Isolation and Functional Analysis of Mouse UbA52 Gene and Its Relevance to Diabetic Nephropathy*

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In delineating the mechanism(s) of diabetic nephropathy various novel genes have been isolated, whereas others remain to be discovered. We identified several up-regulated genes in the kidneys of diabetic newborn mice. Among them was UbA52, a ubiquitin ribosomal fusion protein. Its mRNA expression in the kidney was proportional to blood glucose levels. By in situ hybridization and immunohistochemistry, UbA52 was exclusively localized to renal tubules, and its expression was markedly increased in diabetic mice. The up-regulated UbA52 mRNA and protein expression were also observed in Madin-Darby canine kidney cells, a tubular cell line, treated with 30 mM glucose in both cell lysates and ribosomal fractions. To explore the mechanism(s) of its increased expression, UbA52 genomic DNA was isolated. A transcription start site at −22 bp from the initiation codon was identified and confirmed by primer extension analysis. The UbA52 promoter region included glucose response-related E-box sequences and stress response elements (STRE). Unlike in humans, mouse UbA52 gene had no introns in the coding or 5′-ATG-flanking regions. To identify the DNA segment with maximal promoter activity, deletion constructs were prepared using a pSEAP vector system and transfected into COS7 kidney cells. Maximal activity was confined to −198 to +68 bp, which included E-boxes and STRE motifs. A dose-dependent increase in the promoter activity was observed in cells exposed to high glucose. Mutations in the first E-box (CAGCTG → TGGCTG) or STRE (CCCCT → CATCT) resulted in a decrease in the SEAP activity under high glucose ambience. Given the presence of glucose-responsive motifs in the promoter region and decrease in the SEAP activity in E-box mutants in the presence of glucose, these data suggest that UbA52, a ribosomal fusion protein, may be relevant in the pathogenesis of diabetic nephropathy.

Diabetic nephropathy is the most common cause of end stage renal disease in the United States. It is characterized by thickening of basement membranes and mesangial expansion with progression to glomerulosclerosis, tubular atrophy, and interstitial fibrosis, ultimately resulting in renal failure (1). A wide variety of mechanisms in the pathogenesis of diabetic nephropathy have been proposed. They include accumulation of nonenzymatic glycated end products in the kidney, oxidation of renal glycoproteins by reactive oxygen species, intracellular accumulation of sorbitol generated by the reduction of glucose by aldose reductase, activation of protein kinase C-diacylglycerol pathway, involvement of mitogen-activated protein kinase and growth factors, e.g., insulin-like growth factor and transforming growth factor-β, and alterations in renal hemodynamics (2–7). Involvement of such diverse mechanisms would indicate that a vast number of molecules and different signal transduction pathways are involved in its pathogenesis. In this regard, newer molecules exhibiting a transcriptional response to hyperglycemia are being discovered at a rapid pace during the last decade, and their identification has given new insights in the pathogenesis of diabetic nephropathy. The identification of these molecules has been facilitated by the use of various molecular biology techniques that employ the isolation of differentially expressed genes in the hyperglycemic versus the normoglycemic state. Such techniques include gene discovery array, representational difference analysis of cDNA, traditional subtractive or differential hybridization, and differential display (8). The use of these techniques has led to the successful isolation of several known human and mouse genes as well as the unknown genes available in the expressed sequence tag mouse or human NCBI data base. Most of these methods are not well suited for the identification of rare messages because they require large amounts of mRNA. Moreover, if the amount of mRNA is limited, it would require several rounds of amplification and hybridization, resulting in false positive signals. More recently, subtraction suppression hybridization-PCR (SSH-PCR)1 (CLONTECH), a procedure based on the selective amplification of differentially expressed genes in response to a given experimental stimulus, has become available, and it equalizes for the relative abundance of cDNAs within a target population and minimizes the false identification of irrelevant genes.

Utilizing SSH-PCR procedure, nine differentially expressed genes from the kidneys of newborn diabetic mice were isolated. Among them was UbA52, a ubiquitin fusion protein. UbA52, a 128-amino acid fusion protein, is made up of a 52-amino acid 60 S ribosomal protein attached to a 76-amino acid ubiquitin peptide (9). The ubiquitin is highly conserved in various species and is generated in cells by proteolysis of larger proteins containing either polyubiquitin chains or ubiquitin fused to carboxyl extension proteins. In humans, two ubiquitin-carboxyl extension protein genes, UbA80 and UbA52, code for ubiquitin

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1 The abbreviations used are: SSH, subtraction suppression hybridization; MDCK, Madin-Darby canine kidney; RT, reverse transcription; SEAP, secreted alkaline phosphatase promoter; DC, deletion construct; STRE, stress response element.
fused to ribosomal proteins S27a and L40, respectively (9). The relevance of ubiquitin in muscle wasting in diabetes is known, and activation of ubiquitin-proteasome system has been well described in the literature (10). However, the relevance of ubiquitin or the ubiquitin fusion protein UbA52 in diabetic nephropathy remains to be investigated. This paper describes the isolation of mouse UbA52 gene, its expression in the kidney of diabetic mice, and characterization of some of the molecular features of its promoter region that may be relevant in the pathogenesis of diabetic nephropathy.

MATERIALS AND METHODS

Animals and Induction of Diabetes—A hyperglycemic state was induced in pregnant female ICR mice (Harlan Co.) on day 13 of gestation by injection of streptozotocin (200 mg/kg body weight; Sigma). At day 16, blood glucose levels were measured, and mice with blood glucose >250 mg/dl were selected for continuation of their pregnancy. Following birth, blood glucose levels and body weights of newborn mice of diabetic and non-diabetic mothers were determined, and their kidneys were harvested.

