Altered Mouse Cholinephosphotransferase Gene Expression in Kidneys of Type 2 Diabetic KK/Ta Mouse

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It is generally considered that genetic factors may contribute to the susceptibility of type 2 diabetic nephropathy. The purpose of the present study is to identify molecules that contribute to the development and/or progression of this disease. Differential display was performed to isolate genes in the kidney using the KK/Ta mouse model of type 2 diabetes. The differential expression of 8 randomly chosen candidate genes (DN1-8) were verified by reverse-transcriptase polymerase chain reaction (RT-PCR) or Northern blot analysis. DN1-3 (Zn-α2-glycoprotein, vascular endothelial growth factor receptor [VEGFR]-2, and lactate dehydrogenase [LDH]) were overexpressed and DN7-8 (peroxisome proliferator-activated receptor [PPAR]-interacting protein [PRIP], unknown) were underexpressed in the KK/Ta mouse kidney. DN4-6 (Ezrin, transcobalamin 2, aldo-ketoreductase) did not differ between KK/Ta and control (BALB/c) mice. DN8 only showed no significant sequence similarity to previously reported genes. Molecular cloning revealed that full-length DN8 shares 89% identity with human cholinephosphotransferase 1 (*hCHPT1*), and we designated it as "putative" mouse cholinephosphotransferase 1 (*mCHPT1*). The putative *mCHPT1* gene was most closely mapped to the *D10Mit94* locus with the highest logarithm of odds (lod) score. In situ hybridization revealed the levels of glomerular putative *mCHPT1* in BALB/c mice tended to be slightly higher than those in KK/Ta mice. The altered renal mRNA expression of these genes may be involved in the development and/or progression of diabetic nephropathy.

DIABETIC NEPHROPATHY is a major cause of end-stage renal failure in patients with both type 1 (insulin-dependent) and type 2 (non–insulin-dependent) diabetes. Almost 30% of type 1 or type 2 diabetic patients develop diabetic nephropathy despite strict blood glucose and/or blood pressure control.[1] Since diabetic nephropathy occurs in familial clusters[2-4] and not all patients with poor metabolic control develop nephropathy, genetic factors may contribute to susceptibility to this disease. However, in general, the evidence for genetic susceptibility to type 2 diabetic nephropathy in humans is still not clear. Isolation of these genes may play an important role in clarification of the pathogenesis of type 2 diabetes and related disorders. Differential display was performed to isolate genes that show transcriptional changes in the kidney using the KK/Ta mouse model of type 2 diabetes. Since KK/Ta mice had a clearly different genetic background in terms of body weight, blood glucose, impaired glucose tolerance (IGT), urinary albumin excretion, and serum triglyceride in comparison with BALB/c mice,[5] we investigated the altered gene expression that occurs in the kidney during development or progression of type 2 diabetes using KK/Ta mice. Renal lesions in KK/Ta mice closely resemble those in human early diabetic nephropathy.[6,7] Therefore, KK/Ta mice may serve as a suitable model for the study of type 2 diabetes and early diabetic nephropathy in humans. In the present study, we focused on expression in the kidney using differential display polymerase chain reaction (DD-PCR) to investigate the candidate gene of diabetic nephropathy.

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MATERIALS AND METHODS

RNA Preparation

Tissues (brain, heart, lung, liver, spleen, kidney, intestine, and muscle) were removed from male KK/Ta and BALB/c mice at 20 weeks of age. Total RNA was isolated from mouse tissues by the acid-guanidinium isothiocyanate-phenol-chloroform method using Trizol (Gibco-BRL, Rockville, MD). To remove contaminating DNA from the RNA preparations, samples were incubated with RNase-free DNase I (Amersham Pharmacia Biotech, Buckinghamshire, England) at 37°C for 30 minutes. After phenol/chloroform extraction and ethanol precipitation, an aqueous diethyl pytocarbonate (DEPC) suspension of total RNA was prepared.

