Renal Tubule-specific Transcription and Chromosomal Localization of Rat Thiazide-sensitive Na-Cl Cotransporter Gene*

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Yoshihiro Taniyama‡, Kazunori Sato‡, Akira Sugawara, Akira Uruno, Yukio Ikeda, Masataka Kudo, Sadayoshi Ito, and Kazuhiisa Takeuchi§

From the Division of Nephrology, Endocrinology, and Vascular Medicine, Department of Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

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The molecular mechanism underlying the renal expression localization of the thiazide-sensitive Na-Cl cotransporter (TSC) gene was studied. The TSC gene was localized to chromosome 19p12–14. In cultured cells, tissue-specific transcription activity of the 5′-flanking region of the rat rTSC gene (5′FL/rTSC) was demonstrated, and the major promoter region was located between position −580 and −141. To further examine the tissue-specific transcription, transgenic rats harboring the 5′FL/rTSC fused upstream of the LacZ gene were generated. Immunohistochemical analysis clearly showed that LacZ gene expression was co-localized to distal convoluted tubules (DCT) with TSC, indicating that the 5′FL/rTSC regulates the renal tubule-specific TSC expression. Because a transcription factor, HFH-3 (hepatocyte nuclear factor-3/folk head homologue-3), had also been localized to DCT, a possible role of the putative cis-acting element (HFH-3/TSC, −400 to −387 position) for HFH-3 binding in the tissue-specific transcription was examined. Deletion and mutation analyses suggested that transcription of the HFH-3/rTSC was actually responsive to HFH-3, and electrophoretic mobility shift assay showed a direct binding of in vitro synthesized HFH-3 to the HFH-3/rTSC. In conclusion, the rTSC gene is localized to rat chromosome 19p12–24. The transcription regulatory region of the TSC gene confers DCT-specific gene expression. DCT-specific transcription factor HFH-3 may be involved in the renal tubule-specific transcription of TSC gene.

Thiazide-sensitive Na-Cl cotransporter (TSC) is an important molecule for reabsorption of NaCl in the kidney and a target of thiazide diuretics. TSC cDNAs were cloned, and the TSC function was characterized (1–3). Mutations possibly leading to loss-of-function in the human TSC gene have been shown to cause Gitelman’s syndrome, which is characterized by dehydration, hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria (4, 5). Recently, TSC-deficient mice were generated, and their phenotype was shown to bear a good resemblance to Gitelman’s syndrome (6). Thus, TSC plays an important role not only in NaCl metabolism but in acid-base balance and in the metabolism of other electrolytes such as potassium, magnesium, and calcium.

TSC mRNA expression has been shown to be localized to the distal convoluted tubule (DCT) in the kidney by in situ hybridization histochemistry (7, 8) as well as reverse transcription and polymerase chain reaction (RT-PCR) with microdissected nephron segments (9). Immunohistochemistry using a specific antibody against TSC has also shown that immunoreactive TSC is localized to DCT (10, 11). However, no study to uncover the molecular mechanism underlying the renal tubule-specific TSC expression has yet been performed in any species.

Recently, a transcription factor, hepatocyte nuclear factor-3/folk head homologue-3 (HFH-3), was isolated and partly characterized (12). HFH-3 belongs to an HFH/winged helix transcription factor family and is identical to FREAC-6 (13). Members of the family have been shown to be involved in tissue- or cell-specific gene expression, as well as cellular differentiation during embryonic development (14, 15). Interestingly, HFH-3 mRNA was reported to be localized to the epithelium of the DCT, where TSC is also localized. We therefore hypothesized that HFH-3 might be involved in the TSC gene expression.

In the present study, we studied the tissue-specific expression of the TSC gene in terms of gene transcription by an in vivo as well as in vitro experiments. The structure of the 5′-flanking region of the rat TSC gene (5′FL/TSC) was revealed, and the rTSC gene was mapped to chromosome 19p12–14 by FISH. The transcription function was shown to be tissue-specific in cultured cells. Moreover, we generated transgenic rats harboring the 5′FL/rTSC fused upstream of the LacZ gene to examine the tissue-specific transcription of 5′FL/rTSC. Immunohistochemical analysis clearly demonstrated that LacZ expression was localized to DCT in the transgenic rat kidney, indicating that the 5′FL/rTSC regulates the DCT-specific TSC localization in vivo. Furthermore, we identified a functional HFH-3 binding site in the major promoter region of 5′FL/rTSC, which was actually bound by in vitro synthesized HFH-3. HFH-3 may be involved at least in part in the DCT-specific expression of TSC.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[TM]/EBI DataBank with accession number(s) AB024534.

‡ These authors were equal contributors to this study.
§ To whom correspondence should be addressed: Molecular Biology Unit, Division of Nephrology, Endocrinology, and Vascular Medicine, Dept. of Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. Tel.: 81-22-717-7163; Fax: 81-22-717-7168; E-mail: kazut2i@mail.cc.tohoku.ac.jp.