Suppression Subtractive Hybridization-PCR—Total RNA was extracted from newborn kidneys of mice by the acid guanidinium thiocyanate-phenol-chloroform extraction method as described previously (11), and poly(A)1 RNA was isolated by employing a Oligotex mRNA kit (Qiagen Co.). First strand and second strand cDNA synthesis, Real endonuclease enzyme digestion, adapter ligation, hybridization, and PCR amplification were performed as described in the PCR-select cDNA subtraction manual (CLONTECH, Ref. 11). The differential PCR products in “tester” cDNA population subtracted from the “driver” were cloned into PCR II vector, sequenced, and subjected to a homology search by using BLAST program (www.ncbi.nlm.nih.gov/BLAST).

Northern Blot Analyses—Total RNA was isolated from kidneys of diabetic and control newborn mice and was subjected to 1.5% agarose gel electrophoresis that contained 2.2 M formaldehyde and capillary transfer onto Hybond N+ nylon membranes (Amersham Biosciences). The prehybridization and hybridization were performed with various [32P]dCTP-labeled (1 × 106 cpm/ml) partial length cDNA fragments derived from SSH-PCR (11). In addition, the total RNA was isolated from kidneys of newborn diabetic mice with different glucose levels in a range from 125 mg/dl to 450 mg/dl, and UbA52 mRNA expression was evaluated by Northern blot using mouse UbA52 cDNA probe.

Isolation of the Fusion Protein and Characterization of the Anti-body-Northern blot analyses several up-regulated genes were isolated by subtractive hybridization from newborn diabetic and control newborn mice. For mRNA expression studies, competitive RT-PCR was utilized. First, a UbA52 competitive PCR template was constructed by using sense (5′-AGA TCT CGT TGA AGA CCC CGA CAT GGA GAG TGC AAG GAG-3′) and antisense (5′-GGG GGG GGA TCC TTT GAC CTT CTT GGG GC-3′) primers. The PCR products were cloned into PCR II vector, sequenced, and subjected to a homology search by using BLAST program (www.ncbi.nlm.nih.gov/BLAST).

For quantitative competitive RT-PCR, aliquots of first strand cDNA, synthesized from RNA of MDCK cells treated with various concentrations of glucose, were mixed with serial dilutions of competitive DNA and co-amplified in the presence of either β-actin- or UbA52-specific primer in a PCR mixture as described previously (14). The PCR products were analyzed by 1.5% agarose gel electrophoreses.

For protein expression studies, immunoprecipitation procedures and Western blot analyses were employed, as detailed previously (11, 12). Briefly, the protein expression was determined in the whole cell lysate as well as in the ribosomal fractions. The MDCK cells treated with various concentrations of D-glucose were lysed with radiolabeled precipitation buffer. The insoluble material was removed by centrifugation at 10,000 × g, and the protein concentration in the supernatant was determined by Bradford assay and adjusted to 1 mg/ml. About 500 μl of the supernatant was mixed with polyclonal anti-UbA52 with gentle agitation at 4 °C for 2 h, followed by the addition of 40 μl of 50% protein A-Sepharose 4B (Amersham Biosciences), and incubation was extended for 2 h at 4 °C. Protein A-Sepharose beads were then washed with radioimmune precipitation buffer. About 30 μl of 2× SDS sample buffer was added to the washed beads, boiled for 5 min, and subjected to 12.5% SDS-PAGE. The gel proteins were transferred onto Nnylon membranes, which were then incubated with rabbit anti-UbA52 polyclonal antibody followed by a second incubation with horseradish peroxidase, and then autoradiograms were prepared by using the ECL detection system (Amersham Biosciences).

Because UbA52 is a protein that is fused with 60 S ribosome, its expression in the ribosomal fraction was assessed in MDCK cells treated with various concentrations of D-glucose. The ribosomes were prepared following the method of Sherton and Wool (15). The ribosomal fractions were extracted from the ribosomes and subjected to immunoprecipitation procedures and Western blot analyses as described above.

Cloning of Mouse UbA52 Genomic DNA—Mouse genomic UbA52 DNA was isolated and cloned in two steps. First, 5′-flanking region of the mouse UbA52 gene was isolated using the mouse GenomeWalker kit (CLONTECH) following the vendor’s instructions. Utilizing primers that were flanked a specific antisense cDNA (5′-GGC TCG ACC TCA AGA GT-3′) and sense primer 5′-GAT GGT CTT GCC CGT CAG GGT CGT-3′, and the PouII DNA library of the kit, a PCR product of ~1 kb was generated, sequenced, cloned into pCR II vector, and designated pCR/UbA52DNA. This PCR product included the 5′-flanking region upstream of the open reading frame and the initiation ATG codon. In the second step, PCR were carried out to isolate the remaining 3′ end of the UbA52 gene. Using the mouse photographic emulsion (Kodak), and the autoradiograms were prepared after 3 weeks of exposure. The control included tissues hybridized with sense riboprobes. For immunofluorescence microscopy, 4-μm-thick cryosections were prepared from kidneys of 3-week-old mice as well. The tissue sections were incubated with rabbit polyclonal anti-UbA52 antibodies that were prepared by a secondary incubation with fluorocyanoate-conjugated goat anti-rabbit IgG antibody. The sections were then examined with a UV microscope.

