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Regulation of Prion Gene Expression by Transcription Factors SP1 and Metal Transcription Factor-1^{*§}

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Prion diseases are associated with the conformational conversion of the host-encoded cellular prion protein into an abnormal pathogenic isoform. Reduction in prion protein levels has potential as a therapeutic approach in treating these diseases. Key targets for this goal are factors that affect the regulation of the prion protein gene. Recent *in vivo* and *in vitro* studies have suggested a role for prion protein in copper homeostasis. Copper can also induce prion gene expression in rat neurons. However, the mechanism involved in this regulation remains to be determined. We hypothesized that transcription factors SP1 and metal transcription factor-1 (MTF-1) may be involved in copper-mediated regulation of human prion gene. To test the hypothesis, we utilized human fibroblasts that are deleted or overexpressing the Menkes protein (MNK), a major mammalian copper efflux protein. Menkes deletion fibroblasts have high intracellular copper, whereas Menkes overexpressed fibroblasts have severely depleted intracellular copper. We have utilized this system previously to demonstrate copper-dependent regulation of the Alzheimer amyloid precursor protein. Here we demonstrate that copper depletion in MNK overexpressed fibroblasts decreases cellular prion protein and *PRNP* gene levels. Conversely, expression of transcription factors SP1 and/or MTF-1 significantly increases prion protein levels and up-regulates prion gene expression in copper-replete MNK deletion cells. Furthermore, siRNA “knockdown” of SP1 or MTF-1 in MNK deletion cells decreases prion protein levels and down-regulates prion gene expression. These data support a novel mechanism whereby SP1 and MTF-1 act as copper-sensing transcriptional activators to regulate human prion gene expression and further support a role for the prion protein to function in copper homeostasis. Expression of the prion protein is a vital component for the propagation of prion diseases; thus SP1 and MTF-1 represent new targets in the devel-

opment of key therapeutics toward modulating the expression of the cellular prion protein and ultimately the prevention of prion disease.

Prion diseases, traditionally known as transmissible spongiform encephalopathies, are invariably fatal, transmissible neurodegenerative disorders that include Creutzfeldt-Jakob disease and kuru in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. According to the protein-only model of prion propagation, these diseases are associated with the conformational conversion of the host-encoded cellular prion protein (PrP^C) into an abnormal pathogenic isoform (PrP^{Sc}) (1). PrP^C and PrP^{Sc} both have the same primary sequence and are encoded for by a single gene, *PRNP* (2). PrP^C expression is an absolute requirement for prion infection, because mice in which *PRNP* has been ablated are completely resistant to infection when inoculated with prions (3), and this protective phenotype can be inhibited when transgenes expressing PrP^C are reintroduced (4).

PrP^C is a cell surface glycoprotein anchored at the plasma membrane by a glycosylphosphatidylinositol anchor (5). Expression is most abundant in the central nervous system, primarily in neuronal (6) and glial cells (7). PrP^C is also expressed in many non-neuronal tissues, including blood lymphocytes, gastroepithelial cells, heart, kidney, and muscle (8, 9). Although this widespread expression pattern has suggested potential functional roles for PrP^C in a variety of cellular mechanisms (10), the cellular functions of PrP^C are still poorly understood.

Several studies have demonstrated that a major function of PrP^C relates to the maintenance of intracellular copper homeostasis. PrP^C contains two distinct copper-binding domains. The primary copper-binding domain is located in the N-terminal region between residues 60–91 (11), consists of four to six octapeptide repeats of the sequence Pro-His-Gly-Gly-Gly-Trp-Gly-Gln (12–14), and binds copper ions with between femto- and nanomolar affinities (15). The secondary copper-binding domain is located between residues 91 and 111 just outside of the octapeptide repeat region (16–18). This domain is coordinated by two histidine residues, His⁹⁶ and His¹¹¹, and is suggested to have a much lower affinity for copper than the primary copper-binding domain (15, 19). These copper-binding regions may have functional significance, because they are very highly conserved among a wide variety of mammalian species (11), and insertions of one or more octapep-

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tide repeat units are associated with familial forms of prion disease in humans (20, 21).

Copper binding to the octapeptide repeat region induces the endocytosis of PrP^C from the cell surface in a reversible manner, which suggests PrP^C may act as a recycling receptor for cellular uptake or efflux of copper (22, 23). Furthermore, it has been shown in *PRNP* null mice that copper levels in cerebellar cells are significantly reduced compared with cells from age- and sex-matched controls (13), suggesting that PrP^C is an important component for maintaining brain copper homeostasis. In addition, the octapeptide region of human PrP^C reduces Cu²⁺ to Cu⁺ *in vitro*, which is an important step required for cellular copper uptake (24, 25). It has also been reported that copper can regulate the expression of the rat prion gene promoter in neurons via putative metal response element (MRE)³ DNA sequences (26). However, the precise transcriptional machinery involved in this regulation has not been determined.

Because copper homeostasis from yeast to mammals is regulated by several cellular mechanisms including copper-dependent transcriptional regulation (27, 28) and the *PRNP* promoter region is highly conserved among several species (29), we propose that copper regulation of the human *PRNP* gene is controlled by metal-regulated or metal-responsive transcription factors via putative MRE sequences.

The human *PRNP* promoter region contains a number of putative transcription factor-binding sites, including the transcriptional activator SP1, AP1, AP2, and a CCAAT box (29). The active promoter region has been determined to be within a 273-bp region, -148 to +125, relative to the cap start site (29, 30). Metal-responsive transcription factor-1 (MTF-1) can bind to MRE sequences to regulate genes encoding metallothioneins (31–34), a family of conserved metal detoxification proteins (35). In *Drosophila*, dMTF-1 can also regulate the expression of copper detoxification metallothioneins (36) and paradoxically control the expression of copper import and export proteins DmCtr1b (37) and DmATP7 (38), respectively. SP1, a general activator of transcription, can also bind to putative MRE sequences, possibly in a negative-regulatory manner in competition with MTF-1 to regulate gene expression (39). The contribution of these transcription factors, SP1 and MTF-1, in copper regulation of the human *PRNP* gene has not yet been determined.

To investigate the role of SP1 and MTF-1 in copper-dependent regulation of the human *PRNP* gene, we utilized a novel human cell culture model system that has previously been used to demonstrate copper-dependent regulation of the Alzheimer amyloid precursor protein (APP) gene (40). This approach involves cultured human fibroblasts overexpressing the Menkes protein (MNK; encoded by *ATP7A*), a major mammalian copper translocating P-type ATPase involved in copper efflux (41–43). Cells lacking the MNK protein show high intracellular copper levels caused by the lack of active copper efflux, whereas

cells transfected and hence overexpressing MNK have markedly reduced copper levels (40, 43–45).

Here we report that transcription factors SP1 and MTF-1 increase both cellular prion protein levels and *PRNP* gene expression under copper-replete conditions. Conversely, reducing levels of SP1 and MTF-1 using siRNA “knockdown” decreases both cellular prion protein and *PRNP* gene expression under copper-replete conditions. In addition, depletion of intracellular copper results in complete reduction of PrP^C protein and *PRNP* gene levels. Overall, these data suggest that SP1 and MTF-1 are required for copper-mediated regulation of the *PRNP* gene and supports the increasing evidence that the cellular prion protein is involved in copper homeostasis.

EXPERIMENTAL PROCEDURES

Cell Lines—Establishment and characterization of human skin fibroblast cells, MNK(Del), MNK(v/o), and MNK(++) has been reported previously (43). Briefly, primary fibroblast cells from a classical Menkes disease patient were immortalized by the SV40 gene transfer to derive Me32aT22/2L (43) and designated MNK(Del). The 4.6-kb cDNA encoding the human MNK protein was cloned into a mammalian expression vector and transfected into Me32aT22/2L, and MNK-expressing clone A12-H9 was designated MNK(++) (43). The empty mammalian expression vector, pCMB77, was transfected alone into Me32aT22/2L to derive Me32aT22/2L(pCMB77) and designated MNK(v/o) (43). The SV40-immortalized human fibroblast cell line, normal human (GM2069), was used as a control.