DD-PCR, Reamplification, and Rapid Selection of cDNA Fragments

DD-RT-PCR was performed using the Fluorescence Differential Display (FDD) Kit (Takara Shuzo, Shiga, Japan) as previously described.[8] Briefly, bands revealed by differential expression were cut out from the gel and then eluted in Tris EDTA (TE) buffer. The eluted solution was reamplified using the same primer. The main band was recovered from the gel and purified using the Qiaprep PCR purification kit (Qiagen, Hilden, Germany). Direct sequencing was performed using the Cloning-Sequencing Primer Set for FDD (Takara Shuzo). Sequence analysis was performed by the Dye Deoxy Termination Cycle Sequencing Kit and ABI PRISM 310 sequencer (Perkin Elmer Applied Biosystems, Norwalk, CT)

Library Screening and Rapid Amplification of 5′ and 3′ cDNA Ends

The BALB/c mouse kidney cDNA library was purchased from CLONTECH, Palo Alto, CA (Mouse Kidney 5′-STRETCH PLUS cDNA). Phages were screened with the cloned DD-PCR fragment as a probe using protocols recommended by the manufacturer. Since cDNA clones isolated from the library did not yield the complete 5′ and 3′ end, PCR-based rapid amplification of cDNA ends (RACE) was performed using a commercially available 3′ or 5′ RACE System (Gibco, Tokyo, Japan).

Table 1. Summary of Identification of Differentially Expressed Clones

Clone	Size of Fragment (bp)	Reason for Excision	Northern blot	RT-PCR	Change in mRNA Expression by RT-PCR	Best Match in GenBank of Mus Musclus	Identity (%)
1	240	Up	KK/Ta > Balb/c	KK/Ta > Balb/c	12.0 fold Up	Zn-α 2-glycoprotein 1	98
2	370	Up	No signal	KK/Ta > Balb/c	4.0 fold Up	Vascular endothelial growth factor receptor (VEGFR)-2	98
3	139	Up	KK/Ta > Balb/c	KK/Ta > Balb/c	2.8 fold Up	Lactate dehydrogenase (LDH) 2, B chain	97
4	106	Down	KK/Ta = Balb/c	KK/Ta = Balb/c	No change	Ezrin	97
5	420	Up	KK/Ta = Balb/c	KK/Ta = Balb/c	No change	Transcobalamin 2	95
6	101	Up	No signal	KK/Ta = Balb/c	No change	Aldo-keto reductase family 1	90
7	495	Down	No signal	KK/Ta < Balb/c	3.2 fold Down	Peroxisome proliferator-activated receptor (PPAR) interacting protein (PRIP)	98
8	346	Down	KK/Ta < Balb/c	KK/Ta < Balb/c	10.5 fold Down	Unknown	99

Relative Quantitative Reverse-Transcriptase PCR

Relative quantitative reverse-transcriptase (RT)-PCR was performed using the Quantum RNA kit (Ambion, Austin, TX). Briefly, DNA free total RNA was reverse-transcribed with Moloney mouse leukemia virus (M-MLV) RT using random decamers (Ambion) as downstream primers. The primers were designed based on cDNA sequence analysis. The PCR product was separated on 2% agarose gel and stained with ethidium bromide (EtBr), and then analyzed by densitometery. A mixed ratio of 18S ribosomal RNA primers and competimers was used to amplify rRNA as an internal control under the same conditions as the gene of interest. The standard curve was obtained by plotting the densitometric signal intensity of the rRNA PCR products versus the primer:competimer ratio. The sample signal intensities were normalized as described in the Ambion manual.

Northern Blot Analysis

Northern blot analysis was performed according to the standard protocols. Total RNA sample (10 μg) was denatured and then separated on 1% agarose gel containing 2.2 mol/L formaldehyde. RNA was transferred to nylon membrane (Gene Screen Hybridization Transfer Membrane; MEN Life Science Products, Boston, MA) at room temperature overnight and exposed to UV light for cross-linking. Membranes were preincubated in 5× sodium saline citrate (SSC), 5× Denhardt's reagent, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and 250 μg/mL salmon sperm DNA (Sigma Chemical Co, St Louis, MO) at 42°C for 4 hours and then incubated with (α-^{32}P) deoxycitidine phosphate (dCTP)-labeled probes prepared by the random prime labeling system (Rediprime II; Amersham Pharmacia Biotech) at 42°C for 18 hours and washed 3 times in 2× SSC, 0.1% SDS at room temperature. Autoradiography was performed using a bioimaging analyzer (BAS-2500; Fuji Film, Tokyo, Japan).

