important role in the regulation of Cu homeostasis. This has been well demonstrated in the yeast Ctr1 system. The expression of yeast *yCtr1* and *yCtr3* is upregulated in response to Cu deficiency but is downregulated in response to Cu overload. Upregulation of *yCtr1* and *yCtr3* under Cu-deprived conditions is mediated by transcription

factor Mac1p, which is rapidly phosphorylated, dimerized, and binds to the Cu-responsive element (CuRE) located in the promoter regions of these genes (Jamison McDaniels et al., 1999; Jensen et al., 1998; Jensen and Winge, 1998; Rutherford and Bird, 2004; Winge, 1998). As cells acquire sufficient Cu, Mac1p activity is specifically inhibited, and expression of these transporters is shut down (Jensen and Winge, 1998; Yamaguchi-Iwai et al., 1997). In addition to this transcriptional regulation, a posttranslational mechanism has been suggested to play a role in regulating yCtr1 levels in response to Cu concentration. It has been reported that membrane-located yCtr1 is degraded when cells are exposed to high concentrations of Cu and that the degradation mechanism is mostly independent of the endocytotic pathway (Ooi et al., 1996). Mac1p also participates in Cu-dependent yCtr1 degradation by interacting with the metal ion binding motif located at the C-terminus of yCtr1 (Yonkovich et al., 2002). Transcriptional regulation of the Drosophila *dCtr1B* gene in response to Cu stress conditions mediated by the metal-responsive transcription factor (MTF-1), which interacts with metal response elements (MRE) located at the promoter of the *dCtr1B* gene has been reported (Selvaraj et al., 2005).

Humans have two *Ctr* genes, *hCtr1* and *hCtr2*, but only hCtr1 exhibits high affinity to Cu(I). Using a cultured cell system overexpressing epitope-tagged recombinant hCtr1, it has been reported that Cu exposure caused a rapid (within 10 min) internalization of hCtr1 from the plasma membrane (Petris et al., 2003). The Cu-dependent internalization of hCtr1 was followed by hCtr1 protein degradation. However, in a study in which a baculoviral vector was used to overexpress hCtr1 in the insect cells, Eisses and Kaplan found no evidence of Cu-dependent internalization of hCtr1 in the same system (Eisses et al., 2005). No CuRE- or MRE-like sequences are present in the promoter of *hCtr1* and no Mac1p-like transcription factor is found in the human genome (Kuo et al., 2007). Therefore, the mechanisms that regulate hCtr1 expression is regulated under Cu stress conditions remain to be investigated.

In the present study we demonstrated that, like yeast *yCtr1* and *yCtr3* and Drosophila *dCtr1B*, expression of *hCtr1* is transcriptionally regulated in response to Cu concentration

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variations in mammalian cells. The regulation of Cu homeostasis is controlled at the homeostatic maintenance of hCtr1 mRNA that is regulated by transcription factor Sp1. To our knowledge, this is the first evidence showing that mammalian Ctr1 is also transcriptionally regulated by a Zn-finger transcription factor.

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MATERIALS AND METHODS

Plasmid DNA

For the expression of hCtr1, we used recombinant plasmids of CIN-HA-pcDNA3-hCtr1wt, CIN-HA-pcDNA3-hCtr1δN1, CIN-HA-pcDNA3-hCtr1δN2, and CIN-HA-pcDNA3-hCtr1δC were used as described previously (Song et al., 2004). For construction of pGL3-hCtr1 (-607), a polymerase chain reaction (PCR) was performed with genomic DNA from HEK293 cells as a template and primers 5'-TTTGCTAGCAGGCAAACCCAGGGCTATCTTCC-3' and 5'-GGGAAGCTTAGCCCCAGTCTTTAACCCTCCAGT-3'. The PCR product was digested with *NheI* and *HindIII*, and then inserted into pGL3-Basic (Promega, Madison, WI). A series of progressively deleted flanking sequences were created by PCR using pGL3-hCtr1(-564) as template and appropriate primer sets (sequences available upon request). The PCR products were cloned into pGL3 basic vector, generating pGL3-hCtr1(-417), pGL3-hCtr1(-227), pGL3-hCtr1(-184), pGL3-hCtr1(-144), pGL3-hCtr1(-84), pGL3-hCtr1(-43), and pGL3-hCtr1(+1) recombinant DNA.

To generate site-directed mutations in the Sp1 binding sites, we used the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, pGL-hCtr1(-227) DNA was used as the template in PCR reactions using various primer sets (available upon request). The methylated, wild-type DNA (template) was digested by *Dpn*I, and the nonmethylated, mutated DNA (PCR product) was transformed into XL1-Blue supercompetent cells (Stratagene) after undergoing heat pulse for 45 s at 42°C. The sequences of all deleted and mutated plasmid DNAs were confirmed. These constructs were used as reporter plasmids for promoter analyses and pRL-SV40 (Promega) was used as an internal control.

Wild-type human Sp1 cDNA and its ZF deletion mutants were synthesized by RT-PCR using appropriate primer sets containing a *NotI* recognition sequence and poly(A)+ RNA from HeLa cells (ATCC, Rockville, MD). The resulting PCR products covering nucleotides 98 to

2455 (wild-type, GenBank No. NM138473 as a reference), 98 to 2149 (M1, deletion of one ZF domain), 98 to 2059 (M2, deletion of two ZF domains), and 98 to 1723 (M3, deletion of three ZF domains) were digested by *NotI* and each was cloned into the *NotI* site of the CIN-HA-pcDNA3 vector which contains and HA tag, enhancer CIN sequences (Song et al., 2004), and a neomycin resistance marker for transfection selection. All plasmids were confirmed by sequencing.