1 The abbreviations used are: TSC, thiazide-sensitive Na-Cl cotransporter; h, human; r, rat; HFH-3, hepatocyte nuclear factor-3/folk head homologue-3; DCT, distal convoluted tubule; RT-PCR, reverse transcription-polymerase chain reaction; FISH, fluorescence in situ hybridization; EMSA, electrophoretic mobility shift assay; RACE, rapid amplification of cDNA ends; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tk, thymidine kinase; Luc, luciferase; GST, glutathione S-transferase; bp, base pair(s); C/EBP, CCAAT/enhancer-binding protein.
Southern Blot Analysis—To use as a probe in Southern blot analysis of rTSC gene, an rTSC cDNA fragment was first cloned by a PCR-based method with a rat kidney cDNA library (rat kidney Marathon-ready cDNA, CLONTECH) as a template using a pair of primers (sense, 5′-CCC GGA GCA ATG CCA GAG GTA CTA CCG G-3′ (from position 1 to 2 to in previously reported rTSC cDNA (2)); antisense, 5′-CTC AGG GTA TCC CTA GGC-3′ (from position 3 to 2 to in rTSC transcript) under the following PCR condition: 95 °C, 10 s, 90 °C, 30 s, 72 °C, 30 min for 25 cycles. The PCR product was then subcloned into pCR-TOPO (Invitrogen) by a TA cloning method. Sequential PCR was performed to confirm the complete sequence identity with the previously reported rTSC cDNA (2). The BalII digested fragment of the rTSC cDNA (892 bp) was labeled with [α-32P]dCTP by a random primer method (BAMHI, EcoRI, or HindIII). Digested DNA was then resolved on a 0.9% agarose gel and transferred to a nitrocellulose membrane. Hybridization was performed in Rapid-Hyb Buffer (Amersham Pharmacia Biotech) at 65 °C for 1 h, followed by washing with 2× SSC, 0.1% SDS at room temperature for 20 min, 0.1× SSC, 0.1% SDS at 65 °C for 15 min, and 0.1× SSC, 0.1% SDS at 65 °C for 15 min. The blot was exposed to an x-ray film (Kodak) for 72 h.

FISH—Chromosome mapping of the rTSC gene was carried out by FISH based on a previously reported method (17). At first, three different DNA fragments of the rTSC gene were amplified by PCR with three primers (sense-1, 5′-GCC ACA CCC GCA TTA GGA AAG GGC A-3′ (from position 693 to 720 in rTSC transcript); sense-2, 5′-CCT CAT CCT CAT CTA TTA CCT GCT GCG C-3′ (from position 2,570 to 2,599 in rTSC transcript); antisense-2, 5′-CTC GTC TCT GAG CCT CCA TTA TTG AGG C-3′ (from position 2,799 to 2,828 in rTSC transcript) and antisense-3, 5′-CTC CAG CTT CAG GAG AGC ATG AGT TCC GCT C-3′ (from position 3,070 to 3,097 in rTSC transcript) under the following PCR condition: 95 °C, 10 s; 68 °C, 8 min, for 30 cycles, and final temperature 72 °C for 10 min. The PCR products were labeled with digoxigenin and used as probes in FISH.

Cloning of the 5′-Flanking Region of rTSC Gene (5′ FL/rTSC)—The 5′ FL/rTSC was cloned by a PCR-based method using the Genome Walker Kit (CLONTECH) for rat gene cloning. Briefly, the first PCR was carried out based on the manufacturer's instruction with the gene-specific primer (GP1), 5′-ATG GAT GTA AAG TCG CCT GCT ATT G-3′ (designed to hybridize with the region from position 120 to 147 in the cDNA (1), and the adapter primer (AP)-1, 5′-TCA AAT CTC CAT CTG AAT AAG-3′ (supplied in the kit). Nested PCR was then performed with the GP-2, 5′-TGG ACA GAG CAT CCT GGC CCA ATG CAT TGC AGT TCC GCT CGT (sense) for annealing to the region from position 31 to 58 in rTSC cDNA (2)), and the AP-2, 5′-ACT GTA CCC GGC TCG GAG TGG GGT-3′, using the first PCR product as a template. The resultant PCR product was subcloned into pCR-TOPO (Invitrogen), and sequenced in both directions. Putative transcription factor binding sites in 5′ FL/rTSC were identified by the TRANSFAC 3.4 data base using Transcription Element Search Software (TESS) (18).

Primer Extension Method—Primer extension was performed using the primer extension system-AMV reverse transcriptase (Promega) by a random primer method (22, 23). A double strand DNA containing the putative HFH-3-binding site located from position 46 to 70 in hHFH-3 cDNA (12); antisense, 5′-TCTG GTC GAG TCT CAC TGG ACG-3′ (from position 1,148 to 1,171 in HFH-3 transcript)) under the following PCR condition: 95 °C, 10 s; 68 °C, 8 min, for 30 cycles. An expression vector of HFH-3 (pHFH-3) was synthesized by subcloning the HFH-3 cDNA into an expression vector, pcdNA1/Amp (Invitrogen). Sequencing was performed to confirm the complete sequence identity to the one reported previously (12).

Construction of Luciferase Reporter Gene Vectors—To examine the transcription activity of 5′ FL/rTSC, a chimeric firefly luciferase expression vector containing a 5′ FL/rTSC fragment (pHFH-3-Tk-Luc) was constructed. The 5′ FL/rTSC was subcloned into promoter-less firefly luciferase expression vector, PicaGene Basic Vector (Nippon Gene). Deletion mutants of rTSC/Luc were then created with exonuclease III/Mung bean nuclease as described previously (22, 23). A double strand DNA containing the putative HFH-3-binding site located from position −400 to −387 in 5′ FL/rTSC (HFH-3/TSC) was made by annealing oligonucleotides 5′-AAG AGG AAG GTC ATG CTA CAG CTC-3′ (sense) and 5′-TGG AAG GAC ATG CAT ACA AAA AAG GAG CTT GT-3′ (antisense), in which unterline indicates the putative HFH-3 binding site (consensus sequence 5′ GG TCT TGT TTT TTT YET D (12)) located from position −400 to −387 in 5′ FL/rTSC. The product was then subcloned upstream of a thymidine kinase gene promoter in a luciferase expression vector (HFH-3/Tk-TK-Luc). An expression vector carrying the mutated HFH-3-binding site (mHFH-3/Tk-Tk-Luc) was also synthesized with oligonucleotides 5′-ATG TAC AGG GTT CCT TCG ATG TAT ATG CAT TGC-3′ (sense) and 5′-TGG AAG GAC ATG CAT ATA ACC CCA AAG AAG CCT GT-3′ (antisense), in which italic indicates mutated nucleotides and underline indicates the putative HFH-3 binding site. The resultant mutated construct was designated mHFH-3/Tk-Tk-Luc.

