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Transcriptional and Posttranscriptional Regulation of the *CTNS* Gene

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ABSTRACT: Cell cysteine (Cys) levels and/or the [Cys/CySS] redox potential have been shown to regulate mRNA levels of the *CTNS* gene, which encodes for a lysosomal cystine (CySS) carrier that is defective in cystinosis. To investigate the mechanisms involved *CTNS* mRNA regulation, different portions of the *CTNS* promotor were cloned into a luciferase vector and transfected in HK2 cells. A 1.5–2.4-fold increase in luciferase activity was observed when cells were incubated in culture medium containing low CySS concentrations. Conversely, *CTNS* mRNA levels decreased by 47–56% in the presence of *N*-acetyl-L-cysteine (NAC). Chase experiments with actinomycin D (ActD) demonstrated a 3-fold stabilization of the CTNS mRNA when cells were cultured in low CySS medium for 48 h. Treatment of control cells with cyclohexamide (CHX) increased *CTNS* mRNA levels, suggesting that CHX blocked the synthesis of proteins involved in mRNA degradation or in repression of the *CTNS* gene. Finally, *in vitro* binding assays showed increased binding (30–110%) of the Sp-1 transcription factor to two regions of the *CTNS* promotor when cells were incubated in low CySS medium. These results indicate that the *CTNS* gene is actively regulated at the transcriptional and posttranscriptional levels and suggest that CTNS plays a pivotal role in regulating cell thiol concentrations. **(*Pediatr Res* 70: 130–135, 2011)**

Cystinosis is a rare autosomal recessive disease caused by mutations in *CTNS* gene, which encodes for cystinosin (1). In its most severe form, termed nephropathic cystinosis (NC), the disease is characterized by early onset renal Fanconi syndrome leading to renal failure and by corneal cystine (CySS) crystal depositions. Thereafter, other symptoms develop as CySS accumulates in tissues (1) (2).

The *CTNS* gene encodes for a CySS carrier that is primarily localized in the lysosomal membrane (3). A second isoform, termed cystinosin-LKG, originates from a differential splicing of the last exon and has been shown to encode for a protein that is targeted also to other cell compartments (4). Mutations in the *CTNS* gene are detected in the majority of patients with NC (1,5). In addition, mutations in the *CTNS* promotor have been found in three patients (6). In other cell models, cell concentration of amino acids have been shown to modulate gene activity and expression (7,8). For example, amino acid deprivation stimulates the expression of transporters such as the system A subtype, neutral amino acid transporter A2, CD98 light chain, cationic amino acid transporter-1, or the cystine/glutamate transporter (9–12). Transcriptional regulation of these genes is often mediated by specific sequences, termed amino acid responsive elements (AAREs), that are located in the promotor regions and, occasionally, in the first intronic regions (13).

Recently, we have reported that *CTNS* gene expression is regulated by intracellular thiols in HK2 human kidney cells. CySS and cysteine (Cys) depletion for 48 h caused a 2-folds increase in *CTNS* mRNA that paralleled with an increase in the expression of cystinosin (14). Changes in *CTNS* transcript levels were associated with Cys concentrations and with the redox status of the [Cys/CySS] couple. These effects were partially reverted by treatment with *N*-acetyl-L-cysteine (NAC) or with glutathione ethyl ester (14). The aim of this study was to further elucidate the mechanisms that regulate *CTNS* gene expression.

METHODS

Cell culture. Human kidney tubular cells (HK2, ATTCC CRL-2190) were cultured in 95% medium supplemented with 5% FCS (Invitrogen Life Technologies, Milan, IT), ITS Liquid Media Supplement (Sigma Chemical Co.-Aldrich, Milan, IT), and penicillin/streptomycin (Euro Clone, Pavia, IT). Media used in the experiments included regular DMEM containing 200 μM of CySS and a "low CySS DMEM" containing 20 μM CySS. This later medium was prepared by mixing in a 9:1 ratio DMEM without CySS, Cys, and methionine (Sigma Chemical Co.-Aldrich) and regular DMEM.