Chromosome Mapping of Mouse Cholinephosphotransferase 1

Chromosomal assignment of the putative mouse cholinephosphotransferase 1 (*mCHPT1*) gene was performed by PCR analysis of a mouse/hamster T31 Radiation Hybrid Panel (Research Genetics, Huntsville, AL) PCR conditions were 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute, and 72°C for 5 minutes. The PCR product was separated on 2% agarose gel and stained with EtBr. Chromosomal assignments were determined using The Jackson Laboratory web page: http://www.jax.org/resources/documents/cmdata/rhmap/rh.html.

In Situ Hybridization

The coding sequence of the putative *mCHPT1* cDNA fragment was inserted into the *EcoRI* site of pBluescript (Stratagene Cloning Systems, La Jolla, CA). Antisense and sense probes were synthesized using the T7 promotor and SP6 promotor, respectively. KK/Ta and BALB/c mice at 20 weeks of age were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The kidneys were then removed and immersion-fixed in 4% paraformaldehyde/PBS, washed with 30% sucrose/PBS (pH 7.4), for 2 hours, and frozen in liquid nitrogen. Five-micrometer fixed frozen sections were subjected to in situ hybridization performed essentially as described by Hayashi et al.[9]

RESULTS

Identification of Differentially Expressed mRNAs

DD-PCR was performed to identify genes which are over- or underexpressed in mouse kidney using KK/Ta mice. From a total of 75 differentially expressed cDNAs, 8 were randomly selected for detailed characterization. Seven previously reported genes and one novel gene were identified (Table 1). These fragments were designated as diabetic nephropathy–associated genes (DN). The mRNA expression of DN in the kidney in 20-week-old KK/Ta and BALB/c mice, representing hyperglycemia, was confirmed by RT-PCR or Northern blot analysis (Fig 1). DN2, 6, and 7 were only detected by RT-PCR analysis, presumably due to their low levels. DN1-3 (Zn-α2-glycoprotein, vascular endothelial growth factor [VEGFR]-2, lactate dehydrogenase [LDH]) was overexpressed in the diabetic kidney, DN4-6 (Ezrin, transcobalamin 2, aldo-ketoreductase) did not differ between diabetic and control mice, and DN7-8 (peroxisome proliferator-activated receptor [PPAR]-interacting protein [PRIP]and unknown) was underexpressed in the diabetic kidney.

Molecular Cloning of mCHPT1

The 346-bp cDNA (DN8) cloned from DD-PCR fragment was selected for further analysis because its nucleic acid sequence exhibited no significant similarity to the reported cDNA in the international nucleotide database of National Center for Biotechnology Information (NCBI) using the BLAST service (http://www.ncbi.nlm.nih.gov/BLAST). Screening of the BALB/c mice kidney cDNA library with 346-bp partial-length cDNA yielded 1 positive clone of 1,941 bp length. Since neither clone from the cDNA library contained the 5′ or 3′ end of the gene, 5′ and 3′ RACE was performed to obtain additional sequence information. The final nucleic acid sequence of full-length cDNA was derived from about 3,080 bp cDNA and