Transient Transfection and Luciferase Assay—HEK293, A10, and HepG2 cells were cultured in 6-well culture plates before transfection. Transfection was performed with 2 µg/well of luciferase expression vector as well as 0.5 µg/well control β-galactosidase expression vector (pSV-β-galactosidase control vector, Promega) using DOTAP liposomal transfection reagent (Roche). After the transfection, culture medium was replaced with Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum.

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Materials and Methods

Southern Blot Analysis—To use as a probe in Southern blot analysis of rTSC gene, an rTSC cDNA fragment was first cloned by a PCR-based method with a rat kidney cDNA library (rat kidney Marathon-ready cDNA, CLONTECH) as a template using a pair of primers (sense, 5′-CCC GGA GCA ATG CCA GAG GTA CTA CCG G-3′ (from position 1 to 2 to in previously reported rTSC cDNA (2)); antisense, 5′-CTC AGG GTA TCC CTA GGC-3′ (from position 3 to 2 to in rTSC transcript) under the following PCR condition: 95 °C, 30 s, 68 °C, 2 min, for 30 cycles. The PCR product was then subcloned into pCR-TOPO (Invitrogen) by a TA cloning method. The 5′ end of the rTSC cDNA was determined by sequencing.

Cell Culture—HEK293 (a human embryonic kidney epithelial cell line), A10 (a rat vascular smooth muscle cell line), and HepG2 (a human hepatocyte cell line) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

RT-PCR—RT-PCR was performed to confirm endogenous expression of rTSC in HEK293, HepG2, or A10 cells using a One-Step RT-PCR kit (TaKaRa) according to the manufacturer’s instruction. Total RNA was extracted from these cells by RNaseasy kit (Qiagen). Endogenous hTSC mRNA in HEK293 or HepG2 cells were detected with a pair of primers; sense, 5′-AGG CAG GCA TGG TCC TGA CCT G-3′ (from position 519 to 540 in hTSC cDNA (3)); antisense, 5′-GCA GGA TGC TTT AGG TCA CCA G-3′ (from position 608 to 626 in rTSC cDNA (2)); antisense, 5′-GAT GCG GAT GTC ATT GAT GGC-3′ (from position 793 to 812 in rTSC transcript). Human mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also detected with a pair of primers; sense, 5′-CCA AGG AGC AAC AGC AGC AAG GGG CGA 5′; antisense, 5′-GGA CGAG-3′ (from position 371 to 388 in human GAPDH (pSV-β-galactosidase control vector, Promega) using DOTAP liposomal transfection reagent (Roche). After the transfection, culture medium was replaced with Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum.
Renal Tubule-specific Expression of TSC

charcoal/resin-stripped fetal bovine serum. 48 h after transfection, luciferase expression was determined by measuring its enzymatic activity using a luciferase assay system (Promega). Transfection efficiency was normalized by β-galactosidase expression (determined by its enzymatic activity) as reported previously (23). To examine the effect of HFH-3 on the transcription activity of 5′ FL/rTSC, pCAG-HFH-3 or mock DNA was co-transfected with ~580 rTSC/Luc, ~580 (mHFF-3H) rTSC/Luc, HHF-3/cTSC-Luc, or mHHF-3/cTSC-Luc in HepG2 cells, which have been shown to lack HFF-3 expression (12).

Electrophoretic Mobility Shift Assay (EMSA)—An expression construct was designed to synthesize a fusion protein of the HFF-3 DNA binding domain and glutathione S-transferase (GST) (22) as a generous gift from Dr. Robert H. Costa (University of Illinois at Chicago). The GST-HHF-3 fusion protein was isolated from Escherichia coli cultures and purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The purity of GST-HHF-3 was confirmed by SDS-polyacrylamide gel electrophoresis followed by Coomasie staining (data not shown). The double strand DNA of HFF-3/cTSC was radiolabeled and used as a probe. EMSA was performed with the probe and in vitro synthesized GST-HHF-3 as described previously (23, 24). A double strand DNA containing the consensus HFF-3 binding was created by annealing with oligonucleotides 5′-AGC TGC ACG TTC GTT ATT TAT GTA CCG AGC G-3′ (sense) and 5′-TGG AGC CTG GTG ACA TAA ACA ACG AAC GTG C-3′ (antisense), in which underlining indicates the consensus HFF-3 binding sequence, and was used as a competitor. An expression construct of HFH-3 (HFH-3/cTSC oligonucleotides (containing the mutated HFF-3 binding site) were also used as competitor.

Generation of Transgenic Rats Bearing 5′ FL/rTSC-LacZ—The transgene used herein consisted of the 2,110-bp rTSC promoter fused to the LacZ gene. A fragment encoding the rTSC promoter region (~2,110 to +45) was excised from rTSC/Luc with KpnI and subcloned to the KpnI site of pSV-β-galactosidase control vector (Promega). The KpnI site of this plasmid was located 87 bp upstream of the LacZ coding region, and the frame for the intact LacZ gene expression was confirmed by sequencing. The transgene fragment excised by digestion with SalI was purified using the QiAquick gel extraction kit (Qiagen). Transgenic rats were generated using pronuclear microinjection as the standard procedure. The microinjection was performed by YS New Technology Laboratory (Tochigi, Japan). Briefly, the transgene fragment was microinjected to the pronuclei of fertilized single-cell oocytes obtained from Harlan Sprague-Dawley rats. Embryos that survived microinjection were transferred into the oviduct of pseudo-pregnant Wistar rats. Transgenic rats were selected with amplification of LacZ by PCR from genomic DNA extracted from tails. The number of integrated copies of the transgene was estimated by Southern blotting with the transgene fragment as a probe.