Construction of promotor fragments. Overlapping segments of the CTNS promotor (6) were amplified by PCR from human genomic DNA using pairs of primers listed in Table 1 and cloned in the PGL4.17 Firefly Luciferase reporter vector (Promega, Milan, IT). The Homo Sapiens clone RP11-48B14 (GenBank: AC132942.16) was used as reference with the initiation of transcription site located at position 1495 (Fig. 1).

Most amplicons encompass sequences located in the 5′ UTR region. In addition, a 4862-bp fragment, spanning the entire promotor through intron 2 was cloned. This fragment contains a consensus AARE sequence near the 3′ end at position +3329 bp (Fig. 1). A second construct containing the promotor region and the last 400 bp of intron 2 was also engineered. PCRs and insert cloning were performed using standard molecular biology protocols.

Transfections and luciferase detection. HK2 cells were cultured in DMEM, low CySS DMEM, or in DMEM containing 10 mM NAC for 3 d and starved in serum-free medium for 24 h before the experiments. Cells were then transiently cotransfected at 80–90% confluence with the above described

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Abbreviations: AARE, amino acid responsive element; **Act D,** actinomycin D; **CHX,** cyclohexamide; **Cys,** cysteine; **CySS,** cystine; E_h**,** redox potential; **GSH,** reduced glutathione; **GSSG,** oxidized glutathione; **NAC,** *N*-acetyl-L-cysteine

Table 1. *Primer and oligonucleotide sequences*

PGL4 construct	Forward primer	Backward primer	bp
[−769/−1]	gaccggtaccagacttcattgcgggaagggc	aacgctcgagggtcaggtgacagcggacc	769
[−348/−1]	gaccggtaccaggcgagcttccacgcaacct	aacgctcgagggtcaggtgacagcggacc	348
[−316/−1]	gaccggtacccggctataggcggagaggcg	aacgctcgagggtcaggtgacagcggacc	316
[−283/−1]	gaccggtaccctaaaggggccccgccccac	aacgctcgagggtcaggtgacagcggacc	283
[−81/−1]	gaccggtaccgcggaaactacaactcccag	aacgctcgagggtcaggtgacagcggacc	81
[−777/−82]	gactggtaccatagagaacttcattgcgg	atatctcgagcgtcttagacggacagag	696
[−1158/−1]	gaccggtacctctgtggaatgagccaggag	aacgctcgagggtcaggtgacagcggacc	1158
[−1158/+3705]	gaccggtacctctgtggaatgagccaggag	gatcctcgagtctcgatttctcagaactagg	4862
	Oligonucleotide sequences for Sp-1 binding assays		Position
(+) Strand	aggcggagaggcggcggaaggcggggttacataacttacggtaaatggccc		[−308/−284]
(−) Strand	gggccatttaccgtaagttatgtaaccccgccttccgccgcctctccgcct		[−284/−308]
(+) Strand	gcccaggggggcggggcaggggcggggagtcgccgttacataacttacggtaaatggccc		[−440/−407]
(−) Strand	gggccatttaccgtaagttatgtaacggcgactccccgcccctgccccgccccccctgggc		[−407/−440]
(+) Strand	aggaggctcctggaggcggcgcggccccgggttacataacttacggtaaatggccc		[−712/−683]
(−) Strand	gggccatttaccgtaagttatgtaacccggggccgcgccgcctccaggagcctcct		[−683/−712]

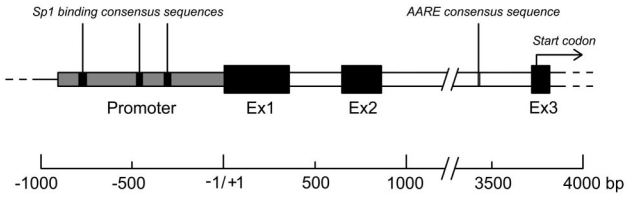

Figure 1. Schematic representation of the initial part of the *CTNS* gene. The promoter region is shown in *gray*, introns are shown in *white*, and exons (Ex) are shown in *black*. The sequences of the promoter corresponding to the oligonucleotides used for Sp-1 binding assays are highlighted in *black* −308/−284, −440/−407, and −712/−683; the −440/−407 oligonucleotide contains two Sp-1 consensus sequences. The putative (5′-TGATGCAAA-3′) AARE in the intron 2 at position +3329 is also highlighted in *black*.