Cell culture and treatments with metal ions and metal chelators

Small cell lung cancer cells (SCLC) (Song et al., 2004) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 ^{o}C in a 5% CO_2 atmosphere. Cells at exponential growth conditions were treated with different concentrations of $CuSO_4$, $FeCl_3$, or various metal chelators (100 μM each, all from Sigma, St. Louis, MO), including bathocuproine disulfonic acid (BCS), bis-cyclohexanone oxaldihydrazone (CUP), bathophenanthroline disulfonic acid (BPS), and deferoxamine mesylate (DEF). Cells were also treated with 100 μM $CuSO_4$ for different time intervals from 4 to 16 hrs.

RNase protection assay

Total RNA was extracted and levels of hCtr1 mRNA were determined by the RNase protection assay. The RNase protection assay was carried out as previously described (Song et al., 2004). In brief, the template DNA was linearized by appropriate restriction digestion and purified using a gel extraction kit (Qiagen, Valencia, CA). Antisense riboprobes were synthesized using T7 or Sp6 polymerase (Roche Applied Science, Indianapolis, IN) in the presence of 50 μCi of [α-^{32}P] UTP (MP Biomedicals, Irvine, CA). We used 20 μg of RNA for the hybridization with a ^{32}P-labeled riboprobe for 16 h at 45 $^{\circ}$C. Unhybridized RNA was degraded by incubation with RNase A and RNase T (Roche Applied Science). Protected RNA fragments were resolved by electrophoresis on denaturing 8% acrylamide/8M urea gel and

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visualized by autoradiography. To determine the endogenous and exogenous hCtr1 mRNA, we constructed a recombinant plasmid containing hCtr1 cDNA (GenBank No. U83460) from +91 to 450 bp using a TA cloning kit (Invitrogen, Carlsbad, CA). The riboprobe synthesized from this template annealed to the endogenous and transfected (exogenous) hCtr1 mRNA and gave rise to protected fragments of 360 and 300 nucleotides, respectively. In all cases, the hybridization conditions were in large excess of the radioactively labeled probe (2×10^5 cpm in approximately 1μg) in reference to endogenous and exogenous hCtr1 contents. The autoradiographic signals were quantified with an image analyzer (Image Quant 5.0).

Measurement of Cu uptake.

Intracellular accumulation of Cu was measured according to the procedure previously described (Song et al., 2004). Briefly, SCLC cells (10^6 cells) were plated in a 12-well plate. After 24 hr, the uptake of $^{64}CuCl_2$ (100 nM, MIR Radiological Sciences, St. Louis, MO) was initiated by incubating the cells at 37°C on 5 % CO_2 up to 30 min. After incubation, plates were placed on ice and rinsed three times with 3 ml ice-cold phosphate-buffered saline (PBS). Cell lysis buffer (0.1% Triton-X 100 and 1% SDS in PBS) in a volume of 300 μl was added to the wells, and the radioactivity of cell lysates was determined by scintillation counter. Aliquots of cell lysates (5 μl) were used to determine the protein concentration using Bio-Rad DC protein assay kit (Bio-Rad laboratories, Hercules, CA). Intracellular Cu uptake was normalized by the protein amount of the cells.

Luciferase assay

SCLC cells (2×10^5 cells) were seeded in 24-well plates, and 24 h later, 0.2 μg of recombinant pGL3 basic vector containing various lengths of the promoter region of hCtr1 sequence and 25 ng of pRL-SV40 renilla luciferase vector (Promega) were cotransfected into cells using 4 μg Lipofectamine. After 6 h of incubation, the medium was replaced with the

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regular medium. After 24 h of transfection, cells were treated with 100 μM Cu or 100 μM BCS for 16 h, washed twice with 1× PBS and lysed with 100 μl of passive lysis buffer (Promega). Firefly and renilla luciferase activity of the aliquot (10 μl) of cell lysate was measured by adding LAR II reagent and Stop and Glo reagent, respectively, using a luminometer (TD 20/20, Turner Designs, Sunnyvale, CA).

Western Blot analysis

SCLC cells were treated with Cu (100 μM) and BCS (100 μM) for 16 h, washed three times with PBS and lysed with 400 μl RIPA buffer and centrifuged at 12,000 rpm for 10 min at 4^{o}C. The supernatants were used for Western blot analysis. Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories). Aliquots (40 μg) of protein were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then subjected to primary and secondary antibodies with blocking using 5% nonfat milk. The signals were developed with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Rabbit polyclonal antibodies against Sp1 (Santa Cruz, 1:500 dilution) and hCtr1 (1:1000 dilution) (Klomp et al., 1997) and mouse monoclonal antibodies against AP2 (Upstate, Charlottesville, VA), β-actin (Pierce) and HA tag (Sigma) were used.

Electrophoretic Mobility Shift Assays

Double-stranded oligonucleotides were labeled with γ-32P-ATP using polynucleotide kinase and purified by 8% PAGE. The labeled probes (20,000 cpm) were incubated at ambient temperature with 3 μg of nuclear extract and 3 μg of poly dIdC in a binding buffer containing 10 mM Tris HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 5% Glycerol, 0.1 mg/ml BSA and 1 mM

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$MgCl_2$. For the super-shift assay, 0.5 μg of anti-HA antibody was added to the reaction mixture. The reaction mixture was separated by 4% PAGE. The shifted bands were visualized by autoradiography.

siRNA Transfection

SCLC cells (5-10 × 10^5 cells) were transfected with hCtr1-specific siRNA, Sp1-specific siRNA and a control (scrambled) sequence at a 100 nM concentration or without any siRNA by using Lipofectamine 2000 (Invitrogen). Transfected cells were maintained in regular culture medium for 2 days and transfection was repeated once as described earlier. Cells were harvested and expression of hCtr1 and Sp1 were determined by western blot analyses.

Statistical Analysis

Statistical significance was analyzed using an unpaired *t*-test, and $p < 0.05$ was considered to be statistically significant. The reproducibility of the results was confirmed by at least three separate experiments. Data are expressed as means±SD.