Effect of High Glucose on UbA52 Expression in Madin-Darby Canine Kidney Cells—Madin-Darby canine kidney (MDCK) cells (ATCC), a well differentiated renal tubular epithelial cell line, was used for further studies. The expression of UbA52 was examined in high glucose ambiance. The MDCK cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone Labs), 100 units/ml penicillin, 100 units/ml streptomycin (Invitrogen) in an atmosphere of 5% CO2 and 95% air at 37 °C. At the cell confluency of 70–80%, the concentration of fetal bovine serum was reduced to 0.5%. The concentration of D-glucose in the medium was adjusted to 5–30 mM, and the cultures were maintained for 24 h. 1-Glucose (30 mM) was used as control. The cells were then collected and processed for UbA52 mRNA and protein expression studies.

For mRNA expression studies, competitive RT-PCR was utilized. First, a UbA52 competitive PCR template was constructed by using sense (5′-AGA TCT CGT TGA AGA CCC CGA CAT GGA GAG TGC AAG GAG-3′) and antisense (5′-GGG GGG GGA TCC TTT GAC CTT CTT GGG GC-3′) primers. The PCR products were cloned into PCR II vector and designated the competitive PCR template. Using this competitive mutant plasmid DNA, the expected sizes of the PCR products for UbA52 and β-actin were 217 and 274 bp, respectively. Whereas in wild type cDNA, the expected respective sizes of the PCR products were 434 and 430 bp. For quantitative competitive PCR, aliquots of first strand cDNA, synthesized from RNA of MDCK cells treated with various concentrations of glucose, were mixed with serial dilutions of competitive DNA and co-amplified in the presence of either β-actin- or UbA52-specific primer in a PCR mixture as described previously (14). The PCR products were analyzed by 1.5% agarose gel electrophoreses.
genomic 129 Svl DNA (Jackson Laboratories), Ub-C(SE) sense (5’-CAG ACC CCA ACA TGC AGA TCT TCG TG-3’) and Ub-C(AS) antisense (5’-ACC ACA GCT TTA TTT GAC CTT CTT CTG GGC TCG ACC TCA AGA GT-3’) primers, a PCR product of ~0.4 kb was generated, which represented the 3’ end of the ~1.4-kb UbA52 gene. The PCR product was cloned, sequenced, and analyzed for various motifs using the NCBI web site (www.motif.genome.ad.jp/MOTIF.html).

**Primers and Site-Directed Mutagenesis**

 Primer Extension Analysis—To determine the transcription start site(s) of the mouse UbA52, a primer extension reaction was carried out. The total RNA was extracted from mouse kidney, and after determining its integrity, mRNA was isolated (11). An antisense primer (UB-G1N (AS), 5’-GAT GGT CTT GCC CGT CAG GGT CTT-3’) was derived from downstream of the 5’ end of open reading frame of UbA52, was synthesized. The primer (10 μmol/μl) was labeled with [γ-32P]dATP (3,000 Ci/mmol; Amersham Biosciences) and purified by ethanol precipitation. After annealing the radiolabeled primer (~10,000 dpm) with mRNA (0.5 μg) at 58°C for 20 min, the extension reaction was carried out at 42°C for 30 min using 1 unit of avian myeloblastosis virus-reverse transcriptase (Promega) in a reaction buffer containing 2 mM dNTP and dithiothreitol in a total volume of 20 μl. The reaction products were separated by 8% PAGE under denaturing conditions, and autoradiograms were prepared. Various primer extension reaction products were eluted from the gel, purified, cloned into pCR II vector, and then sequenced.

**UbA52 Gene Promoter Analyses**—To identify the minimal promoter region and to understand the mechanism of glucose-induced up-regulation of UbA52, a GreatEscarAPE™ SEAP system (CLONTECH) and various deletion constructs were employed. For generation of deletion constructs, first, five sense Ub-G1(SE) (5’-GGG CTC GAG TCG ACG GCC CCG GCT GGT-3’), Ub-G2(SE) (5’-GGG CTC GAG ATG CAA CTA GAG ACA CA A GCT C-3’), Ub-G3(SE) (5’-GGG CTC GAG TGT ATT TGC CAG GCA CTG GCC-3’), Ub-G4(SE) (5’-GGG CTC GAG GCC TGG ATA GAG TCA TCT G-3’), and Ub-G5(SE) (5’-GGG CTC GAG TTT AGC CTT GAT GAC AAC CT-3’) and an antisense Ub-G1(AS) (5’-GGG GAA GGT ATG TGC TCA GTC GGG GCC TCG ACC TCA AGA GT-3’) primers were synthesized. Xbol (CTC GAG) and HindIII (AAG CTT) sites were included in the sense and antisense primers, respectively. These sites are underlined in the various primer sequences. Using these primers and pCR II UbA52gDNA (see above), various PCR products were generated and cloned into Xbol- and HindIII-digested pSEAP2-Enhancer plasmid vector (CLONTECH) and sequenced. COS7 cells (a cell line derived from African monkey kidney; ATCC) were transfected with various plasmid deletion constructs using LipofectAMINE™ 2000 reagent, and the minimal promoter activity of the UbA52 gene was measured using a GreatEscarAPE™ SEAP fluorescence detection kit (CLONTECH) in the supernatant of the cell cultures. The activities of various deletion constructs were expressed as the percentages of the activity in the deletion construct with the highest promoter activity, which was designated as being 100%.