reporter vectors and with the Renilla-Luciferase vector [pGL4.74(hRluc/TK), Promega], which served as control to assess transfection efficiency using Lipofectamine 2000 (Invitrogen Life Technologies). After 24 h, cells were seeded in 96-well culture plates (Nunc, Rochester, NY) at a density of 1.9×10^4 cells per well. At 48 h, luciferase activity was assayed using the Dual GloTM Luciferase Assay System (Promega) according to the manufacturer instructions on a Wallac Victor (2) 1420 Multilabel Counter (Perkin Elmer, Life Sciences, Shelton, CT). Promotor activities were normalized by dividing the Firefly luciferase luminescence with the Renilla luminescence. All experiments were performed at least four times in duplicate.

CTNS mRNA stability analysis and cyclohexamide chase assay. The half-life of *CTNS* mRNA was estimated using the transcription inhibitor actinomycin D (Act D; Sigma Chemical Co.-Aldrich) at a 5 μg /mL concentration. The effects of protein synthesis inhibition were assessed by measuring mRNA levels in media containing 5 μg/mL of the translation inhibitor cyclohexamide (CHX; Sigma Chemical Co.-Aldrich).

Total RNA was isolated with TRIzol Reagent (Invitrogen Life Technologies). Oligo-p$(dT)_{15}$ primed cDNA strings were obtained with the First-strand cDNA synthesis kit for RT-PCR (Roche Applied Science, Indianapolis, IN). *CTNS* transcripts were amplified by PCR using the following pair of primers: 5′-ccctgaagctcgtagagaaatg-3′ (forward) and 5′-ggaggaacatctgcaggagg-3′ (backward). Glyceraldehyde 3-phosphate dehydrogenase was used as a control housekeeping gene and was amplified using the following pair of primers: 5′-ctgcaccaccaactgcttag-3′ (forward); 5′-aggtccaccactgacacgtt-3′ (backward). PCR amplicons were resolved in a 1.2%-agarose gel and measured by densitometry with the Typhoon 8600 Variable Mode Imager (Amersham Pharmacia Biothec, Pittsburgh, PA) and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Sp1 binding assay. Nuclear fractions were isolated and processed using the Nuclear Extract and Sp-1 TransAM kits (Active Motif, Carlsbad, CA) that allows detection of Sp-1 binding by ELISA. Briefly, multiwell plates were coated with 10 μM of custom designed biotinylated double-strand oligonucleotides, spanning the *CTNS* promotor regions −308/−284, −440/−407, or −712/−683 (Table 1, Fig. 1). These regions contain consensus sequences for the SP1 transcription factor at positions −299/−290, −437/−429, −426/−418, and −723/−715. Nuclear extracts were added to coated wells and were incubated for 1 h at room temperature. Primary anti-Sp1 antibodies and secondary anti-rabbit-HRP-conjugated antibodies were then applied in sequence for 1 h at room temperature. Sp-1 binding was measured by colorimetry with the Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories) at 450 nm, using a 655-nm reference wavelength. Binding specificity was assayed by competition experiments, where 20 μM of unlabeled double-strand oligonucleotides were added to coated wells.

Measurement of intracellular thiol levels and calculation of redox potentials. Intracellular thiols were measured by HPLC as previously reported (15). Theoretical redox potentials (16) of the GSH/GSSG and Cys/CySS couples were calculated using the Nernst equation, $E_h = E_0 + RT/nF \ln([\text{disulfide}]/[\text{thiol}]^2)$, where E_0 is the standard potential for the redox couple (−264 mV for GSSG/GSH^2), −250 mV for CySS/Cys^2), R is the gas constant, T is the absolute temperature, n is the number of transferred electrons (*e.g.* 2), and F is Faraday's constant (16). Calculations were made assuming a dilution volume for Cys/CySS and GSH/GSSG of 5 μL/mg of protein.

Statistical analysis. Statistical analyses were performed using SPSS for windows 11.0 software (SPSS Inc, Chicago, IL). All continuous data were compared with the Mann-Whitney U test. All tests were two sided and considered significant for p values <0.05.