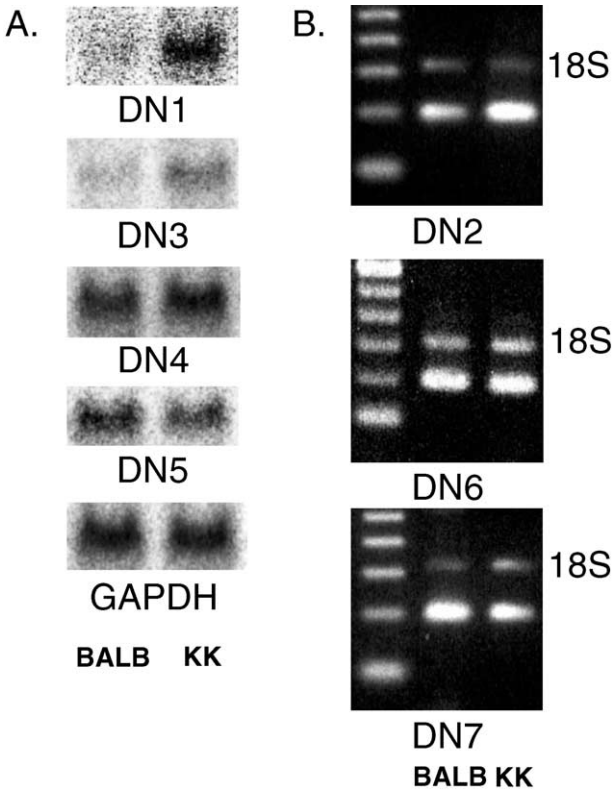

Fig 1. The mRNA expression of DN1-7 in kidneys of 20-week-old BALB/c (BALB) and KK/Ta (KK) mice. (A) Northern blot analysis of kidney probed with DN1, 3-5 and GAPDH. Each lane contains 10 μg of RNA from total kidney of BALB/c (left lane) and KK/Ta (right lane) mice. (B) Competitive RT-PCR analysis of DN2, 6, and 7. The upper bands are competitors (324 bp), and the lower bands (BALB, left lane; KK, right lane) are genes of interest.

comprises an open-reading frame of 398 amino acid residues (*mCHPT1* GenBank accession no. AY445814). A search of homology in the international protein database of NCBI using the BLAST service with the predicted protein sequence revealed that DN8 shares a high degree of homology with human cholinephosphotransferase (*hCHPT1*). This full-length cDNA shares 89% identity with *hCHPT1* (Fig 2), and we designated it as "putative" *mCHPT1*.

Tissue Distribution and Chromosome Mapping of mCHPT1

To determine whether the diabetes-induced decrease in the putative *mCHPT1* mRNA levels was specific to the kidneys (Fig 3), RNA from other organs in diabetic and normal mice was examined using Northern blot analysis. As shown in Fig 3, putative *mCHPT1* mRNA levels of BALB/c kidney (left lane) were greater than those in KK/Ta kidney (right lane). The levels of putative *mCHPT1* mRNA were similar in the brain, heart, lung, liver, spleen, intestine, and muscle.

In situ hybridization revealed glomerular putative *mCHPT1* mRNA expressions. The levels in BALB/c mice tended to be slightly higher than in KK/Ta mice (Fig 4). A T31 radiation hybrid mapping panel revealed that the putative *mCHPT1* gene was most closely mapped to the *D10Mit94* locus with the highest logarithm of odds (lod) score. The best-fit location was between *D10Mit118* (proximal) and *D10Mit209* (distal). This chromosomal region corresponds to the contig NT-009743.11 on human chromosome 12.

DISCUSSION

DD-PCR was performed to identify genes which are over- or underexpressed in mouse kidney using KK/Ta and BALB/c mice. From the total of 75 differentially expressed cDNAs, 8 (7 previously reported genes and 1 novel gene) were ramdomly selected for detailed characterization (Table 1). Since blood glucose at 0 minutes (fasting blood glucose [FBG]) and blood glucose at 120 minutes (intraperitoneal glucose tolerance test [IPGTT]) levels in KK/Ta mice were significantly greater than those in BALB/c mice at 20 weeks of age (data not shown), these genes might be related with the control of blood glucose or glucose tolerance. These fragments were designated as diabetic nephropathy–associated genes (DN). DN1-3 (Zn-α2-glycoprotein, VEGFR-2, LDH) was overexpressed in the diabetic KK/Ta kidney, DN4-6 (Ezrin, transcobalamin 2, aldo-ketoreductase) did not differ between diabetic KK/Ta and control mice, and DN7-8 (PRIP and unknown) was underexpressed in the diabetic kidney. Since the amplification of cDNA depends mainly on the primer sequence and not on the template concentration, false-positive clones were identified in some cases.