Cell Culture Conditions—MNK(Del) and normal human fibroblast cells (43) were maintained in Eagle's basal medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine, 20 mM HEPES. The MNK(v/o) and MNK(++) cell lines were maintained in 10% Eagle's basal medium prepared as above with the addition of 500 µg/ml geneticin (Invitrogen). All of the cells were incubated at 37 °C in a 5% CO₂ atmosphere.

Cellular Metal Analysis—Copper, zinc, and iron levels were determined in MNK(Del), MNK(v/o), MNK(++), and normal human fibroblasts according to established procedures (40).

Preparation of Expression Constructs—Full-length True-Clone™ cDNA expression constructs (Origene) for SP1 (SC116396) and MTF-1 (SC101137) were prepared for transfection using a plasmid DNA maxi kit (Promega) according to the manufacturer's instructions.

Transient Transfection and Lysate Preparation—The cells were seeded at ~1 × 10⁵ cells/well in 6-well plates 24 h prior to transfection in basal medium. All of the cells were transfected with FuGENE® 6 (Roche Applied Science) according to the manufacturer's instructions. Transfection mixture was prepared containing 4 µg of pSP1 and/or 4 µg of pMTF-1/well. 24 h post-transfection, the medium was changed to either basal medium or 100 µM CuCl₂, 50 µM ZnCl₂, or 50 µM FeCl₃ supplemented medium. 48 h post-transfection the cells were washed with ice-cold PBS several times, lysed, and extracted for total protein or total RNA using the PARIS™ kit (Ambion) according to the manufacturer's instructions. The protein concentrations were measured using a BCA protein assay (Pierce).

³ The abbreviations used are: MRE, metal response element; APP, amyloid precursor protein; MNK, Menkes protein; MTF-1, metal transcription factor-1; MLS, MRE-like sequence; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; ANOVA, analysis of variance.

Total RNA was analyzed for quality and quantity by standard spectrometry procedures.

Antibodies—Anti-MNK polyclonal antibody raised to the MNK N-terminal region (42) was diluted 1:2500. Anti-APP (WO2) monoclonal antibody raised to the amyloid- β region (46) was diluted 1:10000. Anti-PrP (3F4) monoclonal antibody raised to human PrP^C region was diluted 1:10000 for Western blot analysis, whereas Anti-PrP (ICSM-18; D-Gen) was diluted at 1:250 for immunofluorescence. Anti-MTF-1 (clone ab55522) and anti-SP1 (clone ab58199) monoclonal antibodies (Abcam) were diluted at 1:500. Anti-GAPDH monoclonal antibody, clone 6C5 (Ambion) was diluted at 1:20000. Anti-mouse horseradish peroxidase secondary antibody (GE Healthcare) was diluted at 1:15000.

Confocal Microscopy—The cells were seeded onto glass coverslips and grown for 48 h prior to being fixed in 3.2% paraformaldehyde/PBS and permeabilized in 0.1% Triton X-100/PBS. The coverslips were then placed in blocking solution containing 10% goat serum (Invitrogen)/2% bovine serum albumin in PBS at 4 °C overnight. PrP^C was detected with primary antibody ICSM-18 and secondary antibody Alexa-488 conjugated to goat anti-mouse (Molecular Probes) diluted at 1:250 and 1:500 in blocking solution, respectively. Nuclear staining was performed with 4',6'-diamino-2-phenylindole (Sigma) diluted at 1:1000 and co-incubated with the secondary antibody. Coverslips were mounted on glass slides in DABCO (Sigma) and scanned using a Leica DMIRE2 confocal microscope under identical exposure conditions.

Western Immunoblot Analysis—Protein extracts were fractionated on NuPage™ Bis-Tris (4–12%) gradient acrylamide gel (Invitrogen) and electroblotted to nitrocellulose filters or polyvinylidene difluoride filters. Detection of protein was performed using an ECL chemiluminescence kit (GE Healthcare), according to the manufacturer's instructions.

Quantitative Real Time RT-PCR—1 μ g of total RNA extracted from cell lysates was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real time RT-PCR samples were then prepared using Taqman® gene expression master mix and human-specific Taqman® gene expression assays (Applied Biosystems) for PRNP (Hs00175591_m1) with endogenous controls for human GAPDH (Hs99999905_m1) and RPLP0 (Hs99999902_m1) according to the manufacturer's instructions. Real Time RT-PCR samples were then run on a RotorGene 3000 (Corbett Research). The data were analyzed and quantified using the DeltaDelta CT method (47).

siRNA—Silencer® predesigned siRNA for SP1 (SP#1, identification code 116547; SP#2, identification code 1165476; SP#3, identification code 143158), MTF-1 (MTF1#1, identification code 3247; MTF1#2, identification code 3337; MTF1#3, identification code 107900), and negative control 1 siRNA (NEG#1) was purchased from Ambion. siRNA transfection reagent siPORT NeoFx was purchased from Applied Biosystems.

siRNA Knockdown—The cells were post-seeded at $\sim 1 \times 10^5$ cells/6 wells and either mock transfected or transfected with siPORT NeoFx transfection reagent and negative control #1 siRNA or Silencer® predesigned siRNAs for SP1 and MTF-1 at a final concentration of 30 nM using the reverse transfection method

according to the manufacturer's instructions. 48 h post-transfection total protein and RNA were extracted and analyzed as described above.

Bioinformatics—The human PRNP gene promoter sequence (GenBank™ accession number AJ289875) was analyzed for the presence of metal response element (48) (MRE consensus sequence 5'-TGCRCNC-3') consensus sequences using TESS: Transcription Element Search Software (49). In addition, promoters were searched for MRE-like sequences (MLS), with no more than one base mismatch from the last three MRE consensus residues (5'-TGCRCNC-3').

Statistical Analysis—The results were expressed as the means \pm S.E. Statistical analysis involving two groups was performed by unpaired *t* test, whereas one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison of mean's post-test was performed to compare more than two groups using Prism 4 for Macintosh (GraphPad Software Inc.). Statistically significant was defined as $p < 0.05$.

RESULTS

The Human PRNP Gene Promoter Contains a Number of Putative Metal Regulatory Sequences—The human PRNP gene promoter (GenBank™ accession code AJ289875) was analyzed for the presence of MREs and MRE-like sequences found in the mammalian copper detoxification gene, metallothionein (*MT*) promoter (48). Utilizing this search criteria, we identified three consensus MTF-1-binding sites, several potential MRE-like sequences (Fig. 1). In addition, multiple SP1-binding sites in the human *PRNP* promoter were also identified (Fig. 1). Of interest, the 273-bp (-148 to +125) active promoter region (29) contained tandem MTF-1- and SP1-binding sites immediately prior to the suggested 5'-untranslated region cap start site (30). These observations supported our hypothesis that transcription factors SP1 and MTF-1 may mediate copper regulation of the human *PRNP* gene.