**Mutation Analysis and Dose-dependent Effect of Glucose on Promoter Activity**—The promoter analysis (see above) indicated that the highest activity was confined to deletion construct 5 (DC5, ~1.98, +68 bp to ~198 bp). Thus, this construct was used to assess the dose-dependent effect of glucose (5–30 mM) on the promoter activity and for the mutational analysis experiments. The SEAP activity of culture supernatants was determined as described above. Next, promoter activity was assessed after creating mutations in the motifs that are relevant to the glucose regulation. The two motifs that were present in the mouse UbA52 promoter and may be involved in the glucose regulation included E-box (CACCTG, ~129 bp to ~104 bp) and putative stress response element (STRE, CCCC, ~154 bp to ~158 bp). Three mutant plasmids were constructed by using a QuickChange site-directed mutagenesis kit (Stratagene). For E-box MUT1, the CAGCTG motif was changed to CAGTGG; for E-box MUT2, CACCTG was changed to TGGCCT; and for STRE, CCCC was changed to CATCT. After confirming the sequence of mutant plasmids, they were then transfected into COS7 cells as described above, and the promoter activity was determined in cells exposed to either 5 or 15 mM glucose for 48 h.

**RESULTS**

**Isolation and Characterization of Differentially Expressed Genes in Streptozotocin-induced Diabetic Mouse Newborn Kidney**—The respective glucose levels of diabetic newborn versus control mice were: 421 ± 29 versus 121 ± 12 mg/dl (n = 50), respectively. Their respective body weights were: 0.58 ± 0.05 versus 1.3 ± 0.07 grams. The SSH-PCR followed by Northern blot analyses revealed nine different cDNA fragments with up-regulated gene expression to varying degrees in the hyperglycemic state (Fig. 1). Nucleotide sequence analysis of cDNAs indicated that all of them are known genes as follows: UbA52 (clone 1), heat shock protein 70 (clone 2), lactate dehydrogenase (clone 3), Rap1b (clone 4), nuclear ribonucleoprotein (clone 5), ferritin L subunit (clone 6), Na,K-ATPase (clone 7), renal-specific oxido-reductase, RSOR (transcript size, ~1.5 kb); column 9, inner mitochondrial membrane, Tim44 (transcript size, ~2.0 kb); column 10, β-actin (transcript size, ~2.2 kb, control).

**FIG. 1. Northern blot analyses of differentially expressed genes in kidneys of normal and diabetic mice.** Nine cDNA fragments were isolated by SSH-PCR and used as hybridization probes for Northern blot analysis of RNAs isolated from normal (N) and diabetic (D) newborn mice. Column 1, ubiquitin, Ub/60S (transcript sizes, ~2.8, ~1.7, and ~0.7 kb); column 2, heat shock protein 70, Hsp (transcript size, ~2.7 kb); column 3, lactate dehydrogenase, LDH (transcript size, ~1.7 kb); column 4, Rap1b (transcript size, ~2.3 kb); column 5, nuclear ribonucleoprotein, Nu-Rb (transcript size, ~1.4 kb); column 6, ferritin-L subunit, Ferritin (transcript size, ~1.2 kb); column 7, Na,K-ATPase, ATPase (transcript size, ~4.0 kb); column 8, renal-specific oxido-reductase, RSOR (transcript size, ~1.5 kb); column 9, inner mitochondrial membrane, Tim44 (transcript size, ~2.0 kb); column 10, β-actin (transcript size, ~2.2 kb, control).
cific oxido-reductase (clone 8), and inner mitochondrial membrane (Tim44) (clone 9). Among all these genes, the up-regulation in the mRNA expression of nuclear ribonucleoprotein (clone 5) was only minimal in the hyperglycemic state. Because SSH-PCR analysis revealed that UbA52 is up-regulated in hyperglycemic state, considerations were given to study the mechanisms involved in the up-regulation of UbA52 by glucose.

**Relationship of UbA52 mRNA Expression with Hyperglycemia—**To investigate a possible relationship between the degree of hyperglycemia and renal UbA52 mRNA expression, Northern blot analyses were performed on the kidneys of newborn mice with different blood glucose levels. Four transcripts of −0.7, −1.7, −2.8, and −4.5 kb were detected under basal conditions, i.e. mice with a blood glucose level of 125 mg/dl. Their sizes corresponded to known sizes of transcripts of UbA52 (0.7 kb), ubiquitin B (1.7 kb), and ubiquitin C (4.5 and 2.8 kb) genes (Fig. 2A, lane 1). Their expression increased proportionally to the blood glucose levels (Fig. 2A, lanes 2–4) and a ~5-fold increase in expression in diabetic mice with a blood glucose level of 450 mg/dl, suggesting a relationship between hyperglycemia and renal ubiquitin expression in the diabetic mice kidneys. No change in the β-actin expression was observed (Fig. 2C).

**Characterization of Its Recombinant Protein and Kidney Expression—**A high level of protein expression was observed in Escherichia coli BL21(DE3) cells that were transfected with pET15b/UbA52 plasmid when induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside. Multiple bands were observed in total cell lysate subjected to SDS-PAGE, and a prominent band of ~17 kDa was observed as well (Fig. 3A, CLONE.1 and CLONE.2). This band was not visualized when vector alone was used for transformation (Fig. 3A, VECTOR). Following the purification of proteins in the lysate by nickel column chromatography, a single band of ~17 kDa was observed (Fig. 3B, CLONE.1 and CLONE.2). The excess ~2 kDa of mass is presumably derived from the His6-tag. The fusion protein was used for generation of a rabbit polyclonal antibody, and its authenticity was confirmed by Western blot analysis. A major band of ~17 kDa was observed for the recombinant protein generated from two different cDNA clones (Fig. 3C, CLONE.1 and CLONE.2), suggesting that the antibody is specific for the UbA52 fusion protein.

**Tissue Expression of UbA52 in Kidneys of Streptozotocin-induced Diabetic Mice—**In vivo expression of UbA52 in the kidney of diabetic mice was evaluated by *in situ* hybridization and immunohistochemistry. Although the kidneys of nondiabetic control animals showed a restricted hybridization signal to mid-cortical renal tubules (Fig. 4, A and B), UbA52 mRNA expression in diabetic newborn mice kidneys increased notably and was seen throughout the cortex (Fig. 4, E and F). Immunofluorescence studies revealed a parallel increase of UbA52 protein expression in the renal tubules of diabetic mice (Fig. 4, G and H) compared with control (Fig. 4, C and D). The UbA52 expression was not observed in glomeruli and medullary tubules.