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RESULTS

Steady-State hCtr1 mRNA Levels Are Regulated by Copper Concentration

The steady-state levels of hCtr1 mRNA in SCLC treated with various concentrations of Cu(II) and Fe(III) were determined by the RNase protection assay using a probe that specifically hybridized with hCtr1 mRNA but not with hCtr2 mRNA (unpublished results). Extracellular copper exists in its oxidized form which is reduced to Cu(I) by membrane-bound FRE1 and FRE2 cupric reductase prior to transport (Georgatsou et al., 1997; Hassett and Kosman, 1995). Densitometric analyses of results from three independent experiments showed about 30% reduction of hCtr1 mRNA in 25 μM Cu(II)-treated cells and 60% reduction in cells treated with high concentrations (100 μM) of Cu(II). Whereas no significant reduction of hCtr1 mRNA was seen in cells treated with 100 μM Fe(III) (Fig. 1A). To determine whether reduction of hCtr1 expression in Cu-treated cells was associated with reduced Cu transport activity, we carried out ^{64}Cu uptake experiments. Fig. 1A also shows that rates of ^{64}Cu uptake in the Cu-treated cells were reduced accompanying with the reduced expression of hCtr1 mRNA. In time-course experiments, we found that significant reduction of hCtr1 expression levels and rates of ^{64}Cu uptake in the 100 μM Cu-treated cells was seen at 8-12 hrs after the treatment (Fig. 1B). These results showed that levels of hCtr1 mRNA expression were suppressed by Cu in cultured cells.

The intracellular Cu concentration in SCLC is 7.14 ± 0.08 μM as measured by inductively coupled plasma mass spectrometry (our unpublished data). We observed that hCtr1 mRNA levels started to reduce at 25 μM Cu (Fig. 1A). This concentration is consistent with those in many previously studies that are associated with Cu toxicosis disorder such as Wilson's disease (Gitlin, 2003).

To investigate whether depleting copper concentrations from the cultured medium would upregulate hCtr1 mRNA expression, we treated SCLC cells with BCS, a chelator of Cu(I). For comparison, cells were also treated with CUP, BPS, or DEF, chelators of Cu(II), Fe(II) or Fe(III),

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respectively (Chakravarty et al., 2004). We demonstrated that hCtr1 mRNA levels were increased about two-fold in cells treated with BCS compared with those in the untreated cells. Levels of hCtr1 mRNA were slightly increased in cells treated with CUP but not with BPS and DEF (Fig. 1C). These results demonstrated, for the first time, that hCtr1 mRNA expression is increased in cells cultured under Cu(I)-depleted conditions and to a lesser extent, Cu(II)-depleted conditions.

Steady-State Levels of hCtr1 mRNA are Self-regulated

We reasoned that Cu deprivation-induced hCtr1 mRNA expression would enhance Cu transport activity, resulting in increased intracellular Cu content that would in turn down-regulate the expression of hCtr1 mRNA. To test this hypothesis, we first performed transient transfection experiments using CIN-HA-pcDNA3-hCtr1 recombinant plasmid DNA. The recombinant cDNA construct encodes the entire coding region but lacks the 5'-untranslationed region (UTR) and 3'-UTR (Fig. 2B, WT). Lacking 5'-UTR in the transcribed hCtr1 mRNA allowed us to design a probe in the RNase protection assay to differentiate between the exogenous (exo-) (transcribed from the transfected cDNA, 300 nt) and the endogenous (endo-) hCtr1 mRNA (360 nt) resolved by gel electrophoresis (Fig. 2C). Fig. 2A shows that increased expression of exo- hCtr1 mRNA resulted in reduced endo-hCtr1 mRNA expression in a range of 2.5 μg to 20 μg of recombinant hCtr1 cDNA transfected.

The hCtr1 contains 190 amino acid residues. The N-terminus of hCtr1 contains two conserved Met-rich sequences ($M^{7}GMSYM^{12}$ and $M^{40}MMMPM^{45}$) (Puig et al., 2002). We previously demonstrated that deleting either the first M-rich domain or both abolished hCtr1's transport function, whereas deleting the C-terminal C/H residues had only a minimal effect as measured by the rates of ^{64}Cu and cisplatin uptake. The reduced rates of cisplatin uptake are reflected in the reduced sensitivity of the transfected cells to platinum-based antitumor agents. Since the expression levels of exo-hCtr1 protein in the cells stably transfected by plasmid DNA lacking sequences encoding these residues had been measured by the western blotting using

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anti-HA antibody in the previous study (Song et al., 2004), we analyzed the endo-hCtr1 mRNA levels (Fig. 2B). Endo-hCtr1 mRNA levels were drastically reduced in WT-hCtr1 cDNA-transfected cells. Levels of endo-hCtr1 mRNA were partially reduced in C-terminus-deleted mutants. In contrast, N-terminus-deleted mutants (δN1 and δN2), like the untransfected control or empty vector-transfected cell lines, did not show a reduction in the endo-hCtr1 mRNA levels (Fig. 2D). These results demonstrated that only expression of functional hCtr1 mRNA suppressed endo-hCtr1 mRNA expression. Taken together, these results demonstrate that increased intracellular Cu concentration mediated by overexpressed full-length hCtr1 protein plays a role in regulating hCtr1 mRNA. These results also suggest that intracellular Cu concentrations are regulated by homeostatic regulation of hCtr1 mRNA.