Immunohistochemistry—The transgenic and nontransgenic rats (16-week) were anesthetized with diethyl ether, and perfused with saline containing 100 U/ml heparin. The kidneys were removed, sectioned, and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). The tissues were embedded in paraffin blocks and incubated with Antisera against Sry (gift from Dr. Steven C. Hebert (11), was used at a dilution of 1:250. Staining was performed using an RTU Vectastain universal avidin-biotin complex kit (ABC, Vector) and a VIP substrate kit (Vector). Sections were counter-stained with hematoxylin.

Data Analysis—p < 0.05 by Student’s t test was considered statistically significant.

RESULTS

Southern Blot Analysis and Chromosome Mapping of rTSC Gene—A single band was observed in either the BamHI or HindIII digest of rat genomic DNA (~6.5 kb and ~4.2 kb, respectively), suggesting that rTSC is a single copy gene (Fig. 1). Additionally, two bands in the EcoRI digest suggested the presence of EcoRI site in the intron between TSC exons complementary to the probe. To determine the chromosomal localization of rTSC gene, we next performed FISH using three different DNA fragments of rTSC gene as probes. After hybridization, one hundred metaphases were analyzed by recording the number and position of fluorescent spots on chromosomes. Twin fluorescent spots were identified in eighty three metaphases at chromosome 19, and single fluorescent spot at the same chromosome in twenty metaphases. A typical metaphase showing the presence of twin fluorescent spots at chromosome 19 is shown in Fig. 2A. The position of fluorescent spots were shown in Fig. 2B, and these spots were clustered at 19p12–14 (p < 0.01).

Structure of 5′ FL/rTSC—A 2.1-kilobase pair fragment of 5′ FL/rTSC was isolated and sequenced (DBJ/EMBL/GenBank™ data bases with accession number AB024534). 5′ FL/rTSC (Fig. 3) contains several putative consensus transcription factor recognition sequences such as: a TATA box at 42 bases upstream (~42) of the transcription initiation site; three SRY binding sites (SRY) at position ~1,931, ~1,728, and ~189; five Pit-1 binding sites (Pit-1) at positions ~1,486, ~1,328, ~1,219, ~990, and ~809; a CCAAT-enhancer-binding protein (C/EBP) family binding site at position ~463 and three C/EBPβ binding sites (C/EBPβ) at positions ~1,461, ~1,118, and ~1,011; two AP-1 binding sites (SP-1) at positions ~153 and ~131; two glucocorticoid-responsive elements (GRE) at positions ~1,091 and ~579; cAMP-responsive element (CRE) at position ~1,021; an AP-2 binding site (AP-2) at position ~788; a c-MYC binding site (c-myc) at position ~141; and an HFF-3 binding site at position ~393.

Transcription Initiation Site of rTSC Gene—The transcription initiation site of the rTSC gene was determined by both the primer extension method and 5′-RACE. Total RNA extracted from rat renal cortex was reverse transcribed using the 5′-end labeled DNA primer (designated to be a complement to the region from position 21 to 60 of rTSC cDNA). As shown in Fig. 4A, the longest extended product had 67 bases in length, indicating that the transcription initiation site of the rTSC gene is located 8 bases upstream of the 5′-end of the cloned cDNA (2) (Fig. 4C). Moreover, we performed 5′-RACE to confirm further the transcription initiation site. A PCR product 720 bp in length was subcloned, and the 5′-end was determined by sequencing. As shown in Fig. 4B, the 5′-end was completely identical to the transcription initiation site determined by the primer extension method (Fig. 4C).

Transcription Activity of 5′ FL/rTSC—Luciferase reporter gene assay was performed to examine the transcription activity of 5′ FL/rTSC. HEK293 cells (in which endogenous TSC mRNA expression was detected) were transfected with either luciferase expression vector alone (control) or rTSC/Luc. Luciferase expression with rTSC/Luc was significantly greater than with control (Fig. 5A: 26.2 ± 1.8-fold compared with control) in transfected HEK293 cells, suggesting that the 5′ FL/rTSC is transcriptionally active. We next conducted the deletion analysis of the 5′ FL/rTSC. As shown in Fig. 5B, transfection of the
the FISH.

dred myeloblasts in the metaphase were screened by FISH with three
mosome region in 12 metaphases. These spots are shown to be clus-
spots. Twin spots were detected at chromosome 19 in 83 of 100
5
9
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...cell. We therefore used these cell
lines to examine the tissue-specific transcription activity of
rTSC was thus indicated to be expressed in HEK293 cells but
not in either HepG2 or A10 cells. We therefore used these cell
lines to examine the tissue-specific transcription activity of
5’FL/rTSC estimated by luciferase expression was pronounced in
HEK293 cells, whereas it was modest in both HepG2 and
A10 cells. It was thus suggested that the 5’FL/rTSC is able to
regulate the tissue-specific gene transcription.

Establishment of Transgenic Rats Bearing 5’FL/TSC-
LacZ—227 microinjected oocytes were transplanted into eight
Wistar rats. 58 rats were born, seven of which had the trans-
gene. The copy number of the integrated transgene was esti-
ated as three to six copies by Southern blotting. Every trans-
genic rat grew normally in appearance. The transgene was
inherited in the Mendelian fashion.

Expression Co-localization of LacZ with TSC—The trans-
genic rat harboring six copies of the transgene was subjected to
immunohistochemistry. In the cortical region of the transgenic
rat kidney, immunoreactive β-galactosidase was observed at
cortical distal tubules (Fig. 7A), whereas no stain was observed in
the control rat (Fig. 7B). Using the sequential sections from the
transgenic rat kidney, we observed co-localization of immu-
noreactive β-galactosidase (arrows, Fig. 7, C and E) and TSC
(arrowheads, panels D and F). No immunostaining was ob-
erved in the medullar region (Fig. 7G).