Zn-α2-glycoprotein (Azgp1) is a glycoprotein with a low molecular weight of 38,000 to 41,000 in serum. Previously, Shibata et al[10] indicated that this protein plays an important role as a carrier protein of nephritogenic renal glycoprotein. In 1998, Hirai et al[11] reported that since exogenous lipid-mobilizing factor, which is homologous with the plasma protein

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Human1   MAAGAGAGSAPRWLRALSEPLSAAQLRRLEEHRYSAAGVS-LLEPPLQLYWTWLLQWIPLW   60
Mouse1   MAAGAGARPAPRWVKALGEPLSAAQLRRLEEHRYTAVGES-LFEPPLQLYWTWLLQWIPLW   60
Yeast1                                MRIARIVKHLYQSDDRSFLSNHVLRPFWRKFATIFPLW   38

Human61  MAPNSITLLGLAVNVVTTLVLISYCPTATEEAPYWTYLLCALGLFIYQSLDAIDGKQARR   120
Mouse61  MAPNTITLIGLAINLVTTLVLIFYCPTVTEEAPYWTYLLCALGLFIYQSLDAIDGKQARR   120
Yeast39  MAPNLVTLLGFCFIIFNVLTTLYYDPYFDQESPRWTYFSYAIGLFLYQTFDACDGMHARR   98
                                  *******
Human121 TNSCSPLGELFDHGCDSLSTVFMAVGASIAARLGTYPDWFFFCSFIGMFVFYCAHWQTYV   180
Mouse121 TNSCSPLGELFDHGCDSLSTVFMAIGASIAVRLGTHPDWLFFCSFVGMFMFYCAHWQTYV   180
Yeast 99 TGQQGPLGELFDHCIDSINTTLSMIPVCSMTGMG-YTYMTIFSQFAILCSFYLSTWEEYH   157
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Human181 SGMLRFGKVóD VTEIQIALVIVFVLSAFGGATMWDYTIPILEIKLKIVPVLGFLGGVIFSC   240
Mouse181 SGVLRFGRV–DVTEIQVALVIVFMLSTFGGATMWDYTIPILEIKLKIVPVLGVVGGLIFSC   240
Yeast158 THKLYLAEFCGPVEGIIVLCISFIAVGIYGPQTIWHTKVAQFSWQDFVFDVETVHLMYAFCT   219

Human241 SNYFHVILHGGVGKNGSTIAGTSVLSPGLHIGLIIILAIMIYKKSATDVFEKHPCLYILM   300
Mouse241 SNYFHVILHGGVGKNGSTIAGTSVLSPGLHIGLIIILAIMIYKKSATNMFEKHPCLYTLM   300
Yeast220 GALIFNIVTAHTNVVRYYESQSTKSATPSKTAENISKAVNGLLPFFAYFSSIFTLVLIQP   279

Human301 FGCVFAKVSQKLVVAHMTKSELYLQDTVFLGPGLLFLDQYFNNFIDEYVVLWMAMVISSF   360
Mouse301 FGCVFAKVAQKLVIAHMTKSELYLQDTVFIGPGLLFLDQYFNNFIDEYVVLWIAMVISSF   360
Yeast280 SFISLALILSIGFSVAFVVGRMIIAHLTMQPFPMVNFPFLIPTIQLVLYAFMVYVLDYQK   339

Human361 DMVIYFSALCLQISRHLHLNIFKTACHQAPEQVQVLSSKSHQNNMD   406
Mouse361 DMMIYFTSLCLQISRHLHLNIFKTSCQQAPEQVYKHID   398
Yeast340 GSIVSALVWMGLGLTLAIHGMFINDIIYDITTFLDIYALSIKHPKEI   386
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Fig 2. Comparison of amino acid sequences among murine, human and yeast *CHPT1*. Conservative amino acid substitutions are indicated in bold letters. Asterisks represent the CDP-alcohol phosphotransferase motif (Asp-Gly-X_2-Ala-Arg-X_8-Gly-X_3-Asp-X_3-Asp).