RESULTS

CTNS mRNA changes in different cell culture conditions. As stated above, we have previously reported that CySS deprivation increases *CTNS* mRNA levels. Complete CySS deprivation, however, decreases substantially protein synthesis, preventing accurate measurements of luciferase activity. We have therefore conducted preliminary experiments using various concentrations of CySS in the culture medium. This allowed defining a minimum concentration of 15–20 μmol/L of CySS that induced maximal luciferase activity at 48 h. At lower concentrations, cell growth and luciferase activity decreased substantially (data not shown). On these bases, experiments in "low CySS DMEM" were carried at 20 μmol/L of CySS (*e.g.* 10 times less than regular DMEM). Under these conditions, cell growth was slowed by a mean of 34 ± 4% after 48 h ($n = 4$), when cells were plated at 20–30% confluency (data not shown). Therefore, cells cultured in low CySS medium were plated at higher density to reach a similar confluence to cell cultured in regular DMEM when luciferase activity was assessed.

CTNS mRNA levels, cell Cys, CySS, GSH, GSSG, and E_h for the [Cys/CySS] and [GSH/GSSG] redox couples under different culture conditions are reported in Table 2. As shown, *CTNS* mRNA increased by 2.3-folds in cell chronically cul-

Table 2. *Changes in cell thiol levels and redox potentials in different experimental culture conditions*

Variables	Units	DMEM	Low CySS DMEM	*p*	DMEM + NAC	*p*
CTNS mRNA	Relative values	1.00 ± 0.19	2.26 ± 0.45	<0.01	0.58 ± 0.25	<0.05
Cys	nmol/mg protein	18.8 ± 1.2	7.2 ± 0.5	<0.0001	76.3 ± 3.0	<0.0001
CySS	nmol/mg protein	0.4 ± 0.2	0.4 ± 0.1	NS	2.1 ± 0.4	<0.0001
E_h [Cys/CySS]	mV	−189 ± 35	−137 ± 16	<0.05	−232 ± 25	<0.05
GSH	nmol/mg protein	195.3 ± 12.1	80.5 ± 5.0	<0.0001	578.2 ± 48.2	<0.0001
GSSG	nmol/mg protein	12.1 ± 0.3	12.4 ± 0.3	NS	17.0 ± 1.1	<0.01
E_h [GSH/GSSG]	mV	−250 ± 17	−196 ± 12	<0.01	−305 ± 23	<0.01

tured in low CySS medium. In parallel, the concentration of reduced thiols decreased significantly, whereas CySS and GSSG levels remained stable; this resulted in decreased E_h for both redox couples.

Approximately the opposite effect was obtained after culturing cells in medium containing NAC. As indicated in Table 2, *CTNS* mRNA decreased by 42%. Lower CTNS mRNA levels were associated with a significant increase in all measured thiol concentrations and E_h of both redox couples.

CySS deprivation increased CTNS promotor activity. To investigate the mechanisms underlying the regulation of *CTNS* mRNA levels, the promotor activity was assayed using luciferase as a reporter gene. Five overlapping fragments of the *CTNS* promotor (starting from bp −1) were cloned into the reporter vectors (Table 1, Fig. 2*A*). As shown in Fig. 2*A*, the luciferase activity increased 1.5 to 2.4-folds in all tested promotor segments in low CySS medium. Specifically, the luciferase activity driven by the longer promoter fragment (769 bp) increased from 331 ± 68 to 814 ± 88 Relative Luminescence Units (RLU; $p < 0.001$), whereas cells transfected with the shorter 81 bp fragment responded to low CySS concentration by an increase in luciferase activity from 117 ± 9 to 215 ± 18 RLU ($p < 0.001$, Fig. 2*A*). Longer *CTNS* fragments (769 bp, 348 bp, and 316 bp) containing the first 316 bp (3′ to 5′) of the promotor induced higher luciferase activity than shorter fragments (283 bp, 81 bp), indicating that the region located between bp −283 and bp −316 is critical to enhance promotor activity. All five tested promotor segments responded similarly to CySS depletion, raising the hypothesis that the first 81 bp may contain a thiol-sensitive region. To test this hypothesis, a sixth construct encompassing *CTNS* sequences located between bp −81 and bp −769 was generated. As shown in Fig. 2*A*, the luciferase activity driven by this latter plasmid was similar to the activity driven by the full-length promotor (−1/−769 bp).