**UbA52 Expression in MDCK under High Glucose Stimulation—**To assess whether the high glucose ambience can directly affect the UbA52 expression, MDCK cells were exposed to high glucose, and UbA52 mRNA and protein expression was determined. The mRNA expression was assessed by competitive RT-PCR. The quantitative RT-PCR analysis revealed a linearity in the ratios of PCR products of wild type UbA52 cDNA to competitive plasmid DNA when plotted against $10^{-1}$–$10^{-7}$ serial logarithmic dilutions of the competitive plasmid DNA, as described previously (14). For β-actin control, a ratio of one was obtained at dilutions of $10^{-7}$–$10^{-4}$ of the competitive plasmid DNA (Fig. 5A). This ratio was similar for cDNA prepared from MDCK cells exposed to 30 mM glucose (Fig. 5B), indicating no change in the β-actin mRNA expression in high glucose ambience. For UbA52 control (5 mM L-glucose or 30 mM D-glucose), a ratio of 1 was obtained at dilutions of $10^{-4}$–$10^{-5}$ of the competitive plasmid DNA (Fig. 5C), although for cDNA prepared from MDCK cells exposed to 30 mM glucose the ratio of 1 was obtained at $10^{-3}$–$10^{-4}$ dilutions of the competitive DNA (Fig. 5D), suggesting a 10–100-fold increase in the UbA52 mRNA population in high glucose ambience. Next, the UbA52 protein expression was assessed in the MDCK whole cell lysate and ribosomal fractions. Immunoprecipitation and Western blot analysis of the whole cell lysate revealed a smear of ubiquinated proteins ranging from ~25 to ~100 kDa. Also, a distinct band of ~16 kDa, corresponding to the molecular mass of UbA52 protein, was observed (Fig. 5E). The intensity of the smeared band was increased to a mild degree for the MDCK cells exposed to 30 mM compared with the control. However, the intensity of the ~16-kDa band was distinctly increased and was ~5-fold higher in cells exposed to high glucose ambience compared with the control. Immunoprecipitation and Western blot analyses of ribosomal fractions prepared from the MDCK cells exposed to normal and high glucose ambience revealed similar results, as observed with the whole lysate (Fig. 5F). The intensity of ~16-kDa band in ribosomal fraction was ~10-fold higher in MDCK exposed to 30 mM glucose compared with the control.

**Characterization of Mouse UbA52 Gene and Mapping of the Transcription Initiation Site—**Using the PouII mouse DNA library provided in the GenomeWalker kit and PCR, a 1289-bp genomic DNA product was obtained, and it contained a 891-bp stretch of 5′- untranslated region and 384-bp segment of the translated region of the UbA52 gene (Fig. 6A). The 5′- untranslated region had no homology with the human UbA52 gene. Analysis of the 5′- untranslated promoter region of UbA52 revealed several consensus sequences and binding sites including AP1, AP2, TATA box, NF-κB, GC box elements, glucose response-related E-box sequences (CAGCGT), and STREs (CCTCC). Another E-box with a palindromic consensus sequence (CAGGTA) with a 4 out 6 match was present 6 nucleotides apart. The total stretch of 18 nucleotides included the E-boxes spanning from ~117 to ~134 bp. Other motifs included GATA-binding factor 2, heat shock factor, CCAAT/enhancer-binding protein, Ras-responsive element-binding protein, and Octamer factor-1 (Fig. 6A). Interestingly, by comparing the cDNA and genomic DNA sequences of mouse UbA52, no introns were found in the 5′-flanking region or within the coding segments of the gene (Fig. 6A).

Primer extension analysis revealed three extension products when Ub-G1N(AS) primer and mouse total RNA or mRNA were used (Fig. 6B). No extension product was observed when tRNA was used. The control RNA provided in the kit revealed an expected 87-bp primary extension reaction product. Among the three extension products, the middle one of 62 bp yielded a distinct band, the analysis of which revealed a major putative transcription start site with the CGGCGG sequence (Fig. 6B, inset). It was located at ~22 bp from the ATG initiation codon and ~200 bp downstream of the TATA box (Fig. 6, A and C). The transcription start site sequence (CGGCGG) of the mouse UbA52 gene was similar to that of humans, but in the latter it was located ~959 bp upstream from the initiation ATG codon (Fig. 6C).

**Promoter Activity Analysis—**To identify the minimal promoter region that regulates the constitutive expression of the mouse UbA52 gene, a series of deletion constructs were generated and designated DC1 ~891, DC2 ~741, DC3 ~592, DC4 ~401, DC5 ~251, DC6
The highest SEAP activity was observed in DC$_5$–198, which included sequences –198 bp of 5’-flanking region +68 bp from the initiation ATG codon. The activity was about ~32 times that of basic pSEAP vector with no insert. Because the highest basal promoter activity was confined in DC$_5$–198 bp that contains STRE and glucose response-related E-box sequences, this construct was used to assay the SEAP activity under different concentrations of glucose ranging from 0 to 30 mM. A dose-dependent increase in the SEAP activity was observed (Fig. 8), and the maximal activity was observed at 15 mM glucose concentration. The latter concentration was used for the mutational analysis of the UbA52 promoter.