A Role of HFH-3 on Transcription Activity of 5’FL/rTSC—To
examine the effect of HFH-3 overexpression on the transcription
activity of 5’FL/rTSC, hHFH-3 cDNA was isolated, and an
expression vector carrying the cDNA (pcHFH-3) was synthe-
sized. In vitro translated HFH-3 with this construct was re-
solved in SDS-polyacrylamide gel electrophoresis, and we iden-
tified a new band with the expected size corresponding to
HFH-3 protein (data not shown). As shown in Fig. 8, lucifer-
ase expression in HepG2 cells transfected with −2,093 rTSC/
Luc was significantly higher by cotransfection with pcHFH-3
than with mock (2.28 ± 0.14-fold expression compared with
mock; p < 0.01). This enhancement of transcription with
pcHFH-3 was also observed in transfection with −1,139
rTSC/Luc and −580 rTSC/Luc, whereas it was not observed with
−141 rTSC/Luc. Thus, the HFH-3-responsive element was
suggested to be present between position −580 and
−141, and we identified an element homologous to HFH-3
binding consensus sequence (DBD TRT TTR YDT D) at position
−393 (TCC TTG TTA TA)

To evaluate the transcription responsiveness of HFH-3 to
putative HFH-3 binding site, we mutated this site and exam-
ined the effect of HFH-3 overexpression on its transcription
activity. In HEK293 cells expressing HFH-3 mRNA (data not
shown), transcription activity of −580 (mHFH-3) rTSC/Luc was
reduced markedly (Fig. 9, open column) compared with
that of −580 rTSC/Luc (Fig. 9, hatched column).

Functional Characterization of HFH-3 Binding Site in 5’FL/
rTSC—We next characterized the HFH-3 binding site in 5’FL/
rTSC (HFH-3/rTSC) by a luciferase reporter gene assay using a
heterologous promoter. In HepG2 cells (which lacks HFH-3
mRNA expression (data not shown)) transfected with HFH-3/
rTSC-tk-Luc, luciferase expression was increased according to
the amount of transfected pcHFH-3 (Fig. 10, hatched column).
The overexpression of HFH-3 induced 2.78 ± 0.09-fold in-
creases in luciferase expression at the maximum compared
with control (mock transfection) (p < 0.01), whereas no in-
crement in luciferase expression was observed in cells transfected
with mHFH-3/rTSC-tk-Luc (Fig. 10, open column).

Direct Binding of HFH-3 to HFH-3/rTSC in EMSA—EMSA
was performed to examine a direct binding between HFH-3 and
HFH-3/rTSC. As shown in Fig. 11, in vitro synthesized HFH-3
protein reacted with the radiolabeled HFH-3/rTSC and formed a
protein-DNA complex (Lane 1). Using unlabeled HFH-3/rTSC
as a cold competitor, the formation of protein-DNA complex
was significantly inhibited (Lane 2). Moreover, the complex
FIG. 3. Nucleic acids sequence of the 5' flanking region of the rTSC gene. An asterisk and position +1 indicate the transcription initiation site. An underline indicates the protein coding sequence. Double underlines indicate the following putative cis-acting elements: TATA, TATA box; SRY, SRY biding site; Pit-1, Pit-1 binding site; GRE, glucocorticoid-responsive element; CRE, cAMP-responsive element; AP-2, AP-2 binding site; c-MYC, c-MYC binding site. The sequence has been deposited in the DDBJ/EMBL/GenBank™ data bases with accession number AB024534.

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ACTATAGGCG ACCTGCTGCT GACGCGCGCG GCTGTGACTT GGGAGCACCG GGGAGGAGGA ACTGAGTTT GAGTCCGGG