AARE sequences are located in promotor or intronic regions of genes encoding for amino acid carriers and have been shown to regulate gene transcription in response to changes in amino acid concentrations. By electronic screening, we have identified a putative AARE sequence (5′-TGATGCAAA-3′) in intron 2, near exon 3 (Fig. 1). To test whether this sequence is involved in the regulation of *CTNS* gene transcription, a construct spanning the promotor region to the end of intron 2 was engineered (Fig. 2*B*). A second construct containing the promotor region and the last 400 bp of intron 2 was also engineered to test the effects of the putative AARE sequence when inserted closer to the promotor (Fig. 2*B*). The first construct elicited very low luciferase activity; the luminescence elicited by the second construct was higher but significantly lower than the luminescence elicited by plasmids containing only the full-length promotor (Fig. 2*B*). The AARE sequence added no noticeable sensitivity to changes in CySS concentration of the culture medium.

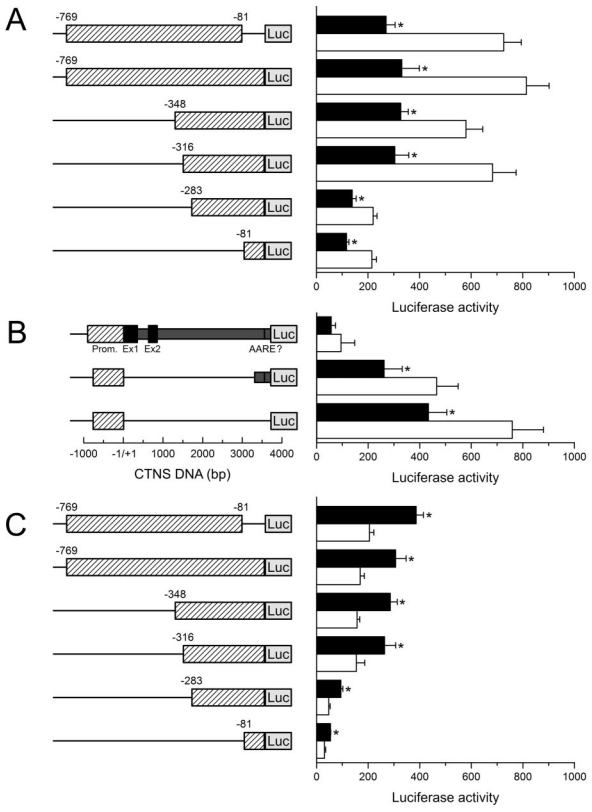

Figure 2. Luciferase activities of *CTNS* promotor constructs. Promotor constructs are represented on the *left*. *Hashed bars* indicate the promotor regions that are cloned into each construct. *Gray bars* indicate the luciferase gene (LUC). DNA coordinates are indicated in numbers and in the *x* axis (*B*). (*A* and *B*) Luciferase activity in control cells and cells cultured in low CySS medium (20 μmol/L). (*C*) Luciferase activity in control cells and cells cultured in medium containing 10 mmol/L of NAC. ■: DMEM; □: "low CySS DMEM" in *A* and *B* end "DMEM + NAC" in *C*; LUC = luciferase. *$p < 0.001$.

To confirm the *CTNS* promotor sensitivity to cell thiols, HK2 cells were cultured in medium containing 10 mM NAC. These results are reported in Fig. 2*C*. As shown, addition of NAC to the culture medium caused a 47–56% decrease in luciferase activity. The degree of inhibition of luminescence was comparable, in relative values, for all tested constructs. In absolute values, differences between constructs paralleled those observed in low CySS medium.