MNK Fibroblasts Have Altered Cellular Copper Levels—MNK encodes a P-type copper-transporting ATPase (50, 51). Mutations in the MNK gene, *ATP7A*, lead to Menkes disease in humans associated with increased intracellular copper in cultured cells from Menkes patients (52), whereas overexpression of MNK results in copper resistance and reduced intracellular copper (42). Immortalized human fibroblasts isolated from a Menkes disease patient (43), herein referred to as MNK(Del) cells, and MNK(Del) cells stably transfected and overexpressed with the MNK efflux protein or the empty mammalian expression vector (43), herein referred to as MNK(++) and MNK(v/o) cells, respectively, represent powerful tools to manipulate intracellular copper concentrations. Analysis of total cellular copper, zinc, and iron levels demonstrates that MNK(Del) and MNK(v/o) control cell lines have significantly increased copper levels, whereas MNK(++) cells have significantly decreased copper levels compared with normal fibroblasts (Fig. 2A). No difference is observed for zinc and iron levels (Fig. 2, B and C, respectively). This is in agreement with previous studies that have consistently demonstrated that MNK(Del) and MNK(++) cell lines have contrasting high and low intracellular copper levels, respectively (40, 43–45). This

SP1 and MTF-1 Regulate Prion Gene Expression

-1500 TGCCCTTCCC CAGCAGACCT TTCAAGTGT CACCCACACT CACATAAACCA TGGCCCAGGC
MLS1/MLS2
-1440 ACTGTTTACA GCAGCTCTCC **TCTGTGCA** TCTCTGGCTT CTCCCTGTC TCAGCCATTG
-1360 TACCCCTCCC CTGCAACTTG AGTGACAGTG ATTGGTCCTG AGATGCAAAT ATTTGA**TGCA**
MLS3
-1300 **CATATGTTA** CAA**TGCA**GCC TCAGCTTTT TTTTTTTTT TGTAAGAGA CAAAGAGTGA
MTF-1 MRE
-1240 GACAGGGTCT TGGCCTATAG CCCTGTGGCC CAGGCTGGAG **TGCA**GTGGCA CAATTAAAGC
MLS5
-1160 TCAC**TGCA**G **CT**TACCTCC TGGGCTCAAG CAATCCTCCC ATTCAGCC CCCCCAGTAGC
-1100 TGGGACTACA GGACTGTGCC ACCGTTCCA GCAATTTTT TTATTTTTTG TAGAAATGGG
-1040 GTCTCACTAT GTTGCTCAA ACTCCTGAGC TCAAGCAATC TTCCTGCCTT
MLS6
-960 GGCCTCTGAA AGTGCTGGGA TTACAGGCCT GAGGCC**TGCA** ACCTGGCTGT CAACATTCTT
-900 AAATCTCTTT CCTTACATGC TTCCTAAACC TCTCACCCAA AACTAGGAGA CTAGATGTCC
SP1
-840 TATTTTCCCC AGGGCATGCC TGGTT**ACGC** CCATTTCACT TTAAAAGTGC CCAATTTGGG
-760 TAATAATTTA TAAGATCCCC CTCCCTCTAA ATCCCTGCTT TCTATCACTT CATCCTTCGC
MLS7
-700 TCTCCTTAA AATGAGACAG TTGTCAAGAG GAATC**TGCG** CAAGAACACA CCACCCCTGTT
MLS8/MLS9
-640 TCATAGAAGA TATCTCAGGT **AATGCA**AA CACGGGTTT TAAACGG**GAGC** GCATTTTCT
-560 CATTGTTAA TATCACCAAC TAAATCATCT CTTGCCTAAA ACAAGGAGTA GAAAGTGAAT
-500 GAAGGAAGGA ACAGGTGATG GTCAAGTGTCC TTTCTACGCC TCAAAATTAA AGAGTTTATG
-440 TGAAAATTCA TAAATATTAA TCTCAATCCA GGTAAAGCAA AATTTTTGC TCTCCTCTT
-360 AGAAATTCT GTTGCCTAA GTTCCAGAAA TTGCTTCCTC ATTCCTGAGC CTTTCATT
-300 CTCGATTCT CCATTATGTA ACGGGGAGCT GGAGCTTTGG GCCGAATTTC CAATTAAAGA
-240 TGATTTTAC AGTCAATGAG CCACGTCAAG GAGCGATGGC ACCCGCAGGC GGTATCAACT
-160 GATGCAAGTG TTCAAGCGAA TCTCAACTCG TTTTTTCCGG TGACTCATTG CGGGCCCTGG
MTF-1 MRE
-100 **TTGGCAGCG** **TGCA**CCCTT AACTAAACC TCGGCCGG**CC** GCGGCCGGG GGCACAGAGT
MLS10
-40 **GTGCGCCGG** CGCGCGGCA ATTGGTCCCC GCGCCGACCT **CCGCGCG** GCGCCGCGC
SP1
+21 **TTC**CCCTTCCC CGCCCCCGCGT CCCTCCCCCT CGGGCCCGCG CGTCGCTGT CCTCCGAGCC
SP1
+81 AGTCGCTGAC AGCCGCGGCG CCGCGAGCTT CTCCCTCCT CACGACCGAG GCAGGTAAAC

FIGURE 1. Putative SP1- and MTF-1-binding sites present in the human prion gene promoter. The human *PRNP* gene promoter (GenBank™ accession number AJ289875), -1500 to +140, relative to the transcription start site at +1 (30) is indicated. The active promoter region, -148 to +125 (29), is shown *underlined*. The putative transcription factor-binding sites for SP1, MTF-1 metal response elements (MRE consensus sequence 5'-TGCRNC-3') and MLS, with no more than one base mismatch from the last three MRE consensus residues (5'-TGCRNC-3'), are indicated in *bold type*.

facilitates investigation of the hypothesis that copper levels modulate human *PRNP* gene expression in this study.

MNK Overexpressed Fibroblasts Have Decreased Cellular Prion Protein Levels—MNK(Del), MNK(v/o) control, and MNK(++) cell lines were analyzed for MNK, APP, and PrP^C protein expression via Western blot analysis. MNK protein was not detectable in the MNK(Del) and MNK(v/o) control cell lines, whereas MNK protein was overexpressed in the MNK(++) cell line (Fig. 3A), as previously reported (40, 43–45). APP protein was detected in MNK(Del) and MNK(v/o) control cell lines and not detectable in the MNK(++) cell line (Fig. 3A). This is consistent with our previous work with this model MNK cell line system where we demonstrated copper-dependent regulation of the human *APP* gene (40).

PrP^C protein was detected in MNK(Del) and MNK(v/o) control cell lines as immunoreactive bands between 38 and 17 kDa (Fig. 3A). Interestingly, PrP^C protein was undetectable in MNK(++) cells (Fig. 3A). PrP^C protein expression in fibroblast lines was also examined using immunofluorescence analysis of confocal microscopy images. Although MNK(Del) and MNK(v/o) control fibroblasts all had detectable PrP^C levels, PrP^C was not detectable in MNK(++) cells (Fig. 3B).

MNK Overexpressed Fibroblasts Have Decreased Cellular Prion mRNA Levels—To determine whether decreased PrP^C levels observed in MNK(++) cells is a result of decreased mRNA transcript levels, we performed quantitative real time RT-PCR (Fig. 3C). Quantitation of human *PRNP* mRNA levels in MNK(Del), MNK(v/o), and MNK(++) cells demonstrated that *PRNP* expression is undetectable in MNK(++) cells compared with MNK(Del) cells (Fig. 3C). Because MNK(Del) and MNK(++) cells have high and low copper levels, respectively, and no change in zinc and iron levels (Fig. 2), these results are consistent with the hypothesis that copper levels regulate the expression of the human *PRNP* gene. Furthermore, the MNK fibroblast cell lines represent an ideal cellular system to test the hypothesis that transcription factors MTF-1 and SP1 can regulate the expression of the human *PRNP* gene under varying copper conditions.

Transcription Factors SP1, MTF-1, and Extracellular Copper Increase Cellular Prion Protein Levels—To investigate the role of the transcription factors SP1 and MTF-1 in regulating *PRNP* gene expression, MNK(Del) and MNK(++) cells were

transiently transfected in basal media for 48 h with SP1 and MTF-1 (Fig. 4A).