GATGGAGG CAGCTCTGCT CTACAAAGTC AGTTAGCGA CAGCGGAGGC TAGTAGACTA CAGAGAAAAC CTGCTCAGAA

AAACAGAAAAT TATCTTGCAG GTAAATAAAA AGCTCCCATAC CAAATTTAAC CATACCAGCA AATAGTACAA ACTTATGAA

CTTCTTCTTA TACAGGAGTA ATGGGTTAGA AAATGGAGGA TCCGAGCCTT CAGATTTATA TACACGTATAT GCTGAATTG

GATTTTGTGC AATTGCTCCC CATCCACCTC AAAACAAAAA CCAAACAAAC ACAGAAAGGC CTGAGACATG AAGACTAGG

AAACATTGAC CTTGGCCCC TTTCTGCTGC TGGCCTGTC TCAAGCCCTC CCCATCCCTG TAGCTCAGGC TTCTCCTGAC

TTTCTTCTCA TTATAGTCG AGCCAGGAGA GACTTGGCGT TTATACATA CATAGACTG TTGGCTGATA TTCCAAGATG

GGGAGAGCT GGTCTTTATT TCTGAGATGG TGAGCTTCAA CTTATATCCC ATCTTCGAGC CATCCCATTC TCTGAAACAT

Pit-1

TTAAACCTGGA TAGCTGAGTA GCATGGATAT ATATAATTTA TACATACATA TATGGGTTTG TATACACATA TACATACATA

TATATACTAC ATATAATATT GTCTCATATAT ATATGAGAT TTATATCCA TTTCTGTTG GTGGAGCAAA GTCAAATCCA

Pit-1

AATTATTTTT AGAGCTCATA AAGAGCAGCT AAAATCAGCAG ACCTCTCTGG TTTCTTTTTTT TTTTTAAAG ATTTATTCAT

TTTATATATA TAGTACACT GTAGCTCTCT TCAGACACAC CAGAAAAGG CATCAGAATC TTATAAGATG GGTGTTGAGG

CACCATGGGG TGGCTGAGA ATGACATTGA CGACCTCACT GGGCTCCTAA CCAATGACCC ATCCGTCCAG

CRE

C/EBP

GGGGAGCTTCTG AGTTAGCCTC CTTGAGCGTCA CTGAGGACA AACTGAGAGC AAATCTCCG CAGTGCTTCT

CRE

Pit-1

AGGGCAGAGG AAGCCAGCTT TTAGGAGGCC CTCAAGCCCT TAGTGATCCA GCAGAAAGCG GGTTAGACCT TCAGAGCTCA

GRE

GCAGATATG CAGCTTCTTA TGCTCAGAG CTATAGTTGA TTGGATGCCC AGCTTCCCGG GAGAAAGATG TCTGCTGAGG

GCTATGAG TACGGGGTCA CTCTGCAGG AACTGAGCC TCCGCCCTTA GAAATATCG AGAATACCT

Pit-1

AP-2

ACTTCATCGA TCAAGCCCA GGGGTGTTCT TTACACAGGG CACTGCCCT GCCGCTGATA ATAGAGGCTC TCAGGAAACAC

GCATCTCAGG GTCCAGGCGA GAATTCTGAG GACTCTAACAT TATGAGCTCT TAGGGCCGCC CTAATGATGC AGAAGTCAGA

GRE

TATGGGACTG GCCCTGATTG TCCTGAGGG CAGTGGACGG GAGCAAAAA TCAAGGTTTG TCAGGGAGGG GTGGCCTCCA

GGTTGCTCT

GGACTGGCTT GAGGCGACTG CTATAATGTC ATATGGAATT GTATGCGAG AGAGCCCGAG ACGTGGAGAG CAGAGACCTG

C/EBP

HA-3

AGGAAGTTTA AACCTGAGGT TAGTGGGCAG CTGGCCAGCA GGCTCTCTCT GTGGCTCCCT GCAGTCTGTAG

GCTTGAAGAG GCTGGCTGAG GTGGCTAACG GTGGCTTAA GGCCTCTCCT GCTCTAAAGT GGCCTCTGTA

SR-Y

TCAAGGAATC GATACTAGG GCCAGGAGAG GAGCTGGTAC GCAGTCCTGC CCAGCTTACA AGAGACCGCT CTGGTTTGCC

SP-1

c-MYC

SP-1

ATGGGCTGTC TGAGAAGACC TTCCGCTCTTC TGCCAGCTT GCAGAGACCT CTTGAGGTC GTGCCCCTAC

TATA

CTGCTCCTGC TTCTGGCAGC GCCCTGAGCA AGAGTCACCTA GGGAGGAGGA CAGAGAAGGC GGGAGAGGA GATCGAGGTG

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disappeared with an excess of oligonucleotides containing the HFH-3 binding consensus sequence as a cold competitor (lane 3). However, the protein-DNA complex was kept unchanged with an excess of oligonucleotides of mHFH-3/rTSC as a competitor (lane 4). These results indicate that HFH-3/rTSC is actually bound by HFH-3 and the binding is sequence-specific.

DISCUSSION

The rTSC gene was shown to be localized to 19p12–14 by FISH. In either human or mouse, the TSC gene has also been mapped to chromosome 16q13 (3, 4, 25) or 8 (26), respectively. These results suggest the segmental chromosome similarity in their regions.

Genes of the Na-K-Cl cotransporter (NKCC2) (2), sodium phosphate cotransporter (NPT2) (27), chloride channel CLC-K1 (28), and kidney-specific cadherin (Ksp-cadherin) (29) have been shown to be expressed specifically in the kidney. The transcription regulatory regions of those genes have already been shown to depend on an interaction between a transcription factor, MAZ, and a transcription repressor, KKLF (33). In the present study, we analyzed the transcriptional function of the rTSC gene in terms of tissue-specific gene expression.

The 5'FL/rTSC contains two putative glucocorticoid-responsive elements (Fig. 3). It has been reported that glucocorticoids and mineralocorticoids stimulate the thiazide-sensitive sodium transport at DCT, inducing an increase in the number of thiazide diuretic binding sites (34, 35) or in TSC protein expression (36). The glucocorticoid-responsive elements may possibly be involved in the steroid-induced TSC expression at the gene transcription level. A putative cAMP-responsive element was also identified, implying that TSC transcription could be regulated by intracellular cAMP formation by an agent such as calcitonin, which has been shown to increase the number of renal thiazide diuretic binding sites (37). Moreover, a couple of putative C/EBP binding sites were identified. Because C/EBPβ is expressed in kidney and induced by interleukin-6 mediating an inflammatory response (38), TSC gene transcription may possibly be affected by inflammation via interleukin-6.

Luciferase reporter gene analysis was performed with the chimeric reporter expression vector containing 5'FL/rTSC (rTSC/Luc). When rTSC/Luc was transfected into HEK293 cells (which express TSC mRNA), marked luciferase expression was observed (Fig. 5A). Additionally, the transcription activity is dependent upon the length of 5'FL/rTSC, indicating that the 5'FL/rTSC has a significant transcription function. Deletion analysis also showed a marked decrease (75%) in the transcription activity between position −580 and −141 (Fig. 5B), suggesting that a major promoter is located between these positions.

Tissue-specific transcription of TSC gene was focused in the present study. As shown in Fig. 6A, transcription activity of 5'FL/rTSC was pronounced in HEK293 cells expressing TSC mRNA, whereas it was suppressed in either HepG2 or A10 cells lacking TSC mRNA expression (Fig. 6), suggesting that the 5'FL/rTSC plays a crucial role in the tissue-specific TSC gene transcription. To further study the tissue-specific transcription of TSC in kidney, we generated the transgenic rats with the 5'FL/rTSC fused upstream of the LacZ gene as a transgene.
shown in Fig. 7, immunoreactive β-galactosidase was present at the same tubular region as TSC, probably at DCT in the renal cortical region, and neither was detected in the other renal tissues. Thus, this in vivo gene transcription model has clearly demonstrated that DCT-specific TSC gene expression is dependent on the 5′-FL/rTSC, and it is suggested that the region contains some important elements responsible for the DCT-specific transcription.