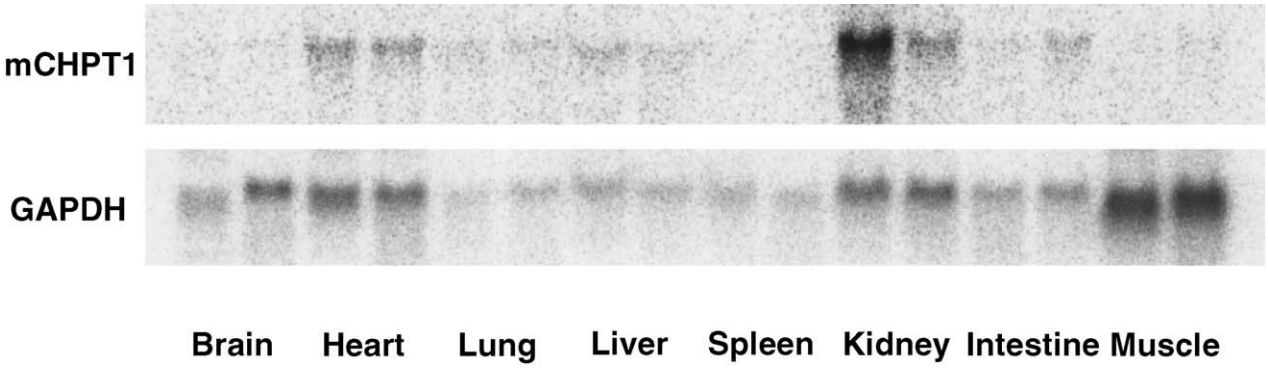

Fig 3. Northern blot analysis of putative *mCHPT1* mRNA expression in the brain, heart, lung, liver, spleen, kidney, intestine, and muscle of BALB/c and KK/Ta mice. Each lane contained 10 μg of RNA from total kidney of BALB/c (left lane, 20 weeks of age) and KK/Ta (right lane, 20 weeks of age) mice.

Azgp1 produces cahectic symptoms such as decreases of body weight or blood glucose and increases of glycerol in the ob/ob mouse model of type 2 diabetes, overexpression of this gene might ameliorate diabetic conditions such as obesity, hyperglycemia, and dyslipidemia.

Page et al[12] reported that LDH mRNA levels in the kidney in the Goto-Kakizaki (GK) rat model of type 2 diabetes at 26 weeks of age was higher than those at 6 weeks of age. This data agrees with the results of the present study. Previously, Nair et al reported that the LDH isoform pattern has changed in patients with diabetic vascular complications.[13] Major disturbances in intracellular lactate/pyruvate metabolism in type 2 diabetes have been reported.[14] Therefore, LDH may also be a candidate gene.

VEGF is a cytokine that potently stimulates angiogenesis and microvascular hyperpermeability.[15,16] VEGFR-1 (fms-like tyrosine kinase receptor, Flt) and VEGFR-2 (fetal liver kinase 1, Flk-1, kinase insert domain-containing receptor, KDR) are receptors of VEGF. Upregulation of VEGF may play a critical role in the progression of diabetic nephropathy. VEGF expression was identified in glomerular and tubular epithelial cells. Treatment with monoclonal anti-VEGF antibody decreased hyperfiltration, albuminuria, and glomerular hypertrophy in streptozotocin (STZ)-induced diabetic rats.[17] The blockade of VEGF receptors may be useful as a therapeutic strategy for treatment of diabetic nephropathy.

Recently, PRIP has been identified as a coactivator of PPARγ.[18] PPARγ is a member of the nuclear hormone receptor superfamily and is secreted in adipose tissue. PPARγ plays a pivotal role in adipocyte differentiation and regulates lipid and glucose homeostasis.[19,20] Recently, thiazolidiones were shown to bind to PPARγ with high affinity and showed efficacy as antidiabetic agents. In mice, the antidiabetic effects are proportional to their binding affinity to PPARγ.[21] Human studies have shown that thiazolidinediones decrease insulin resistance and hypertriglyceridemia.[22]