Transiently transfected cell lysates from MNK(Del) “high copper” and MNK(++) “low copper” cell lines were analyzed for SP1, MTF-1, and PrP^C protein expression via Western blot analysis. Basal levels of SP1 protein were detected, at the expected apparent molecular size of ~95–105 kDa (53), in mock transfected MNK(Del) and MNK(++) fibroblast cell lines (Fig. 4A, lanes 1 and 5, respectively). Transfected SP1 protein was detected in MNK(Del) (Fig. 4A, lanes 2 and 4) and MNK(++) cell lines (Fig. 4A, lanes 6 and 8). Transfected MTF-1 protein was detected, at the expected apparent molecular size of ~100 kDa (54), in MNK(Del) (Fig. 4A, lanes 3 and 4) and MNK(++) cell lines (Fig. 4A, lanes 7 and 8). PrP^C protein bands were detected between 38 and 17 kDa. Interestingly, transfection of SP1 and/or MTF-1 resulted in an increase in PrP^C protein levels in MNK(Del) cells compared with mock transfected cells (Fig. 4A, compare lane 1 with lanes 2–4). However, PrP^C immunoreactive protein bands were undetectable in MNK(++) cells that were transfected with either SP1 and/or MTF-1 (Fig. 4A, lanes 5–8).

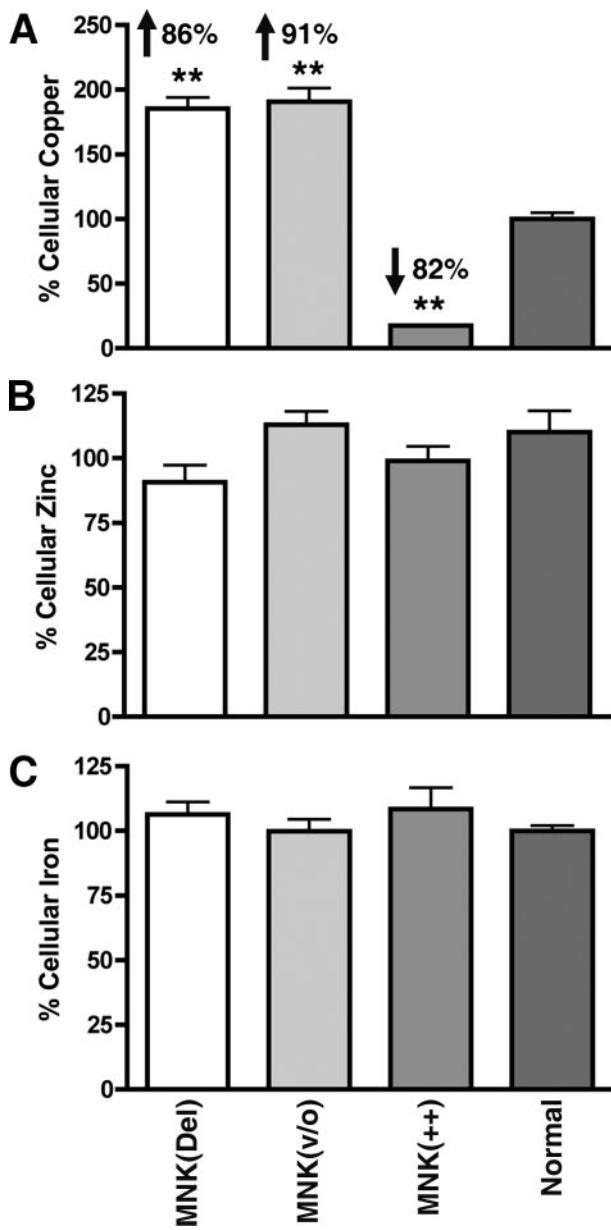


FIGURE 2. Basal metal levels in human MNK deletion and MNK overexpressing fibroblasts. *A*, total cellular copper levels as measured by 24 h of accumulation in MNK(Del), MNK(v/o), MNK(++) and normal human fibroblast cells using both Cu⁶⁴ and atomic absorption analysis. The percentage of change in copper levels from normal fibroblast cells is indicated. *B*, total cellular zinc levels as measured by 24 h of accumulation with atomic absorption analysis. *C*, total cellular iron levels as measured by 24 h of accumulation with atomic absorption analysis. The bars represent the means \pm S.E. of four separate experiments (ANOVA, Bonferroni's post test; **, $p < 0.001$ compared with normal human fibroblasts).

To further explore the role of copper, SP1, and MTF-1 in regulating *PRNP* gene expression, MNK(Del) and MNK(++) cells were transiently transfected with SP1 and MTF-1 in 100 μ M copper-supplemented medium (Fig. 4*B*). In MNK(Del) cells, transfection of SP1 and/or MTF-1 in 100 μ M copper-supplemented medium resulted in an increase in PrP^C levels compared with copper-supplemented mock transfected cells (Fig. 4*B*, compare *lane 1* with *lanes 2–4*). PrP^C remained undetectable in MNK(++) cells when transfected with SP1 and/or MTF-1 in 100 μ M copper-supplemented medium (Fig. 4*B*,

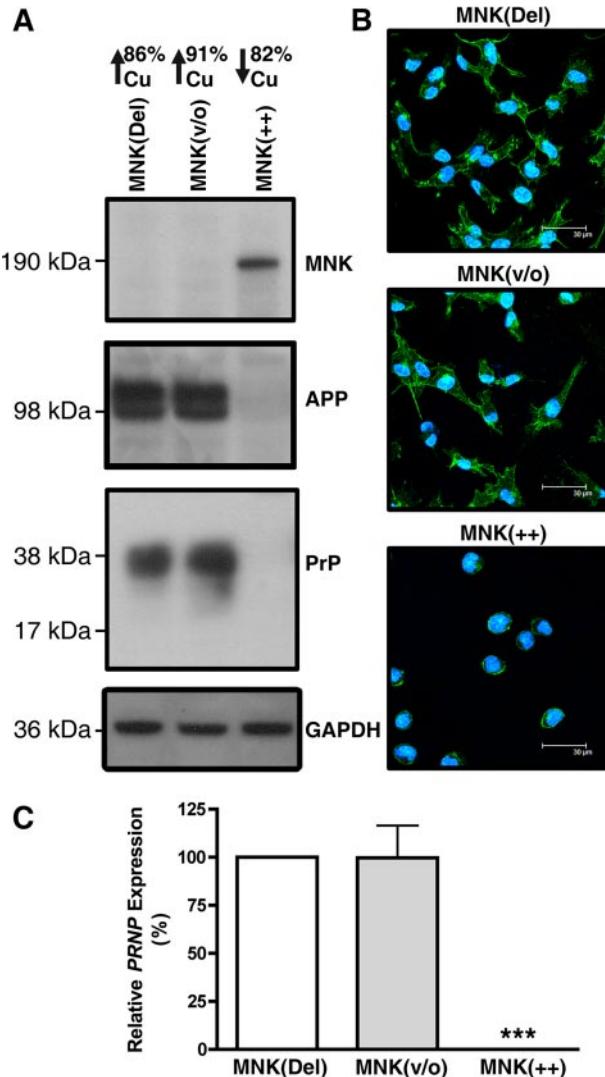


FIGURE 3. Endogenous basal PrP^C protein levels and PRNP levels in human MNK deletion and MNK overexpressing fibroblasts. *A*, Western blot detection of endogenous MNK, APP, and PrP^C protein. 25 μ g of whole cell lysates from MNK(Del), MNK(v/o) control, and MNK(++) human fibroblasts were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to MNK, APP, and PrP. Changes in total cellular copper levels are indicated. Reprobing with GAPDH served as a control for loading. Representative Western blots from four identical experiments are shown. *B*, immunofluorescence expression of endogenous PrP^C protein. Immunostaining was performed with a primary monoclonal antibody (ICM5-18) that detects both mouse and human PrP^C and visualized with Alexa-488™ (green). The cell nucleus was detected by TOPRO-3 staining (blue). The images were taken under identical exposure conditions by confocal microscopy. *C*, quantitative real time RT-PCR detection of endogenous PRNP mRNA levels. 1 μ g of total RNA isolated from MNK(Del), MNK(v/o) control, and MNK(++) human fibroblasts was converted to cDNA and then analyzed by quantitative real time RT-PCR for human PRNP expression. GAPDH and RPLP0 were used as endogenous internal reference genes. The data were analyzed and quantified according to DeltaDelta CT method. The bars represent the means \pm S.E. of four separate experiments. ***, no detectable PRNP mRNA expression in MNK(++) cells.

lanes 5–8). This may result from a lack of available copper in the pool responsible for transcriptional activation of SP1 and/or MTF-1 and could be due to sequestration of copper via MNK in intracellular compartments or vesicles in MNK(++) cell lines (55, 56).