As indicated above, the DC$_5$–198 deletion construct included STRE (CCCCT) and glucose response-related E-box sequences (CAGCTG); thus, mutations were created in these motifs (Fig. 8). The mutant plasmids were transfected into COS7 cells, and the SEAP activity was determined under 5 and 15 mM glucose concentrations in the medium. Mutation in the STRE motif (CCCCT to CATCT) induced a remarkable decrease in the SEAP activity at 15 mM glucose concentration compared with the basal activity of the promoter. Mutation in the first E-box (MUT1, CAGC to CAGT) did not result in any significant change in the SEAP activity compared with the basal promoter activity. However another mutation in the first E-box (MUT2, CAGC to TGCT) resulted in a notable decrease in the SEAP activity (Fig. 8). These findings suggest that both of these motifs are involved in the up-regulation of UbA52 in high glucose ambience.

**DISCUSSION**

Diabetic nephropathy plays an important role in the development of end stage renal disease. However, pathophysiologic mechanisms are still incompletely understood. At the molecular level, several genes and their functions in diabetic nephropathy have been characterized, whereas others remain to be discovered. So far, during the last decade, various techniques that are modifications of the original method of differential display have been used to identify the glucose-regulated genes in different organ systems affected by diabetes mellitus. The cells or organs investigated by the use of such modified methods include retinal pericytes, aortic smooth muscle cells, cardiac myocytes, and the kidney (8, 11, 16). Lately, to study the gene regulation in diabetic nephropathy, we have successfully employed a versatile technique, SSH-PCR, yielding 10% spurious signals, and a number of differentially expressed genes have been identified. The current study extends the exploration of the genes that are relevant to diabetic nephropathy, and several genes exhibiting a transcriptional response to hyperglycemia are described in this paper (Fig. 1). Some of them (i.e. Rap1b, a small G-protein; RSOR, a renal specific oxido-reductase; and Tim44, an inner mitochondrial membrane translocase) have been described in our previous publications (11–13).

Interestingly, these genes and the other genes identified in this study, like UbA52, are transcriptionally responsive to various forms of stresses, including oxidant and carbonyl stresses. Both of these stresses are relevant to the pathobiology of diabetic nephropathy and apoptosis, as recently alluded to by Brownlee (17). The isolation of genes responsive to a common transcriptional stimulus, i.e. hyperglycemia, with the use of SSH-PCR underscores its utility in the exploration of differential gene regulation. In this investigation, we focus on the biology of the ubiquitin fusion protein UbA52 with regard to its relevance to diabetic nephropathy.

About 20 years ago, a protein that promoted the differentiation of lymphocytes was isolated, and it is now known as ubiquitin (9). The ubiquitin gene typically exists in two states.

**Fig. 2. Relationship between renal UbA52 mRNA expression and blood glucose levels.** Northern blot analysis of kidneys from newborn mice with a blood glucose of 125 mg/dl (lane 1) shows four different sized transcripts representing UbA52 (0.7 kb), ubiquitin B (1.7 kb), and ubiquitin C (4.5 and 2.8 kb). In diabetic mice, renal ubiquitin mRNA expression increases proportionally to the blood glucose levels (A, lanes 2–4). The kidneys of mice with a blood glucose level of 450 mg/dl (lane 4) have a more than 5-fold UbA52 mRNA expression compared with those with a blood glucose of 125 mg/dl. mRNA expression of β-actin is unaffected by the blood glucose levels (C). B depicts the quality and equal loading of total RNA in various lanes as indicated by the 28 and 18 S bands.
First, as a doublet, e.g. ubiquitin B, or linear repeats in a polyubiquitin chain, i.e. ubiquitin C; second, the ubiquitin gene may be fused to ribosomal proteins, i.e. UbA52 and UbA80 (9, 18, 19). Although ubiquitin has no intrinsic proteolytic activity, it plays an important role in the turnover of cellular proteins by closely regulating their degradation. The latter is a multistep ATP-dependent pathway that involves the activation of carboxyl terminus of ubiquitin, its conjugation with a specific protein, and the addition of ubiquitins to form polyubiquitin, which is then followed by degradation of ubiquitin-tagged protein in 26S proteasome with the release of peptide fragments of the protein and ubiquitin to be reutilized (9, 19). The ubiquitin-dependent proteolysis is responsive to the stimuli of a diverse group of molecules, including glucocorticoids and an inhibitor of NF-κB, IκB (20), suggesting that the ubiquitin-dependent proteolytic pathway is relevant in various pathobiological processes. The NF-κB has an important role in cachexia, the state with skeletal muscle loss, and in support of the role of ubiquitin-proteasome pathway in muscle wasting in diabetes is the seminal work of Mitch and co-workers carried out during the last decade (10, 20, 21). These studies suggest that metabolic acidosis and glucocorticoids are the major contributors to ubiquitin-dependent muscle proteolysis in diabetes mellitus along with the increased transcription of ubiquitin. This catabolic state of muscle wasting seems to be the major phenotype in diabetes mellitus, even when there may an abundance of anabolic growth factors in circulation, like in this study where diabetic newborn mice exhibited a substantial loss of body weight (0.58 ± 0.05 g versus 1.3 ± 0.07 g).

In addition to the skeletal muscle, an increased transcription of ubiquitin in cardiac musculature has been reported in diabetes mellitus (22). Moreover, it seems that there is a systemic increase in gene transcription, because high blood levels of ubiquitin have been found, and they have inverse correlation with the decrease in the muscle action potential in diabetes mellitus (23). The above studies mainly address the pathobiology of ubiquitin, whereas the current observations describe the relevance of ubiquitin fusion protein, UbA52, in diabetes mellitus. Moreover, the increased transcription of UbA52 in the kidneys of diabetic mice and its renal mRNA expression rising proportionally to blood glucose levels of newborn diabetic mice (Fig. 2) relate to an important organ-specific complication of diabetes mellitus, i.e. diabetic nephropathy. Therefore, it should be noted that in previous studies a ubiquitin-like pro-
tein, unrelated to UbA52, was found to be differentially expressed in diabetic rats with hyperglycemia (16).