Transcriptional Regulation of hCtr1 Expression by Copper

The observation that steady-state hCtr1 mRNA levels were regulated in response to Cu stress suggested that transcriptional and/or post-transcriptional mechanisms are involved in the regulation of hCtr1. To investigate whether transcriptional regulation is involved, we cloned the promoter sequence of the *hCtr1* gene, from which a series of recombinant plasmid DNA containing various lengths of *hCtr1* promoter sequence in a luciferase reporter vector was prepared. *hCtr1*is located on human chromosome 9q32. Another transcription unit with opposite direction named KIAA0674 which encodes an FK506-binding protein-like transcript (Nakajima et al., 2006) is located -201 bp upstream of the *hCtr1* locus (Fig. 3A). These reporter plasmids were transfected into SCLC cells following treatment with Cu or BCS. Fig. 3B shows that, in the absence of promoter sequence (+1 to +303 Luc), the reporter only showed minimal level of transcriptional activity, regardless whether in the presence or absence of Cu or BCS. Including sequences between -43 to -227 bp in the reporter constructs increased promoter activities in a progressively increased manner except the -184 Luc. Reporter constructs with sequences further upstream to -564 bp showed no additional increases of the transcription activity. In all the

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transfection experiments, treatments with Cu reduced reporter expression; whereas treatments with BCS increased reporter expression. The magnitudes of reduction or increase were no more than 50%, but results were very similar across four experiments. These low levels of change were in agreement with those of steady-state hCtr1 mRNA levels measured by the RNase protection assay (Fig. 1). These results suggest that sequences within -43 bp of the *hCtr1* promoter contain regulatory signals for hCtr1 expression by Cu concentration stress.

Examining the proximal region of the *hCtr1* promoter sequence, we found three putative Sp1 binding sites: $^{-40}$GGGGCGGAG, $^{+6}$GGGGGCGGG and $^{+17}$GGGGCGGGA (Fig. 3A). To determine whether these Sp1 sites were involved in Cu responsiveness, we introduced mutations in these sites and found that mutations at any one of the three sites abolished the responsiveness to Cu and BCS treatments (Fig. 3C). These results demonstrate that these putative Sp1 binding sites are important for the Cu-regulated *hCtr1* expression.

To determine whether these GC-rich sequences were indeed recognized by Sp1, we performed an electrophoretic mobility shift assay (EMSA) using ^{32}P-labeled nucleotides containing either site 1 alone or sites 2 and 3 combined because the latter two sites are too close to generate individual probes without interference to each other (Fig. 3D, right). With both probes, a prominent gel shift signal was noted in the EMSA. This signal could be efficiently competed by the unlabeled oligonucleotides containing the WT sequence but not by those containing mutant sequences (Fig. 3D). Because commercially available anti-Sp1 antibodies performed very poorly for super-shifting Sp1-DNA complex, we prepared nuclear extracts from SCLC cells transfected with HA-tagged Sp1 recombinant DNA. The mobility-shifted signal could be supershifted using anti-HA antibody. These results demonstrated that these sites are indeed recognized by Sp1. We performed chromatin immunoprecipitation (ChIP) assay to determine *in vivo* Sp1 bindings under Cu stress conditions but the results were inconclusive (four experiments). This was probably due to the technical limitation of ChIP when differences in *in vivo* Sp1 engagement were low (less then 2-fold) between two transcriptional statuses.

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Nonetheless, several sets of experiments described below provide additional supports for the involvement of Sp1 in the regulation of hCtr1 mRNA expression.

Sp1 Functions as a Positive Regulator for hCtr1 Expression at Normal Cu Concentration

We transfected increasing amounts of HA-tagged Sp1 recombinant plasmid into SCLC cells and measured the expression levels of hCtr1 mRNA. Increasing expression of Sp1 enhanced hCtr1 mRNA levels in a concentration-dependent manner (Fig. 4A). In contrast, increasing expression of a control transcription factor AP2 did not increase hCtr1 mRNA expression (Fig. 4B). A putative AP2-like sequence is located at ($^{-214}$CCGCCGA) in the promoter region of *hCtr1*. In another experiment, downregulation of Sp1 by siRNA reduced the expression of hCtr1 in a time-dependent manner (Fig. 4C). These results, taken together, supported the positive role of Sp1 in the regulation of hCtr1 at Cu non-stressed concentration.

Sp1 contains three zinc finger domains (ZF1 to ZF3) at the C-terminus that bind to GC-rich DNA sequences and an N-terminally located transactivation domain consisting two serine and tyrosine (S/T)-rich and two glutamine (Q)-rich domains (Fig. 4D). To investigate whether the ZFs in Sp1 were responsible for the transcriptional activation of hCtr1 expression, we prepared three expression recombinants encoding HA-tagged Sp1 in which the three ZFs were progressively deleted, Sp1-M1, Sp1-M2 and Sp1-M3 (Fig. 4D). These recombinant mutants were transfected into SCLC cells and stable Sp1-expressing cell lines were established. Expression of different versions of Sp1 was confirmed by western blot using anti-HA antibody (Fig. 4E, middle). Levels of hCtr1 mRNA were measured by RNase protection assay (Fig. 4E, upper and bottom panels) and by western blotting (Fig. 4E, middle). As consistent with those shown in Fig. 4A, transfection with the Sp1-WT recombinant enhanced levels of hCtr1 mRNA. Deleting the C-terminal ZF (ZF3) motif partially diminished the ability of enhancing endo-hCtr1 expression as compared with that in the Sp1-WT-transfected cells. Removing both ZF2 and ZF3 motifs completely abolished the enhancing expression of endo-hCtr1 (Fig. 4E and Fig.

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4F, lane 1). These results support the roles of ZF motifs in the transcriptional regulation of hCtr1 expression by Sp1.