To control for metal specificity, MNK(Del) cells were transiently transfected with SP1 and MTF-1 in 50 μ M zinc- or iron-supplemented medium (Fig. 4*C*). In MNK(Del) cells, transfec-

SP1 and MTF-1 Regulate Prion Gene Expression

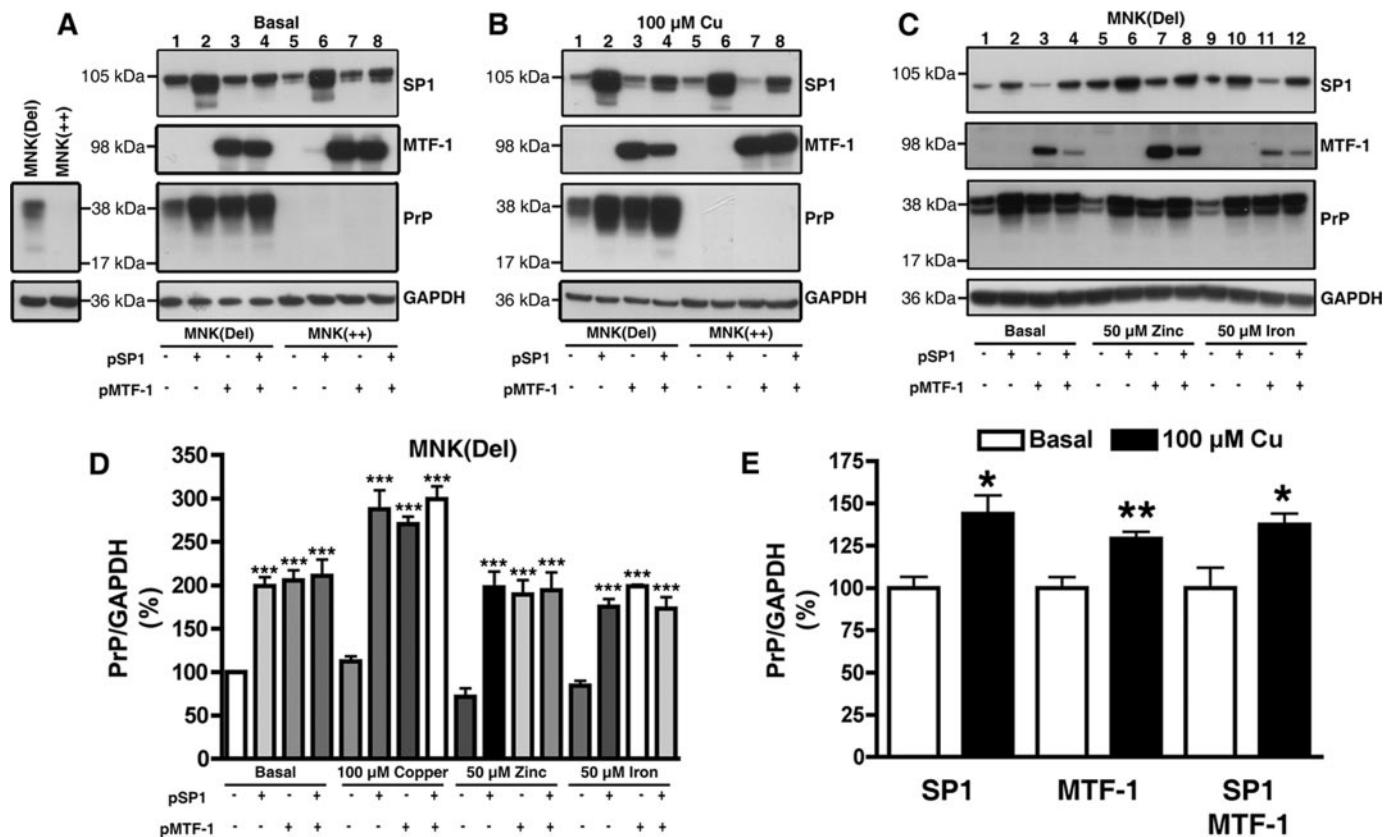


FIGURE 4. **SP1 and MTF-1 increase the PrP^C protein levels in MNK deletion fibroblasts.** *A*, SP1 and/or MTF-1 were transiently transfected into MNK(Del) and MNK(++) fibroblast cells. Whole cell lysates from MNK(Del) (25 μ g) and MNK(++) (50 μ g) cells were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to SP1, MTF-1, and PrP. Reprobing with GAPDH served as a control for loading. Representative Western blots from four identical experiments are shown. For comparison purposes, PrP^C levels from nontransfected MNK(Del) and MNK(++) cells are included in a side panel. *B*, effect of Copper on expression of PrP^C in the presence of SP1 and MTF-1. SP1 and/or MTF-1 were transiently transfected into MNK(Del) and MNK(++) fibroblast cells treated with 100 μ M CuCl₂ for 24 h. Whole cell lysates from MNK(Del) (25 μ g) and MNK(++) (50 μ g) cells were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to SP1, MTF-1, and PrP. Reprobing with GAPDH, served as a control for loading. Representative immunoblots from four identical experiments are shown. *C*, effect of zinc or iron on PrP^C levels in the presence of SP1 and MTF-1. SP1 and/or MTF-1 were transiently transfected into MNK(Del) and MNK(++) fibroblast cells treated with 50 μ M ZnCl₂ or FeCl₃ for 24 h. Whole cell lysates from MNK(Del) (25 μ g) cells were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to SP1, MTF-1, and PrP. Reprobing with GAPDH served as a control for loading. Representative immunoblots from four identical experiments are shown. *D*, quantitation of PrP^C levels in MNK(Del) cells transfected with SP1 and MTF-1 under basal and metal-induced conditions. Densitometry of bands was performed with National Institutes of Health ImageJ software (version 1.38x). PrP^C was normalized to GAPDH levels and expressed as the percentage increase from mock transfected MNK(Del) cells. The bars represent the means \pm S.E. of four separate experiments (ANOVA, Bonferroni's post test; ***, $p < 0.001$ compared with mock transfected MNK(Del)). *E*, normalized expression of Copper induced expression PrP^C in the presence of SP1 and MTF-1. Quantified PrP^C levels transfected under copper induced conditions were normalized against basal transfected conditions for MNK(Del) cells. The bars represent the means \pm S.E. of four separate experiments (*t* test, *, $p < 0.05$ compared with basal SP1; *t* test, **, $p < 0.01$ compared with basal MTF-1; *t* test, *, $p < 0.05$ compared with basal SP1 and MTF-1 transfected).

tion of SP1 and/or MTF-1 in 50 μ M zinc- or iron-supplemented medium resulted in an increase in PrP^C levels compared with zinc- or iron-supplemented mock transfected cells (Fig. 4C, compare lane 5 with lanes 6–8 and compare lane 9 with lanes 10–12, respectively). However, the increase in PrP^C levels from transfection of SP1 and/or MTF-1 in the presence of zinc or iron appeared to be similar to that observed under basal conditions (Fig. 4C, compare lanes 6–8 and lanes 10–12 with lanes 2–4).