HFH-3 belongs to the HFH/winged helix transcription factor family (12), and its family members are involved in tissue-specific gene expression and cell differentiation during embryonic development (14, 15). HFH-3 mRNA is localized to DCT in kidney (12), but the target gene of HFH-3 has not yet been identified. We hypothesized that HFH-3 might be involved in the renal tubule-specific gene transcription of TSC, because a putative HFH-3 binding site (HFH-3/rTSC) at the −393 position (Fig. 3), 5′-TCC TTT TTG TTA T-3′, which bears a homology to the previously reported HFH-3 binding sequence (DBD TRT TTR YDT D) (12) by 10 of 13 nucleotides (77%). To examine the transcription activity of HFH-3/rTSC, the HFH-3/rTSC in −580 rTSC/Luc was mutated, and its transcription function was examined in HEK293 cells (expressing both TSC and HFH-3 mRNAs). By the mutation, we observed a marked loss of transcription function. It is therefore suggested that the HFH-3/rTSC plays an important role in TSC gene transcription in HEK293 cells. We next focused on the transcriptional function of HFH-3/rTSC by examining a functional interaction between HFH-3 and HFH-3/rTSC in HepG2 cells. HFH-3 overexpression activated the transcription of HFH-3/rTSC-tk-Luc in a dose-dependent manner (Fig. 8), whereas the transcription stimulation was not observed in transfected cells with the mHFH-3/rTSC. These results indicate that HFH-3 can stimu-
FIG. 9. Loss of transcription activity by a mutation of HFH-3/rTSC. Transcription activity of HFH-3/rTSC was examined in HEK293 cells (expressing HFH-3 mRNA) using -580 (mHFH-3)/rTSC in which the HFH-3/rTSC was mutated. Bars represent the mean ± S.E. (n = 12; *, p < 0.01).

FIG. 10. Transactivation of HFH-3/rTSC by HFH-3. Luciferase constructs containing the truncated wild-type HFH-3/rTSC (HFH-3/rTSC-tk-Luc) or mutated HFH-3/rTSC (mHFH-3/rTSC-tk-Luc) fused upstream of tk promoter was transfected into HepG2 cells (lacking TSC1/2 but with TSC2 subunit), whereas it is kept unchanged in cells transfected with HFH-3/rTSC-tk-Luc. The luciferase expression with HFH-3/rTSC-tk-Luc is stimulated in proportion to the amounts of transfected pcHFH-3 DNA, whereas it is kept unchanged in cells transfected with HFH-3/rTSC-tk-Luc. Bars represent the mean ± S.E. (n = 10; *, p < 0.01).

FIG. 11. Protein-DNA interaction between HFH-3 and HFH-3/rTSC. EMSA was performed with the in vitro synthesized GST-HFH-3 fusion protein (GST-HFH-3) and [32P]-labeled HFH-3/rTSC fragment (32P-HFH-3-rTSC) as a probe. Formation of a protein-DNA complex is observed (lane 1). The complex formation is significantly inhibited with an excess of unlabeled HFH-3/rTSC fragment (T) (lane 2) as well as oligonucleotides containing consensus HFH-3 binding site (C) (lane 3). On the other hand, no inhibition of the complex formation is observed with an excess of oligonucleotides containing the mutated (m) HFH-3/rTSC (lane 4). Lane 5, radiolabeled probe alone.

late the transcription through an interaction with HFH-3/rTSC. However, the magnitude of the transcription stimulation (−2–3-fold that of mock) was not comparable with the transcription activity of −2,029 rTSC/Luc in HEK293 cells. Some other elements in the 5′ flanking region of the TSC1/2 gene may also be involved in the gene transcription to enhance the HFH-3 transactivation.

EMSA was performed to examine an interaction between HFH-3/2 and HFH-3/rTSC. In EMSA, a protein-DNA complex was formed with the radiolabeled HFH-3/rTSC and in vitro synthesized HFH-3. The complex formation was inhibited with an excess of the HFH-3 consensus sequence oligonucleotides but not with an excess of mHFH-3/rTSC. HFH-3 therefore can actually bind the HFH-3/rTSC and may lead to transactivation of TSC gene. Putative target genes for HFH-3 have been suggested to be the mineralocorticoid receptor, Na+/H exchanger NHE3, Na+/K-ATPase α2 subunit, and Na+/K-ATPase β2 subunit (12), although any interaction with HFH-3 has not been proven even in these the transcription activity of the rTSC gene. We have demonstrated here that HFH-3 can stimulate through the HFH-3/rTSC via an interaction with HFH-3. This is the first report in which a target gene for HFH-3 has been identified.

In conclusion, we have localized the rTSC gene to chromosome 19p12–14 and characterized its promoter region. The promoter determines the DCT-specific gene expression of rTSC, and DCT-specific HFH-3 may be involved at least in part in renal tubule-specific rTSC gene transcription.

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REFERENCES

1. Gamba, G., Saltzberg, S. N., Lombardi, M., Miyashita, A., Lytton, J., Hediger, M. A., Brenner, B. M., and Hebert, S. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2749–2753

2. Gamba, G., Miyashita, A., Lombardi, M., Lytton, J., Lee, W. S., Hediger, M. A., and Hebert, S. C. (1994) J. Biol. Chem. 269, 17133–17122

3. Mastroianni, N., De Fusco, M., Zollo, M., Arrigo, G., Zuffardi, O., Bettinelli, A., Ballabio, A., and Casari, G. (1996) Genomics 35, 486–493

4. Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Korel, P. F., Molina, A. M., Vaara, I., Iwata, F., Cushner, H. M., Koolen, M., Gainza, F. J., Gitelman, H. J., and Lifton, R. P. (1996) Nat. Genet. 12, 24–30

5. Takeuchi, K., Kure, S., Kato, T., Taniyama, Y., Takahashi, N., Ikeda, Y., Abe, T., Narisawa, K., Muramatsu, Y., and Abe, K. (1996) J. Clin. Endocrinol. Metab. 81, 4496–4499