The ZFs in Sp1 Function as Sensors of Cu Stress Conditions

To investigate whether the ZF domains of Sp1 are involved in regulation of hCtr1 expression under Cu stress conditions, we transfected WT- or ZF-deleted Sp1 constructs into SCLC cells followed by treatments with Cu (II), BCS, and Fe (III). Fig. 4F shows that elevated expression of Sp1-WT enhanced the expression of hCtr1 mRNA in cells treated with all these agents, suggesting that Sp1 also functions as a positive regulator even when cells were under Cu stressed conditions. However, levels of hCtr1 mRNA were not changed in SCLC cells transfected with Sp1-M2 or Sp1-M3 recombinant DNA under these conditions. These results strongly suggest that the ZFs in Sp1 function as sensors of Cu-stress conditions. It is interesting to note that the hCtr1 transcription levels were elevated in the Sp1-M2- and Sp1-M3-transfected cells in presence of copper relative to that in the untransfected control (Fig. 4F). The mechanism of this enhancement is not clear at the present. It is possible that the remaining transactivation domains in these Sp1 mutants may have copper-dependent transcriptional activation activity. However, further investigations are needed to address this issue.

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DISCUSSION

We report here, for the first time, that a transcriptional mechanism is involved in the regulation of steady-state levels of hCtr1 mRNA by Cu homeostasis in mammalian cells. Our ability to detect copper concentration-modulated hCtr1 mRNA expression was largely attributed to the use of the RNase protection assay, which provided a sensitive and quantifiable measurement system. While the magnitude of the changes in the hCtr1 mRNA level in response to Cu concentration variation was only moderate, the results were very reproducible. Our previously study using transfected myc-hCtr1 showed that membrane-bound exo-hCtr1 were significantly reduced 10 min. after Cu (100 μM) treatment as analyzed by immunoblotting (Petris et al., 2003). In another heterologous system where hCtr1 were stably expressed in insect cells, internalization of hCtr1 was not observed upon the initial exposure of cells to 100 μM $CuCl_2$ (Eisses et al., 2005). As shown in this study, expression of exo-hCtr1 can influence the expression of endo-hCtr1. Such stably transfected cells may have already preloaded Cu that interferes with the behavior of endo-Ctr1. Regardless, our present results and those published previously combined suggest that many aspects of hCtr1 regulation may have occurred in different cell contexts within different time scales.

We discovered that Sp1 plays an important role in the regulation of hCtr1 expression which is essential for the maintenance of Cu homeostasis. On one hand, hCtr1 can be considered a house-keeping gene that provides the essential Cu for every cell. The involvement of a ubiquitous transcription factor Sp1 in the regulation of house-keeping gene expression is not surprising. On the other hand, hCtr1 expression is regulated by Cu concentration and Sp1 is also involved in the regulation. These observations demonstrate that Sp1 plays dual roles in regulating both basal and Cu-dependent hCtr1 mRNA levels and suggest a complex mechanism of Sp1-mediated *hCtr1* gene regulation, which in turn is concomitantly regulated by the dynamic Cu homeostatic regulation mechanism. This may explain the low magnitude of changes in hCtr1 mRNA expression in response to Cu stress conditions as demonstrated in this

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study.

We observed that deleting the proximal Sp1 binding sites of hCtr1 promoter abolished the basal and Cu-responsive hCtr1 expression in the transient transfection assay. Knockdown of Sp1 expression by siRNA downregulated hCtr1 expression and overexpression of Sp1 by transfection upregulated hCtr1 expression. Further analyses showed that deleting the ZF domains of Sp1 rendered hCtr1 expression insensitive to Cu concentration stress. These results collectively strongly support that ZF in the Sp1 functions as a sensor of intracellular Cu concentration. Cu is a well-known pro-oxidant. It generates hydroxyl radicals (˙OH) from hydrogen peroxide according to the Fenton reaction. Each ZF in Sp1 is composed of two Cys and two His residues that are coordinated by zinc in a tetrahedral conformation. Cys in the ZF domain is particularly redox-sensitive. When a Cys-containing finger structure folds around the Zn^{2+} ion, a protective pocket is formed with no additional redox stress on the Cys residues. However, it has been well-documented that redox-active metal ions can destabilize the structural folding of ZF domain, including displacement of Zn, formation of mixed complexes, or incomplete coordination of metals, resulting in loss of DNA binding activity (for review, see (Hartwig, 2001). Indeed, the toxic effects of copper on zinc fingers in the transcription factors such as estrogen receptor (Predki and Sarkar, 1992) and Sp1 (Thiesen and Bach, 1991), have been mentioned. Within this context, we hypothesize that the mechanism by which hCtr1 expression is downregulated under Cu-replete condition is because of Cu poisoning of Sp1; and that upregulation of hCtr1 under Cu-depleting condition is through the rescue of Sp1 from Cu poisoning. Further investigations on the mechanistic aspects of how Sp1 precisely regulates hCtr1 expression under Cu stress conditions are needed.

Our observation that Sp1 is involved in the regulation of hCtr1 expression is consistent with an evolutionarily conserved role of ZF-containing transcription factors in the regulation of Cu homeostasis from yeast to humans. Like mammalian Sp1, both yeast Mac1p (Heredia et al., 2001) and Drosophila MTF1 contain ZF domains. Besides Cu, Sp1 is also involved in the

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transcriptional regulation of Zip8, which is a recently identified cadmium transporter (our unpublished results). Furthermore, a Zn-responsive transcription activator, Zap1, is also involved in the regulation of Zn homeostasis. This transcription factor controls the expression of genes involved in Zn accumulation in yeast. Zap1 is active in Zn-limited cells and repressed in Zn-replete cells. Like Sp1 (Nicolas et al., 2001). Zap1 controls its own expression through transcriptional autoregulation. Zap1 has two activation domains (AD1 and AD2) and seven ZFs. In all these, ZF in Zap1 functions as a sensor of Zn concentration (Bird et al., 2003; Dhanasekaran et al., 2006). These results collectively demonstrate the importance of ZF-containing transcription factors in the regulation of cellular metal metabolism.

In summary, we have presented the new discovery that mammalian Cu homeostasis is transcriptionally regulated by transcription factor Sp1. This finding presenting a new paradigm for future research into the transcriptional machinery involved in the regulation of mammalian Cu homeostasis.