Densitometric analysis of MNK(Del) cells under basal conditions showed that transfection of either SP1, MTF-1, or SP1 and MTF-1 resulted in a significant increase, by ~100%, in PrP^C protein levels compared with mock transfected cells (Fig. 4D; ***, $p < 0.001$). Furthermore, densitometric analysis of MNK(Del) cells under 50 μ M zinc- or iron-supplemented conditions showed that transfection of either SP1, MTF-1, or SP1 and MTF-1 significantly increased cellular PrP^C levels by ~90 and

80%, respectively, compared with mock transfected cells (Fig. 4D; ***, $p < 0.001$). However, densitometric analysis of MNK(Del) cells under 100 μ M copper-supplemented conditions showed that transfection of either SP1, MTF-1, or SP1 and MTF-1 significantly increased cellular PrP^C levels by ~185%, when compared with mock transfected cells (Fig. 4D; ***, $p < 0.001$).

Therefore, transfection of SP1 and MTF-1 in the presence of extracellular zinc or iron did not result in any significant increase in PrP^C levels, whereas the addition of extracellular copper resulted in increased PrP^C levels from that observed under basal transfection conditions. Similar results were obtained in normal human fibroblasts transfected under the same conditions (supplemental Fig. S1).

To compare the effect of additional extracellular copper on the ability of SP1 and MTF-1 transcription factors to increase PrP^C protein levels, densitometry data were normalized to cells

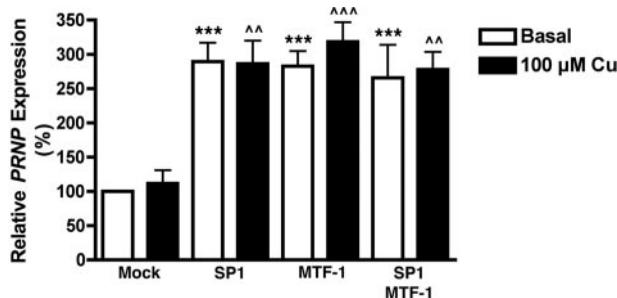


FIGURE 5. Copper induced expression of human *PRNP* in the presence of SP1 and MTF-1. SP1 and/or MTF-1 were transiently transfected in MNK(Del) fibroblast cells untreated (mock) or treated with 100 μM CuCl₂ for 24 h. Quantitative real time RT-PCR analysis was then performed from isolated total RNA for human *PRNP* expression with *GAPDH* and *RPLP0* used as endogenous internal reference genes. The data were analyzed and quantified according to DeltaDelta CT method. The bars represent the means \pm S.E. of four separate experiments (ANOVA, Bonferroni's post test; ***, $p < 0.001$ compared with mock transfected MNK(Del); ^, $p < 0.001$ compared with mock transfected MNK(Del); ^^, $p < 0.01$ compared with mock transfected MNK(Del)).

transfected under basal conditions compared with cells transfected under copper-supplemented conditions (Fig. 4E). Transfection of SP1 in MNK(Del) cells under 100 μM copper-supplemented conditions significantly increased cellular PrP^C levels compared with transfection under basal conditions (Fig. 4E; *, $p < 0.05$). Furthermore, transfection of MTF-1 in MNK(Del) cells under 100 μM copper-supplemented conditions also significantly increased cellular PrP^C levels compared with transfection under basal conditions (Fig. 4E; **, $p < 0.01$). Moreover, co-transfection of SP1 and MTF-1 in MNK(Del) cells under copper-supplemented conditions also significantly increased cellular PrP^C levels compared with transfection under basal conditions (Fig. 4C; *, $p < 0.05$).

SP1 and MTF-1 Increase Human *PRNP* Gene Expression— To determine whether increased cellular prion protein expression is a result of increased transcriptional activation of the human *PRNP* gene expression, we performed quantitative real time RT-PCR analysis. MNK(Del) cells were transiently transfected under basal or 100 μM copper-supplemented conditions with pSP1 and pMTF-1 expression constructs.

Quantitation of human *PRNP* mRNA levels in MNK(Del) cells, under basal and 100 μM copper-supplemented conditions, demonstrated that transfection of SP1 and/or MTF-1 significantly increased *PRNP* expression by ~200% compared with mock transfected cells (Fig. 5; ***, $p < 0.001$; ^, $p < 0.001$; ^^, $p < 0.01$). No significant difference was observed between basal and 100 μM copper-supplemented conditions in mock transfected MNK(Del) cells. Overall, these data provide strong support for the hypothesis that transcription factors SP1 and MTF-1 can modulate expression of the human *PRNP* gene.

SP1 and MTF-1 Knockdown Decreases Human *PRNP* Gene Expression and Cellular Prion Protein Levels— To further support our hypothesis that human *PRNP* gene expression can be modulated by transcription factors SP1 and MTF-1, we performed siRNA knockdown in MNK(Del) cells and determined the relative gene expression levels by quantitative real time RT-PCR. Transfection of two independent SP1 and MTF-1 siRNA sequences resulted in a significant decrease in *SP1* mRNA levels (Fig. 6A; **, $p < 0.01$) and *MTF-1* mRNA levels (Fig. 6B; **, $p < 0.01$). Importantly, *PRNP* mRNA levels were significantly

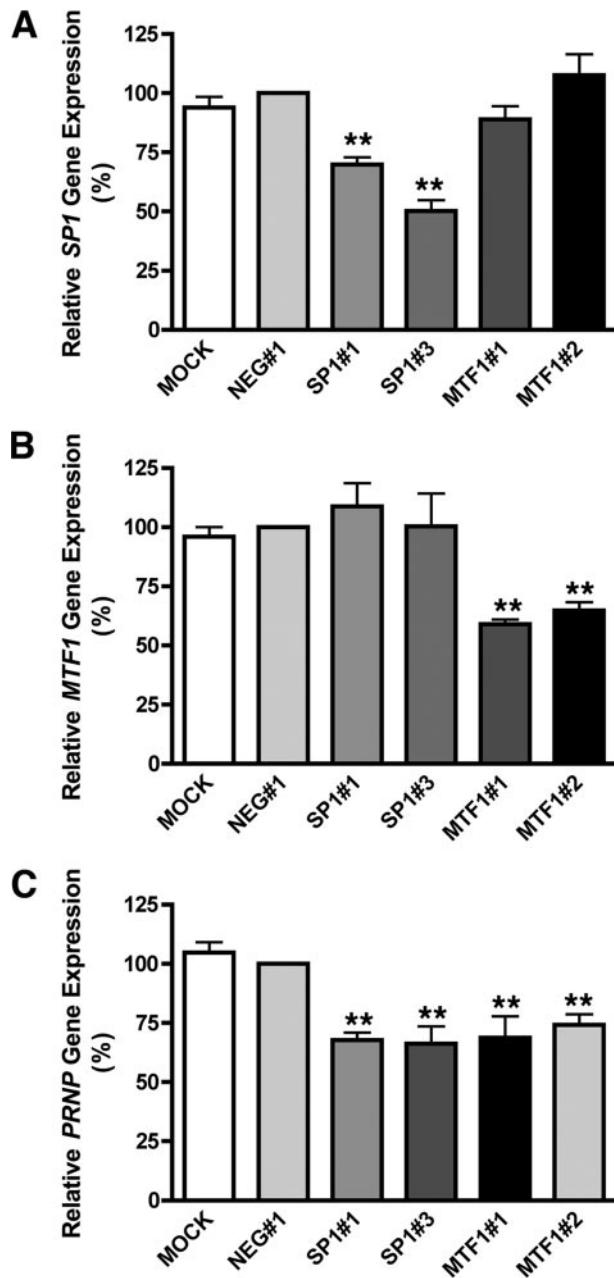


FIGURE 6. Knockdown of SP1 and MTF-1 in MNK(Del) cells decreases *PRNP* mRNA levels. MNK(Del) cells were transfected with either mock, negative control siRNA(NEG#1), SP1 siRNA (SP1#1 and SP1#2), and MTF-1 siRNA (MTF1#1 and MTF1#2) for 48 h. Quantitative real time RT-PCR analysis was then performed from isolated total RNA for human *SP1*, *MTF-1*, and *PRNP* expression with *GAPDH* and *RPLP0* used as endogenous internal reference genes. A, quantitative real time RT-PCR analysis of *SP1* mRNA levels. B, quantitative real time RT-PCR analysis of *MTF-1* mRNA levels. C, quantitative real time RT-PCR analysis of *PRNP* mRNA levels. The data were analyzed and quantified according to the DeltaDelta CT method. The bars represent the means \pm S.E. of four separate experiments (ANOVA, Bonferroni's post test; **, $p < 0.01$ compared with negative siRNA transfected MNK(Del)).