6. Schultheis, P. J., Lorenz, J. N., Meneton, P., Nieman, M. L., Riddle, T. M., Flagella, M., Duffy, J. J., Doetschman, T., Miller, M. L., and Shull, G. E. (1998) J. Biol. Chem. 273, 29150–29155

7. Obermuller, N., Bernstein, P., Velazquez, H., Reilly, R., Moser, D., Ellison, D. H., and Bachmann, S. (1996) Am. J. Physiol. 270, F900–F910

8. Bostanjoglo, M., Reeves, W. B., Reilly, R. F., Velazquez, H., Robertson, N., Litwack, G., Morsing, P., Dorup, J., Bachmann, S., and Ellison, D. H. (1998) J. Am. Soc. Nephrol. 9, 1347–1358

9. Velazquez, H., Naray-Fige-Toth, A., Silva, T., Andujar, E., Reilly, F. F., Desir, G. V., and Ellison, D. H. (1998) Kidney Int. 54, 464–472

10. Bachmann, S., Velazquez, H., Obermuller, N., Reilly, R. F., Moser, D., and Ellison, D. H. (1995) J. Clin. Invest. 96, 2510–2514

11. Plotkin, M. D., Kaplan, M. R., Verlander, J. W., Lee, W. S., Brown, D., Poch, E., Gullans, S. R., and Hebert, S. C. (1996) Kidney Int. 50, 174–183

12. Overdier, D. G., Ye, H., Peterson, R. S., Clevendorn, D. E., and Costa, R. H. (1997) J. Biol. Chem. 272, 13725–13730

13. Pierrou, S., Hellqvist, M., Samuelsson, L., Enerbo, S., Carlsson, P. (1994) EMBO J. 13, 5002–5012

14. Lai, E. K., Clark, K. L., Burley, S. K., and Darnell, Jr., J. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10421–10423

15. Kaufmann, E., and Knochel, W. (1996) Mech. Dev. 57, 3–20

16. Schumock, J., and Overton, G. C. (1997) TESS: Transcription Element Search Software on the WWW, Technical Report CBBL-TR-1997.1001-v0.0, Biocomputational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania

17. Takachiki, K., Takahashi, N., Kato, T., Abe, T., Taniyama, Y., Tsutsumi, E., Ito, O., Nakagawara, K., and Abe, K. (1996) Kidney Int. 55, S183–S186
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19. Takeuchi, K., Alexander, R. W., Nakamura, Y., Tsujino, T., and Murphy, T. J. (1993) Circ. Res. 73, 612–621
20. Tokunaga, K., Nakamura, Y., Sakata, K., Fujimoto, K., Ohkubo, M., Sawada, K., and Sakiyama, S. (1987) Cancer Res. 47, 5616–5619
21. Tso, J. Y., Sun, X.-H., Kao, T.-H., Reece, K. S., and Wu, R. (1985) Nucleic Acids Res. 13, 2485–2502
22. Takahashi, N., Takeuchi, K., Sugawara, A., Taniyama, Y., Kato, T., Wilcox, C. S., Abe, K., and Ito, S. (1998) Biochem. Biophys. Res. Commun. 244, 489–493
23. Ikeda, Y., Sugawara, A., Taniyama, Y., Uruno, A., Igarashi, K., Arima, S., Ito, S., and Takeuchi, K. (2000) J. Biol. Chem. 275, 31142–31150
24. Overdier, D. G., Porcella, A., and Costa, R. H. (1994) Mol. Cell. Biol. 14, 2755–2766
25. Levi, M., Kempson, S. A., Lotscher, M., Biber, J., and Murer, H. (1996) J. Membr. Biol. 154, 1–9
26. Adachi, S., Uchida, S., Ito, H., Hata, M., Hirose, M., Marumo, F., and Sasaki, S. (1994) J. Biol. Chem. 269, 17677–17683
27. Whyte, D. A., Li, C., Thomson, R. B., Nix, S. L., Zanjani, R., Karp, S. L., Aronson, P. S., and Igarashi, P. (1999) Am. J. Physiol. 277, F587–F598
28. Igarashi, P., Whyte, D. A., Li, K., and Nagami, G. T. (1996) J. Biol. Chem. 271, 9666–9674
29. Hilfiker, H., Hartmann, C. M., Stange, G., and Murer, H. (1998) Am. J. Physiol. 274, F197–F204
30. Uchida, S., Rui, T., Yatsushige, H., Matsumura, Y., Kawasaki, S., Sasaki, S., and Marumo, F. (1998) Am. J. Physiol. 274, F602–F610
31. Uchida, S., Tanaka, Y., Ito, H., Saitoh-Ohara, F., Inazawa, J., Yekoyama, K. K., Sasaki, S., and Marumo, F. (2000) Mol. Cell. Biol. 20, 7319–7331
32. Pollak, M. R., Delaney, V. B., Graham, R. M., and Hebert, S. C. (1996) J. Am. Soc. Nephrol. 7, 2233–2248
33. Pathak, B. G., Shaughnessy, J. D., Meneton, P., Greel, J., Shull, G. E., Jenkins, N. A., and Copeland, N. G. (1996) Genomics 33, 124–127
34. Chen, Z., Vaughn, D. A., Blakely, P., and Fanestil, D. D. (1994) J. Am. Soc. Nephrol. 5, 1361–1368
35. Velazquez, H., Bartiss, A., Bernstein, P., and Ellison, D. H. (1996) Am. J. Physiol. 270, F211–F219
36. Kim, G. H., Masilamani, S., Turner, R., Mitchell, C., Wade, J. B., and Knepper, M. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14552–14557
37. Blakely, P., Vaughn, D. A., and Fanestil, D. D. (1996) J. Am. Soc. Nephrol. 7, 1052–1057
38. Lekstrom-Himes, J., and Xanthopoulos, K. G. (1998) J. Biol. Chem. 273, 28543–28548
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