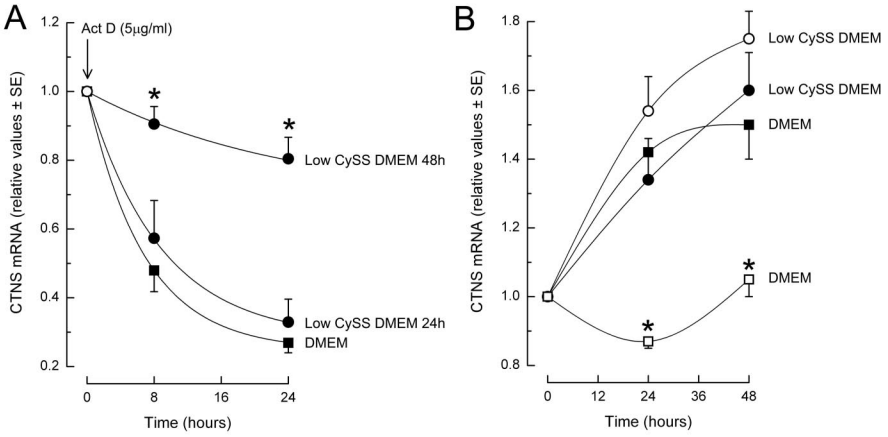

Figure 3. *CTNS* mRNA levels after actinomycin and CHX treatment. (*A*) *CTNS* mRNA was measured after incubating cells in the presence of Act D. Cells were maintained in regular DMEM (*squares*) or were switched to low CySS medium for 24 h or 48 h (*circles*). mRNA was measured by RT-PCR and normalized for GADPH expression. (*B*) *CTNS* mRNA was measured in the presence (*filled labels*) or absence (*empty labels*) of CHX. mRNA was measured as in *A*. $*p < 0.001$.

CySS deprivation increased CTNS mRNA stability. The above data indicate that changes in *CTNS* mRNA levels related to CySS concentrations in the culture media are, at least in part, related to transcriptional regulation of the *CTNS* gene. To test whether posttranscriptional elements are also involved, cells were treated with ActD and with CHX.

As shown in Fig. 3*A*, *CTNS* mRNA levels decreased substantially from 1 ± 0.31 to 0.27 ± 0.27 (relative values) in 24 h, after blocking transcription in cells cultured in regular DMEM. mRNA decay was similar (from 1 ± 0.42 to 0.33 ± 0.12 in 24 h) after switching cells to a low CySS medium for 24 h (+ duration of the chase). Conversely, if cells were switched to low CySS medium for 48 h before inhibiting transcription, CTNS mRNA levels decrease significantly less rapidly (from 1 ± 0.27 to 0.80 ± 0.19, Fig. 3*A*), indicating a substantial stabilization of the mRNA.

Translation inhibition experiments were performed after switching cells to low CySS medium in the presence or in the absence of CHX. As shown in Fig. 3*B*, in the absence of CHX (*empty labels*), mRNA levels remained stable in cells that were maintained in regular DMEM and increase after 48 h by 1.75 ± 0.20-folds ($p < 0.001$) when cell were switched to low CySS medium, as expected. In the presence of CHX, mRNA levels increased in a similar way in the latter group of cells. However, CTNS mRNA increased to levels comparable with low CySS medium in cells that were maintained in regular DMEM in the presence of CHX.

Taken together, these results indicate that CTNS mRNA degradation is an active process and raise the hypothesis that CHX blocks the synthesis of a protein or a group of proteins that are involved in this process.

CySS deprivation modifies Sp-1 binding to the CTNS promotor. The Sp-1 transcription factor has been shown to bind specifically to the *CTNS* promotor and mutations involving consensus sequences for Sp1 have been shown to cause cystinosis (6). We have tested the effects of partial CySS deprivation on Sp-1 binding in three regions of the CTNS promotor that contain four consensus sequences for Sp-1. Nuclear fractions were collected from HK2 cells that were cultured for 5 d in regular DMEM or in low CySS DMEM and were assayed as described in the method section.