decreased as a result of *SP1* and *MTF-1* knockdown (Fig. 6C; **, $p < 0.01$). To confirm that knockdown of SP1 and MTF-1 reduced PrP^C protein levels, siRNA knockdown cell lysates were analyzed for PrP^C protein expression via Western blot analysis (Fig. 7A). Densitometric analysis of MNK(Del) cells knocked down with two independent SP1 and MTF-1 siRNAs resulted in a significant decrease in PrP^C protein levels, com-

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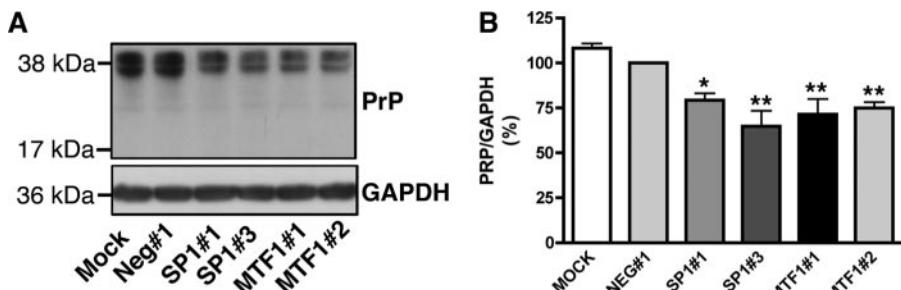


FIGURE 7. Knockdown of SP1 and MTF-1 in MNK(Del) cells decreases cellular PrP protein levels. MNK(Del) cells were transfected with either mock, negative control siRNA(NEG#1), SP1 siRNA (SP1#1 and SP1#2), and MTF-1 siRNA (MTF1#1 and MTF1#2) for 48 h. A, 25 μ g whole cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to PrP. Reprobing with GAPDH, served as a control for loading. Representative immunoblots from four identical experiments are shown. B, quantitation of PrP^C levels in MNK(Del) cells transfected with SP1 and MTF-1 siRNA. Densitometry of bands was performed with National Institutes of Health ImageJ software (version 1.38x), and PrP^C was normalized to GAPDH levels and expressed as a percentage compared with negative siRNA transfected MNK(Del) cells. The bars represent the means \pm S.E. of four separate experiments (ANOVA, Bonferroni's post test; *, $p < 0.05$; **, $p < 0.01$ compared with negative siRNA transfected MNK(Del)).

pared with negative control siRNA cells (Fig. 7B; *, $p < 0.05$; **, $p < 0.01$). Overall, these data provide strong support for copper regulation of human *PRNP* gene expression being mediated by the transcription factors SP1 and MTF-1.

DISCUSSION

An important cellular function of the prion protein has been suggested to be the maintenance of copper homeostasis (11, 13, 22, 23). A recent study has also demonstrated that copper also regulates expression of the rat prion gene via putative MRE DNA sequences (26). However, the transcription factors involved in this copper regulation remained to be determined.

MTF-1 is the most characterized metal-dependent mammalian transcription factor. MTF-1 is activated by a variety of stimuli, including copper and zinc, and binds to MREs to regulate the expression of copper detoxification metallothioneins (31–34). SP1, a general transcriptional activator, can also bind to MREs to regulate the expression of metallothioneins (39). Because bioinformatics analysis of the human *PRNP* gene promoter revealed several putative MTF-1 MREs, as well as a number of predicted SP1-binding sites (Fig. 1), we hypothesized that transcription factors SP1 and MTF-1 play a significant role in copper regulation of the *PRNP* gene.

To investigate the role of copper and the transcription factors SP1 and MTF-1 in modulating human *PRNP* gene regulation, we utilized a system where intracellular copper levels are genetically manipulated through altered expression of the MNK copper efflux protein. Cells lacking a functional MNK protein show high intracellular copper levels because of reduced copper efflux (43). Restoration of MNK function by stable transfection in a MNK deletion background restores copper efflux ability, resulting in dramatically decreased intracellular copper levels and no change in other transition metals such as zinc and iron (Fig. 2).

Therefore, MNK(Del) and MNK(++) cells have contrasting high and low intracellular copper levels, respectively (40, 43–45). Here we report for the first time evidence that copper regulation of the human *PRNP* gene is mediated by the transcription factors SP1 and MTF-1. This demonstrates a previously uncharacterized aspect of regulation of the human *PRNP*

gene and further supports a role for the cellular prion protein in copper homeostasis.

Investigation of PrP^C protein levels in MNK(Del) and MNK(++) cell lines demonstrated that PrP^C is not detectable by both Western blot and immunofluorescence analysis with PrP specific antibodies in the low copper MNK(++) cell line (Fig. 3). These data are consistent with the hypothesis that copper levels regulate the expression of the human *PRNP* gene and also support the findings that elevated copper regulates *PRNP* gene expression (26, 57).

To evaluate the role of transcription factors SP1 and MTF-1, in cop-

per regulation of *PRNP* gene expression, we transfected SP1 and MTF-1 into MNK(Del) and MNK(++) cell lines. In low copper MNK(++) cells transfection of SP1 and/or MTF-1 did not have any effect on restoring cellular prion protein levels under basal or copper-supplemented conditions (Fig. 3, A and B). This may be due to a lack of available copper in the pool required for activating SP1 and MTF-1 or suppression of *PRNP* gene by an unknown mechanism that overrides the activities of SP1 and MTF-1. Moreover, it is possible that other co-factors required for transcriptional activation of the *PRNP* gene are either absent or down-regulated. This is supported by proteomic antibody array analysis, which identified a significant protein expression differential between high copper MNK(Del) and low copper MNK(++) cells (44).

Under basal and copper-, zinc-, and iron-supplemented medium conditions, transfection of SP1 and/or MTF-1 resulted in elevated PrP^C protein levels in MNK(Del) cells (Fig. 4, A–C). PrP^C protein levels were increased by ~100% under basal, zinc, and iron conditions and ~200% under copper-supplemented conditions in MNK(Del) cells (Fig. 4D). Furthermore, transfection of SP1 and MTF-1 under copper-supplemented conditions, either alone or co-transfected, resulted in an additional ~30–50% significant increase in cellular PrP^C levels compared with transfections performed under basal conditions (Fig. 4E). Together, these results strongly suggest that SP1 and MTF-1 expression increases cellular prion protein levels and that copper is required as a specific co-factor.