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FOOT NOTES

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FIGURE LEGENDS

Fig. 1. Regulation of steady-state hCtr1 mRNA levels by the treatments of metal ions and their chelators. (A) Upper panel: Expression level of hCtr1 mRNA was measured after the treatment of different concentrations of Cu and Fe as indicated for 16 h in SCLC cells using RNase protection assay. The expression level of 18S RNA was also measured and used as a control for sample loading. Middle panel: The mRNA levels were quantified and ratios of hCtr1 mRNA/18S RNA (% of control) were plotted versus the concentrations of metal ions. Bottom panel: Intracellular Cu content was assessed by measuring the uptake of ^{64}Cu after Cu treatment for 16 h. Each data point was represented by the mean ± S.D. from three independent experiments. *: $p<0.05$, significantly different compared with control group, using unpaired *t*-test. (B) Upper panel: Time-dependent effect of Cu treatment (100 μM) on the expression level of hCtr1 mRNA was measured by RNase protection assay. Middle panel: Ratios of hCtr1 mRNA/18s RNA (% of control) were plotted against treatment time of Cu. Bottom panel: Intracellular Cu content was assessed by measuring the uptake of ^{64}Cu after Cu treatment for up to 16 h. Each data point is the mean±S.D. from three independent experiments. *: $p<0.05$, significantly different compared with control group, using unpaired *t*-test. (C) Upper panel: Expression levels of hCtr1 mRNA in SCLC cells treated with different metal ion chelators as indicated (100 μM each for 16 h). Bottom panel: Imaging signals in autoradiographs were quantified. Each bar represents the mean±S.D. from three independent experiments. *: $p<0.05$, significantly different from the control group, using unpaired *t*-test.

Fig. 2. Steady-state levels of hCtr1 mRNA Are self-regulated. (A) Autoradiographs of RNase protection assay showing that expression of exogenous hCtr1 mRNA (exo) by transfecting *hCtr1* cDNA recombinant downregulates endogenous hCtr1 mRNA (endo) in a concentration-dependent manner (left panel); quantified results are shown in the right panel. Each bar refers mean ± S. D. from three experiments. (B) Structure of wild-type hCtr1 (WT)

and its N-terminal (δN1) and (δN2) and C-terminal (δC) mutants. (C) Design of hybridization riboprobe in reference to hCtr1 mRNA that allows simultaneous detections of endogenous hCtr1 mRNA transcripts (360 nt signal) and transcripts from different transfected hCtr1 deletion mutants with the indicated specific protection fragment sizes. (D) Autoradiographs of RNase protection assay of the expression of the endo- and exo-hCtr1 mRNA in the transfected cells. Note that levels of endo-hCtr1 mRNA were reduced in the WT-transfected cells.

Fig. 3. Identification of DNA sequences in the promoter region of hCtr1 that are involved in transcriptional regulation of hCtr1 by Cu. (A) Nucleotide sequence of the *hCtr1* promoter region. Long arrows show the direction of transcription; short arrows refer to the borders of the luciferase reporter constructs as shown in B. Boxed nucleotides are the GC-rich, Sp1 binding sites and nucleotides in gray dotted boxes represent Ap2 binding sites. (B) Expression levels of reporter recombinant DNA transfected into SCLC cells that had been treated with Cu or BCS or left untreated. Each was cotransfected with control pRL-SV40 vector. The luciferase activity was normalized to a control renilla luciferase activity and is presented as a fold induction. Each bar represents the mean ± S.D. from three independent experiments. (C) Evaluation of the roles of GC boxes (Sp1 sites) in the regulation of hCtr1 expression by transient reporter expression assay. (D) Electrophoretic mobility shift assay (EMSA) of Sp1 binding to the GC box sequences. ^{32}P-labeled probes containing Site 1 or Site-2/3 sequences as indicated were used in EMSA using nuclear extracts prepared from recombinant HA-Sp1 cDNA-transfected SCLC. DNA containing WT and MT sequences were used in competition assays with the relative amounts as indicated. Supershift was carried out using anti-HA antibody as indicated by arrow.

Fig. 4. Transcription factor Sp1 functions as a positive regulator for hCtr1 expression at normal physiological Cu concentration. (A) Expression of WT Sp1 expression recombinant DNA by transient transfection upregulated hCtr1 expression in a concentration-dependent

manner as determined by an RNase protection assay. (B) Expression of AP2 did not upregulate hCtr1 expression. (C) Treatment of Sp1 siRNA downregulated expression of hCtr1 as determined by western blot analysis. (D) Structure of wild-type Sp1 (WT) and its progressive ZF deletion mutants M1, M2, and M3. The Q-rich and S/T-rich domains are indicated by gray and hatched bars, respectively. The solid bars represent the three ZF domains. (E) Upper panel: Progressive deletions of ZF in Sp1 abolished the positive regulation of hCtr1 in a transfection assay, as determined by an RNase protection assay (top), expression of the transfected Sp1 (middle, indicated by white dots, The 100-kDa and 50-kDa signals were from nonspecific cross-reactivity) and hCtr1 (bottom) are shown below by western blot; Lower Panel: quantitative analyses of results are shown in the upper panel. Each bar represents the mean ± S.D. from three independent experiments. (F) Effects of ZF in Sp1-mediated regulation of hCtr1 expression in response to treatments with Cu, BCS, or Fe. SCLC and stable transfectant cell lines (10^6 cells per plate) were treated with Cu, BCS, or Fe (100 μM each for 16 h), and the expression levels of hCtr1 mRNA and 18S RNA (as a loading control) were measured using RNase protection assay (upper panel) and quantitative results are shown in the bottom panel. Each bar represents the mean ± S.D. from three independent experiments.