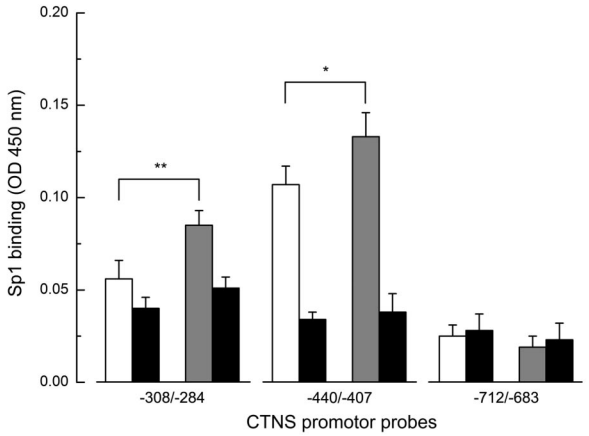

Figure 4. Sp-1 binding assay. Sp1 binding activities of three different *CTNS* promotor regions (positions −316/−268, −450/−400, and −738/−680 from the translation start site). Binding of Sp-1 was assayed by ELISA (see text for details). □: DMEM; ■: "low CySS DMEM." Specificity was assayed by competition assays with unlabeled competitor oligonucleotides (■). $*p < 0.02$, $**p < 0.001$.

As shown in Fig. 4, specific binding was observed in two regions of the promotor that are included in the oligonucleotide probes −440/−407 and −308/−284. After partial CySS deprivation and after subtracting the nonspecific signal obtained after competition with nonlabeled oligonucleotides, Sp-1 binding increased on average by 112% using the probe −308/−284 ($p < 0.001$) and by 30% using the probe −440/−407 ($p < 0.02$).

DISCUSSION

Cystinosis is a monogenic disease that is caused by mutations or deletions of *CTNS* gene that encodes for cystinosin (1,3,5). Until recently, cystinosin was considered to be a constitutive carrier of the lysosomal membrane, allowing the efflux of CySS as it accumulates in lysosomes after protein degradation. We have recently reported that the *CTNS* gene also encodes for cystinosin-LKG, which is also expressed in other cell compartments, and that cell incubation in CySS-depleted medium up-regulates the expression of both *CTNS* isoforms (4,14). By culturing cells in different conditions,

including oxidizing conditions and after inhibiting GSH synthesis, we have shown that changes in *CTNS* mRNA correlate best (negative correlation) with cell Cys concentrations and with the E_h of the [Cys/CySS] redox couple (14).

To date, the physiopathology of cell damage in cystinosis has not been fully elucidated. Studies on human fibroblasts, on proximal tubular epithelial cells or using directly human tissues from patients with cystinosis, have shown that cystinotic cells are more prone to apoptosis and to autophagy, and that cysteinylation of proapoptotic kinases may be involved in this process and that in some cases mitochondrial function is impaired (17–21).

To better understand the role of cystinosin in cells, we have begun analyzing the mechanisms involved in *CTNS* mRNA regulation. Our results indicate that the *CTNS* gene is actively regulated through mechanisms that operate both at the transcriptional and posttranscriptional levels.

Other examples of gene regulation by intracellular amino acids have been reported. These may involve coordinated activation of groups of genes that encode for transcription factors, enzymes, or cell transporters in response to cell stress induced by amino acid deprivation (7,8) or more targeted activation of specific transporters or enzymes. Changes in Cys concentration in HeLa cells, for example, modify the expression of several genes, including the C/EBP homologous protein transcriptional factor and the asparagine synthetase genes (22). Similarly, CySS starvation in human hepatoma cells increases the expression of genes involved in Cys metabolism and transport, in GSH synthesis and in cell growth (23). More specific gene activation often requires the interaction of enhancers with specific AARE sequences that are located in the promotor region or in the first introns of genes (12,13,24,25). Disruption of the AARE sequence of the asparagine synthetase promotor, for example, inhibits gene transcription on asparagine deprivation (25). Similarly, CySS deprivation induces the transcription of the CySS/glutamate transporter through a specific AARE sequence located in its promotor (12). This same AARE sequence is present in the second intron of the *CTNS* gene and was therefore a *cis*-acting candidate region for the regulation of the *CTNS* gene. However, our results do not support this hypothesis. This AARE sequence of the *CTNS* gene may represent a remnant of an ancestral gene; alternatively, other *cis*- or *trans*-acting elements that were not active in our reporter gene system may be required to elicit its function.