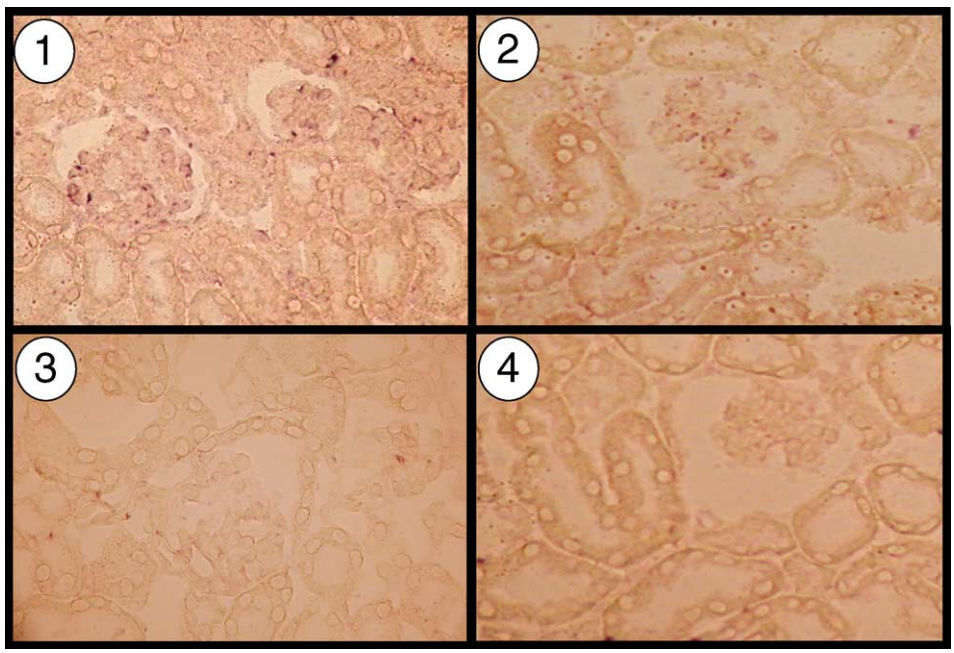

Fig 4. In situ hybridization of putative *mCHPT1* using digoxigenin-labeled cRNA probe (1 through 4). Antisense probe: 1, BALB/c mouse; 2, KK/Ta mouse. Sense probe: 3, BALB/c mouse; 4, KK/Ta mouse.

DN8 is a previously uncharacterized gene with products and roles yet to be determined. We identified the molecular cloning of putative mouse cholinephoshotransferase (DN8), relatively tissue-specific novel gene expression in the kidney. Since the putative *mCHPT1* described here shares 89% identity with *hCHPT1*, this cholinephosphotransferase may be a murine homolog of *hCHPT1*. Although the tissue distribution of putative *mCHPT1* is similar to that of *hCHPT1*, the deduced transcript size of putative *mCHPT1* is about 2.4-fold longer than that of *hCHPT1*. Cholinephosphotransferase catalyzes the transfer of phosphocholine from CDP choline to diacylglycerol (DAG) with the release of cytidine monophosphate (CMP) and the formation of phosphatidylcholine. Recently, Henneberry et al[23] reported that *hCHPT1* synthesizes platelet-activating factor (PAF) and phosphatidylcholine in yeast. However, yeast does not contain ether-linked lipids, and the precise in vivo substrate specificity for the synthesis of ether-linked phosphatidylcholine molecules by *hCHPT1* has not been identified. Since putative *mCHPT1* has the same cytidine diphosphate (CDP)-alcohol phosphotransferase motif as *hCHPT1*, putative *mCHPT1* might also synthesize PAF and phosphatidylcholine. Recently, PAF, a phospholipid mediator of inflammation, has been found to induce enhanced size- and charge-dependent glomerular permeability in experimental animals.[25,26] In KK/Ta mice, putative *mCHPT1* mRNA levels might be decreased to correct this condition. In future, we must determine the specific substrate of putative *mCHPT1* and whether putative *mCHPT1* can actually synthesize PAF. Furthermore, the putative *mCHPT1* gene was most closely mapped to the *D10Mit94* locus (0 centirays from *D10Mit94*) with the highest lod score (lod score > 3.0) and this chromosomal region corresponds to the contig NT-009743.11 on human chromosome 12. *hCHPT1* was positioned on the same chromosome 12. Since Bektas et al[27] reported diabetic susceptibility locus mapped on chromosome 12, putative *mCHPT1* might be a candidate gene for diabetic nephropathy.

In conclusion, using DD-PCR, we identified 5 changes including 1 novel gene in gene expression in the total kidney of the KK/Ta mouse model of type 2 diabetic nephropathy. Identification of these genes and the mechanisms by which their expression is regulated are likely to provide important insights into the overall mechanism responsible for the development of diabetic nephropathy.

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