Fig.1

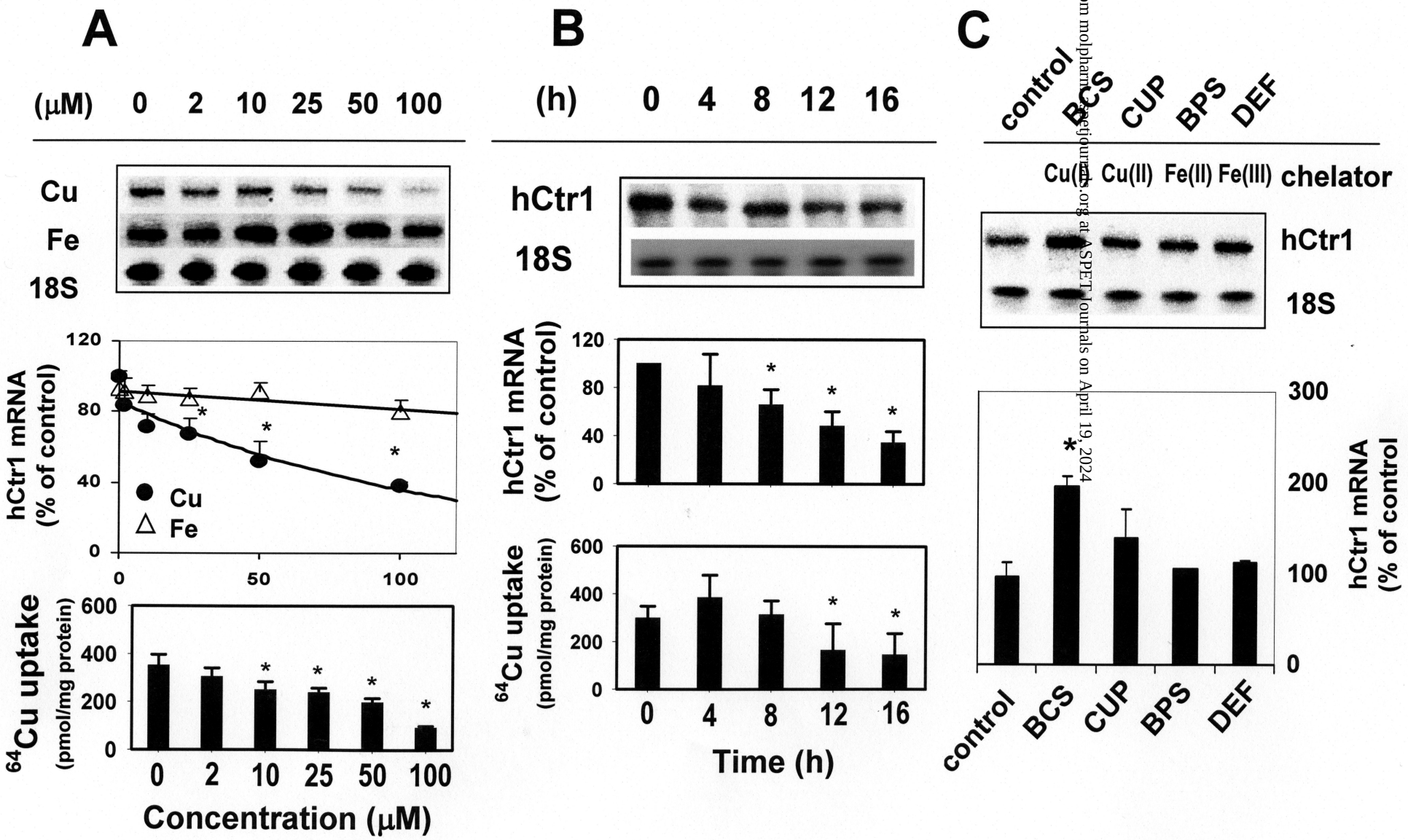

Fig.2