Notwithstanding this result, our data clearly show that the *CTNS* promotor is activated when cells are cultured in low CySS medium, which causes a dramatic fall in cell thiol levels. Although these conditions are unlikely to be observed *in vivo*, the complex regulatory elements that we observed *in vitro* may play a crucial role *in vivo*, in particular, in the proximal tubular epithelium, which is compromised at the early stages of NC and where high amounts of CySS are reabsorbed. In addition, thiol depletion also induces cell oxidation. Although our previous data (14) support the hypothesis that high *CTNS* mRNA levels are driven primarily by Cys depletion rather than reactive oxygen species, we cannot rule out completely that increased *CTNS* mRNA are part of a broader response to oxidative stress.

The comparison of different promoter constructs activity in our study suggests that the thiol sensitivity of the *CTNS* promotor depends on other *trans*-acting elements. DNA strings encompassing bp −283 to bp −316 represent a critical region, as indicated by a marked increase in luciferase activity in transcripts containing this region and confirm the data by Phornphutkul *et al.* (6), who showed that a G > C mutation at position −295 bp causes cystinosis and reduces the binding of the Sp-1 transcription factor (6), which is involved in several cell processes, including apoptosis, cell differentiation, and cell cycle (26).

On these bases, we have tested the impact of changes in medium CySS concentrations on Sp-1 binding to the CTNS promotor. In partial contrast to our gene reporter results, which showed increased promotor activity even in constructs that do not contain Sp-1 binding sites, we observed increased Sp-1 binding to the consensus sequence located at position −308/−283 and to two other sequences that are contained in the −440/−407 oligonucleotide (the presence of two Sp-1 consensus sequences in this region may explain higher activity in our *in vitro* assays). The apparent contradiction between our gene reporter data and the results of our DNA binding experiments do not exclude that both mechanisms may be involved *in vivo*.

Significant posttranscriptional regulation was also demonstrated. Specifically, we observed that culturing cells in low CySS medium induced a very significant stabilization of the *CTNS* mRNA. A thorough description of the mechanisms involved in *CTNS* mRNA stability was beyond the scope of the present work. Our results are reminiscent of several other biological systems, such as the "classic" model of the transferrin receptor, whose mRNA half-life depends on cell iron concentrations (27,28). Mechanisms involved in mRNA stability and degradation include the action of "broad" spectrum RNAses, of gene-specific RNAses and of microRNAs, among others (29). Enzyme-mediated degradation of *CTNS* mRNA is in part supported by our CHX data. Hypothetically, cell culture in low CySS conditions inhibits the synthesis of a specific RNAse that decreases *CTNS* mRNA half-life. Alternatively, CHX could inhibit the synthesis of a repressor protein that operates at the transcriptional level.

Regardless of the underlying mechanisms, the present data demonstrate that the expression of *CTNS* mRNA is heavily regulated. Our previous data suggested that cytosolic Cys levels are the primary stimuli that regulate cystinosin expression (14). This would be consistent with mechanisms of regulation of other amino acid transporters. Once in the cytosol, CySS is reduced into Cys and is maintained in a reduced state by cell reducing systems, mainly by the GSH/GSSG couple. In experimental solutions, however, Cys is readily oxidized into CySS, preventing to test the effects of different Cys concentrations on *CTNS* mRNA synthesis with more direct experimental models, such as nuclear run-on assays.

Similar to iron or calcium, Cys is a highly reactive compound, which is essential to a number of cell processes,

including protein and GSH synthesis but which can also become detrimental, causing cysteinylation of proteins, when it accumulates in excess.

The present data may shed new light in understanding cell damage in cystinosis. In addition to demonstrate that *CTNS* mRNA levels increase when cell need to augment their thiol content, inhibition of *CTNS* gene expression in the presence of excess of thiols may be equally important. Hypothetically, loss of regulation of cell Cys content secondary to *CTNS* mutations may lead to increased cytosolic Cys content and to abnormal cysteinylation of proteins, which would make cystinotic cells more prone to apoptosis (20,21). In this respect, it is interesting to notice that inhibition of *CTNS* mRNA by RNA interference in HK2 cells not only increases cell CySS but also cell Cys concentrations.

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