To confirm that increased cellular prion protein, as a result of expression of SP1 and/or MTF-1 transcription factors, is due to transcriptional activation of human *PRNP* gene expression, we performed quantitative real time RT-PCR analysis (Fig. 5). Quantitation of human *PRNP* mRNA levels in MNK(Del) cells, under basal or copper-supplemented conditions, demonstrated that SP1 and/or MTF-1 significantly increased *PRNP* gene expression by ~200% (Fig. 5). Additionally, siRNA knockdown of SP1 and MTF-1 transcription factors significantly reduced *PRNP* gene expression levels (Fig. 6), resulting in a concomitant reduction in cellular prion protein levels (Fig. 7). Although we were unable to achieve greater than 50% knockdown of transcription factors SP1 and MTF-1 because of cell toxicity when

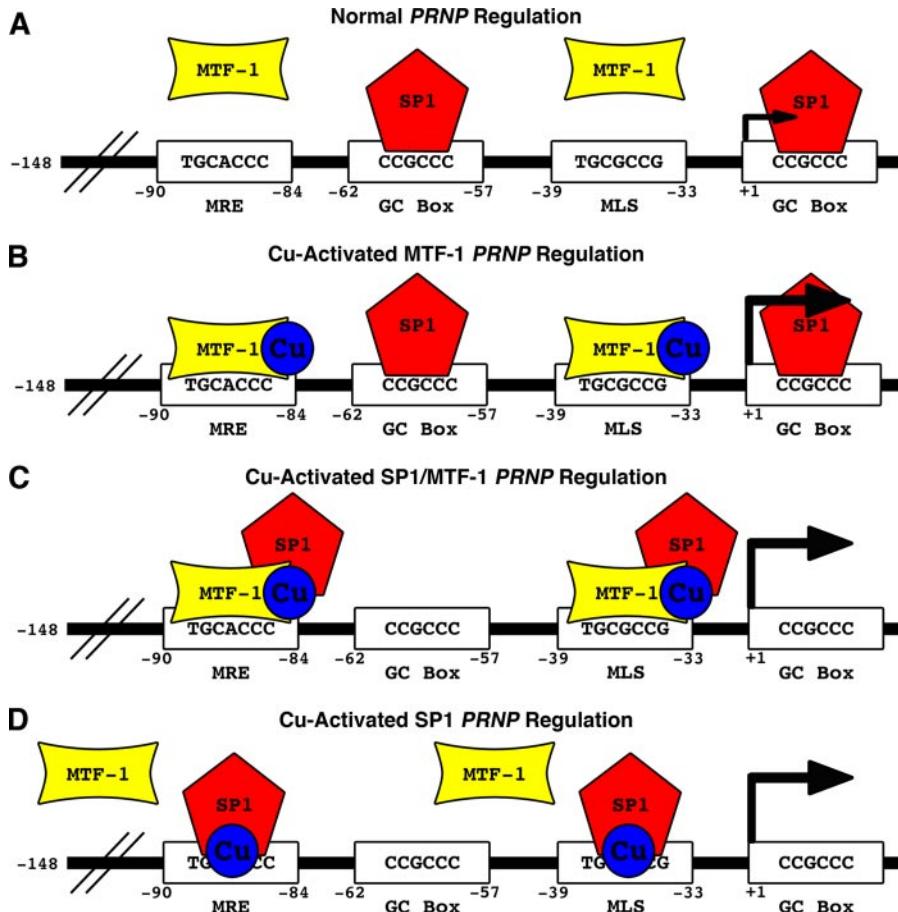


FIGURE 8. Model on copper regulation of human *PRNP* gene expression by SP1 and MTF-1 transcription factors. *A*, under normal conditions, the active promoter region -148 to $+125$ of *PRNP* is activated by SP1 binding to GC box sites. *B–D*, under copper-replete conditions, copper-activated expression of *PRNP* may occur independently or as a co-activator complex. *B*, copper-activated expression of *PRNP* may occur via MTF-1 binding to putative metal response element (MRE) and MLS located in the active promoter region. *C*, copper-activated expression of *PRNP* may occur via SP1/MTF-1 co-activator complexes binding to putative metal response element (MRE) and MLS located in the active promoter region. *D*, copper-activated expression of *PRNP* may occur via SP1 acting as a copper sensing transcription factor and binding to putative metal response element (MRE) and MLS located in the active promoter region, possibly in competition with MTF-1 for weakly activated MRE sequences.

the amount of siRNA transfected was increased above 30 nm, this is consistent with SP1 and MTF-1 being vital for cellular development with SP1 and MTF-1 null mice being embryonic lethal (58, 59). Together, these data provide strong support for the mechanism that increased cellular PrP^C levels are the result of transcriptional activation of the human *PRNP* gene by SP1 and MTF-1.

Overall, the regulation of *PRNP* gene expression described in the current studies suggests that SP1 and MTF-1 function as copper transcriptional activators to regulate *PRNP* expression. We therefore propose that copper regulation of human *PRNP* gene expression occurs by either copper-activated SP1 or MTF-1 binding to putative MREs located in the human gene promoter. This may occur independently or as a co-activator complex (Fig. 8).

This model is strongly supported by two recent studies; first, MTF-1 and SP1 form a co-activator complex to regulate metallothionein gene expression (60), and second, SP1 is a copper-sensing transcription factor responsible for maintaining cellular copper homeostasis by regulating expression of the copper uptake gene *hCTR1* (61). Furthermore, the active promoter

region of human *PRNP*, -148 to $+125$ relative to the $+1$ transcriptional start site, contains tandem MTF-1 MRE- and SP1-binding sites in the human *PRNP* promoter (Fig. 1). Although it is well established that MTF-1 binds to putative MRE sequences to activate gene expression (31–34), SP1 can also bind to weakly activated putative MREs in close proximity to the SP1-binding regions, possibly in competition with MTF-1, to regulate gene expression (39). Based upon sequence homology, this putative MTF-1 MRE, located -90 to -83 in the human *PRNP* promoter, is predicted to be weakly activated by MTF-1 (48).

Therefore, it is plausible that SP1 and MTF-1 compete for the weakly activated MTF-1 MRE located -90 to -83 in the human *PRNP* promoter to modulate copper regulation of human *PRNP* (Fig. 8). Further studies will be aimed at determining the role of SP1 and MTF-1 in activating this promoter region under a variety of copper conditions in both neuronal and non-neuronal cell lines.

These results may also have a significant impact on studies investigating the regulation of the APP. APP and the cellular prion protein are hypothesized to be involved in neuronal copper homeostasis (62).

APP and PrP possess domains, and interactions with metal ions such as copper have important roles in the biology of both proteins. A β -copper interactions have been shown to mediate amyloid plaque formation (63, 64). PrP has been shown to coordinate copper; it has been reported that this interaction modulates PrP aggregation and conversion from PrP^C to PrP^{Sc} (65). APP gene expression is also regulated in a copper-dependent manner (40, 57), with the promoter region of human *APP* containing a number of putative MRE-binding sites that may be regulated by SP1 or MTF-1 (40). Two key proteins, β -amyloid cleaving enzyme (BACE) and Tau, implicated in the pathology of Alzheimer disease are also regulated by SP1 (66, 67). BACE is a key enzyme responsible for cleavage of APP into β -amyloid (68), whereas Tau is a microtubule associated protein that forms the major component of neurofibrillary tangles in Alzheimer disease (69, 70). Because SP1 can act as a copper-sensing transcription factor (61), SP1 may be a global neuronal regulator of proteins involved in mammalian copper homeostasis.

In summary, our results strongly suggest that at least in human fibroblasts, SP1 and MTF-1 are co-factors in copper-regulated

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PRNP gene regulation. The data also further support a role for PrP^C in copper efflux/detoxification. The elucidation of the copper regulation mechanisms of *PRNP* in human neuronal cells lines and prion susceptible cell lines may provide new targets in developing therapeutic strategies in the treatment of prion diseases. These strategies would be designed to reduce the expression of the *PRNP* gene and the ensuing conversion of the cellular prion protein into the abnormal pathogenic PrP^{Sc}.

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