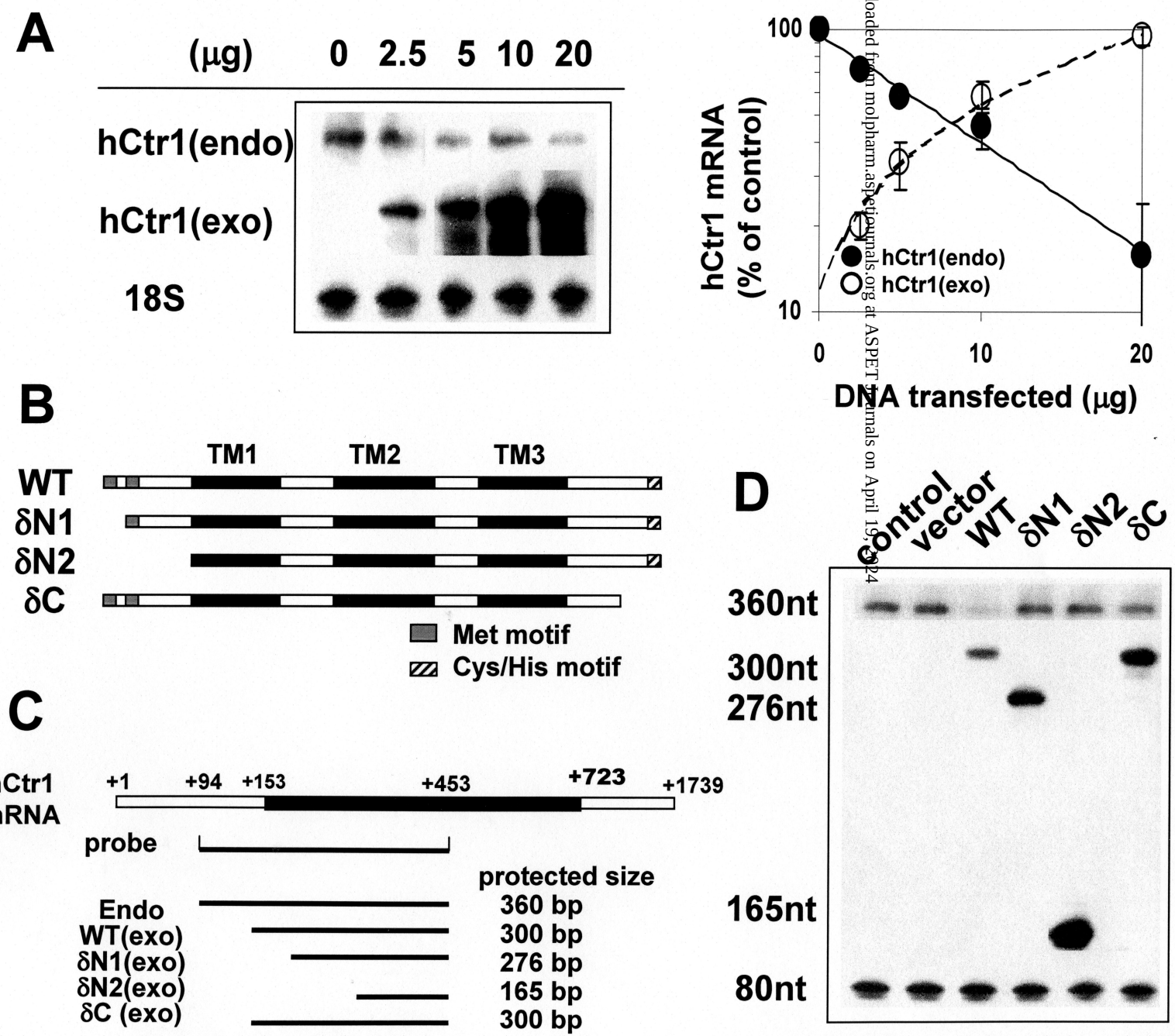

Fig.3

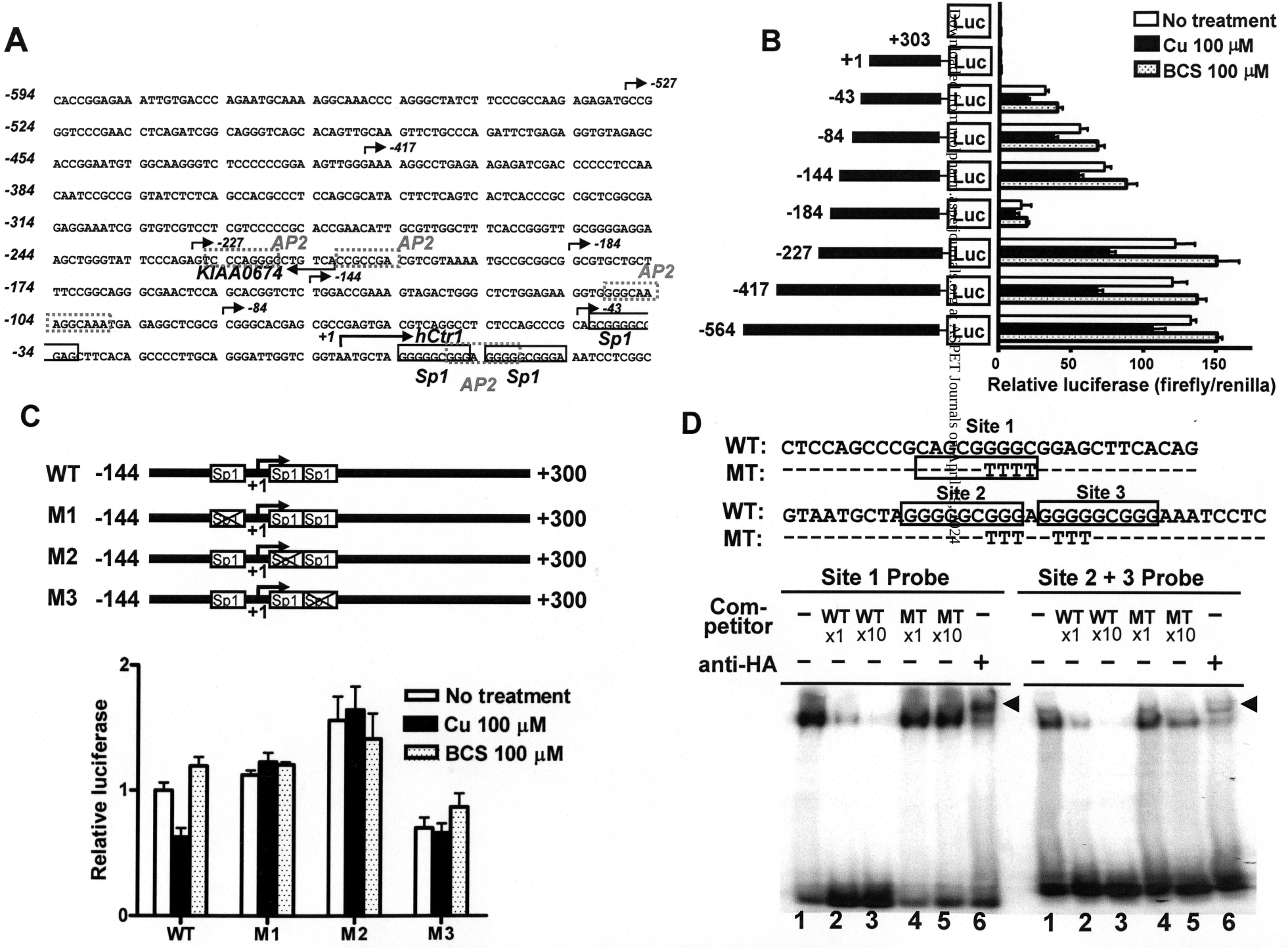

Fig. 4