Aside from the above studies, there is no report available in the literature describing the distribution of UbA52 in the kidney. In the present study, we could show by in situ hybridization and immunofluorescence techniques using an antibody generated against its recombinant protein (Fig. 3) that in vivo expression of UbA52 is confined to the renal tubules (Fig. 4). Moreover, its mRNA and protein expression is notably up-regulated in the tubular compartment of the kidneys of diabetic mice. In context of tubular expression of ubiquitin ligase (24), Nedd4, a protein with WW domain and ligase activity (25), and ubiquitin-conjugating enzyme E2 (26), selective expression of UbA52 in the renal tubules would suggest that the ubiquitin-proteasome proteolytic system is indeed operative in this compartment of the kidney and might play an important role in diabetic nephropathy. To assess whether tubular cells are responsive to high glucose ambience directly, MDCK cells, a tubular epithelium-derived cell line, were employed. By RT-PCR analyses, a 10–100-fold increase in UbA52 mRNA expression was observed at 30 mM glucose in the culture medium (Fig. 5, A–D). In addition to the transcriptional effect on UbA52, increased translation reflected by the increased intensity of the ~16-kDa band in the cell lysates was also noted (Fig. 5E). In addition, increased intensity of the smeared band was observed, which may be due to the cross-reactivity of UbA52 antibody with other ubiquitinated proteins or polyubiquitin. Nevertheless, ~16-kDa band seems to correspond to the UbA52 ribosomal protein, because an identically sized band with increased intensity was observed in the ribosomal fraction of the glucose-treated MDCK cells (Fig. 5F). A similarly sized band, corresponding to UbA52 protein, has been observed in the ribosomal fractions prepared from various stages of development of Drosophila melanogaster (27). The presence of the single ~16-kDa band in our studies is intriguing, because usually during the proteolytic processing the co-synthesized ubiquitin is removed. It is conceivable that this band represents a protein that underwent post-translational conjugation of carboxyl extension protein 52 to ubiquitin.

The next question that needs to be addressed is the mechanism by which glucose increases the transcription of UbA52. It is known that insulinopenia accompanied with acidosis contributes to cachexia (28), although hyperinsulinemia and hyperaminoacidemia ameliorate the loss of skeletal mass and decrease the transcription of ubiquitin (29), indicating thereby that one needs to search for the promoter elements of the gene that could regulate insulin signaling, such as E-box (30). Another rationale to investigate the promoter analysis of UbA52 is based on the studies indicating that insulinopenia and glucocorticoids increase the transcription of ubiquitin C by involving Sp1 promoter site (31). The mouse UbA52 promoter has some of the transcription factors, such as Sp1, Ap1, and NF-kB, which are similar to those in the human homologue (32) and in ubiquitin C0 (33). Unlike the human UbA52 promoter that shares features of many of the ribosomal genes, the mouse UbA52 included canonical TATA box sequences and consensus sequences for STRE, E-box, and heat shock factor elements (Fig. 6A). The transcription start site sequence (CGGCCG) was similar, as assessed by primer extension analysis (Fig. 6B); however, unlike in human UbA2, the mouse UbA52 had no introns downstream of the transcription site (Fig. 6C). Interestingly, no introns were found in the coding region of the UbA52 gene, indicating that the whole mouse gene is made up of a single exon, unlike that of humans, which is made up of three exons (32). The mouse cDNA sequences in the open reading frame revealed 85% homology with human UbA52. Finally, the mouse UbA52 protein sequence was identical to that of the human and also of the UbA80 up to the 60th amino acid residue (34).

For identification of the minimal promoter region, deletion constructs of the 5′-flanking region of mouse UbA52 were prepared and transfected into COS7 cells (a cell line derived from kidney cells), and SEAP activity in the culture medium was determined (Fig. 7). Basal promoter activity was confined to the +68 to –198 bp, i.e., in the DC5–198 construct. This segment included STRE (CCCCCT) and two E-box (CAGCTG and CAGGTA) motifs that are known to be responsive to glu-
FIG. 6. Nucleotide sequence analyses of genomic DNA of mice UbA52 gene. A, nucleotide sequence of the mouse UbA52 genomic DNA. The base pairs downstream from the initiation ATG codon of UbA52 are given as positive numbers. The base pairs upstream of ATG codon are labeled as negative numbers. The mouse UbA52 cDNA sequence is included as the second line under the genomic DNA sequence starting from the $GATA-2$, GATA-binding factor 2; HSF, heat shock factor; C/EBP, CCAAT/enhancer-binding protein; RREB-1, Ras-responsive element-binding protein; Oct-1, Octamer factor-1. 

B, primary extension analysis showing three transcription start sites (arrowheads) similar to that of the human UbA52 gene. Among the three extension products, the middle one of 62 bp yielded a distinct band, and its nucleotide sequence (CGGCCG) is similar to that indicated above (inset).

C, the nucleotide sequence of the putative transcription start site (CGGCCG) of mouse UbA52 gene is similar that of the human. However, it is located $-959$ bp upstream from the initiation ATG codon in human. Also, the mouse UbA52 lacked the 940-bp intronic segment upstream of the 5'-flanking region.
Among these two palindromic E-boxes, the first one has a perfect canonical CAGCTG sequence, whereas the second one has an imperfect consensus sequence with a match of 4 of 6 nucleotides. Relative to the construct DC5/H11002198, 75% of the SEAP activity could be measured in the DC5/H11002592 construct, which may be partly attributed to the presence of additional STRE elements responsive to carbonyl stress (Fig. 7). With the use of the DC5/H11002198 construct, a dose-dependent increase in the SEAP activity was observed (Fig. 8), and like the promoter activity of glucagon receptor, the maximal activity was observed at a 15 mM concentration of glucose (35). This is intriguing because the maximal increase in the UbA52 mRNA expression is observed with 30 mM glucose in the mesangial cell culture. These differences may be attributed to a number of factors, including that the SEAP activity was determined in COS7 cells and that the expression of glucose transporters between these two cell lines may not be comparable.

The stretch spanning the two E-boxes is the major groove in the target DNA, and it serves as the contact site for the interaction between specific amino acids and the nucleotide bases. Such palindromic E-boxes have been reported in promoters of other genes that are transcriptionally regulated by high glucose ambience. They include pyruvate kinase L, Spot14 (a lipogenesis-associated protein), and glucagon receptor (35–37). They are expressed in the liver, the major site for the transcription regulation of glucose. In pyruvate kinase L and Spot14 gene, the E-box core motifs constitute the carbohydrate response elements and are known as GIRE and ChoRE, respectively (36, 37). The location of glucose regulatory elements containing the E-box motifs varies considerably among the three genes, e.g., from −144 to −168 nucleotides in pyruvate kinase L and from −1431 to −1448 nucleotides in Spot14, with an intermediate location for the glucagon receptor, i.e., from −527 to −545 bp. In the UbA52 the stretch of glucose regulatory elements containing E-boxes seems to be confined between −117 and −134 bp (Fig. 6A).

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The presence of STRE and E-boxes in the mouse UbA52 gene led us to define their role in the transcriptional regulation of glucose. In the glucagon receptor gene, the two E-boxes with CAGCTG and CAGCTG sequences are believed to be essential for promoter activity (35), because a mutation in the CAGCTG motif to CAGTTG results in a decrease in the activity of the reporter gene. Similar observations were made for the UbA52 gene.2 L. Sun, X. Pan, J. Wada, C. S. Haas, R. P. Wuthrich, F. R. Danesh, S. S. Chugh, and Y. S. Kanwar, unpublished results.

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**Fig. 7.** Promoter analysis of mouse UbA52 gene. Schematic representation of mouse SEAP activity of UbA52 promoter (bar graphs) corresponding to reporter gene constructs with sequential deletions are given on the left side of the diagram. Five deletion constructs of pSEAP2-enhancer/UbA52 promoter were generated by PCR using sense and antisense primers. 48 h after transfection with the constructs into COS7 cells, the media were collected to measure the SEAP activity. The control included the pSEAP2-basic vector, which contained neither SV40 enhancer nor promoter. The highest enzyme activity of SEAP is seen in the DC5 −198 deletion construct, and it is ~32 times higher than that of the pSEAP2-basic vector. The activity in this construct is significantly higher than other deletion constructs. *, p < 0.01. The data are presented as the means ± S.D. (n = 4).

**Fig. 8.** Effect of various concentrations of glucose on the SEAP activity and mutation analysis of STRE and E-box. The SEAP activities in COS7 cells transfected with DC5 −198 plasmid are given as bar graphs with the means ± S.D. (n = 4). The highest activity is seen at 15 mM glucose concentration in medium. The experiments were performed in quadruplicate. The mutation in the STRE element (CCCTC to CATCCT) resulted in a partial decrease in the SEAP activity. Similarly, the MUT2 mutation in the E-box (CAGCTG to TGCTG) resulted in a significant decrease in the SEAP activity, *, p < 0.01. However, the MUT1 mutation in the E-box (CAGCTG to CAGTTG) did not cause any significant change in the SEAP activity. WT, wild type.
gene, where a mutation in CAGCTG motif to TGGCCTG resulted in a marked decrease in the reporter gene activity at 15 mM glucose concentration, suggesting a role for the E-box in the biology of UbA52 gene relevant to diabetes mellitus (Fig. 8). However, no change in the reporter activity was observed when mutation in the single base pair, i.e., CAGCTG to CAGTTG, was introduced. This may suggest that mutation in two contiguous base pairs or alternatively the purine base substitution is required to perturb the functions of E-box confined to the major groves in the target DNA. In addition to the E-box, mutational analyses were also carried for 18 bp upstream STRE (CCCCCT) motif. Ubiquitin mRNA expression is a major stress-induced transcript in mammalian cells (38). Some of the physiologic and pathologic stresses that increase its expression include exercise, heat, ischemia, uncoupling of oxidative phosphorylation, alterations in pH, calcium and glucose metabolism, and reactive oxygen species-induced proteolysis (9, 10, 19). Given the fact that oxidant stress is believed to be a common denominator in the pathobiology of diabetes mellitus, the role of the STRE motif in the UbA52 reporter gene activity seemed worth investigating. Like in manganese superoxide dismutase gene (39), a mutation in CCCCCT to CACTCT in at 15 mM glucose concentration resulted in a marked suppression in UbA52 reporter gene activity (Fig. 8), suggesting a role for the STRE motif in the hyperglycemic state or in cells subjected to high glucose ambience.

In summary, the findings of this study indicate that the ubiquitin fusion protein UbA52 is another important molecule relevant to the pathobiology of diabetic nephropathy, and its transcription regulation is modulated by the characteristic elements embedded in its promoter region. It is anticipated that the observations made in this study give an impetus to search for novel UbA52-interacting molecules, the biology of which could further enhance our understanding of the pathogenesis of diabetic nephropathy.

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