Role of Upstream Stimulatory Factors in Regulation of Renal Transforming Growth Factor-β1

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We previously identified an E-box to be implicated in high-glucose–induced transforming growth factor-β1 (TGF-β1) gene stimulation in murine mesangial cells. In the present study, we evaluated the role of upstream stimulatory factors (USFs) in mediating glucose-induced stimulation of TGF-β1. Mesangial cells cultured in glucose concentrations exceeding 2.7 mmol/l D-glucose exhibited increased levels of USF1 and USF2 protein by Western analysis and electrophoretic mobility shift assay (EMSA). An E-box element from the murine TGF-β1 promoter revealed USF1 and USF2 binding by EMSA. Chromatin immunoprecipitation assay revealed in vivo binding of USF1 to a glucose-responsive region of the TGF-β1 promoter. Transient cotransfection studies of 293 cells with USF1 led to a twofold increase in TGF-β1 promoter activity and a 46% increase in secreted TGF-β1 protein levels. Wild-type and USF2 knockout mice exhibited a 2.5-fold stimulation of renal TGF-β1 expression upon fasting and refeeding with a carbohydrate-rich diet, whereas USF1 knockout mice exhibited only a minimal increase of renal TGF-β1 upon refeeding. USF1 mRNA levels were increased in mouse kidneys with carbohydrate refeeding, and USF1 protein was increased in diabetic rat kidneys compared with controls. We conclude that USF1 is stimulated by modest increases in glucose concentration in murine mesangial cells, bind to the murine TGF-β1 promoter, contribute to carbohydrate-induced renal TGF-β1 expression, and may play a role in diabetes-related gene regulation in the kidney. *Diabetes* 54:1976–1984, 2005

Alterations in glucose concentration regulate a wide variety of genes in various tissues. Glucose-regulated genes may be related to a physiologic response to a changing environment, as in fasting-carbohydrate refeeding, or may underlie the basis for organ dysfunction when fluctuations in glucose concentration are frequent and of an extreme nature, i.e., diabetes. In the fasting-refeeding model, there is a physiologic stimulation of the liver-type pyruvate kinase (L-PK) gene in the liver to metabolize the increased glucose load. The primary element in the L-PK promoter is a CACGTG element or E-box that is critical to the carbohydrate response element or glucose response element (1).

The upstream stimulatory family (USF) of transcription factors has been considered to play an important role in modulating glucose-induced regulation of L-PK mRNA gene expression in livers of mice undergoing fasting-refeeding (2,3). USFs belong to the basic helix-loop-helix leucine zipper family of transcription factors characterized by a highly conserved COOH-terminal domain responsible for dimerization and DNA binding (rev. in 4). USFs have been implicated in regulating several genes that are considered to be glucose sensitive, including L-PK, S-14 (2), fatty acid lipase (5), and osteopontin (6,7). Recently, the role of the carbohydrate response element–binding protein (ChRBP) was found to be important for regulating hepatic genes by high carbohydrate intake compared with a standard diet (8).

In the context of diabetes complications, the profibrotic cytokine transforming growth factor-β (TGF-β) has been implicated in the development of diabetic kidney disease (9). Elevated levels of high glucose in vitro and in vivo lead to excess mesangial matrix production that is largely due to enhanced production of TGF-β1 (10–12). We have previously demonstrated that mesangial cell gene expression of TGF-β1 is transcriptionally regulated by the ambient glucose concentration (13). The region of maximal glucose responsiveness was between −835 and −406 of the murine TGF-β1 promoter. Within this region, the murine TGF-β1 promoter contains an E-box that exhibits increased binding of transcription factors from cells cultured in high glucose (13). In addition, recent studies have implicated USF factors to be involved in regulation of the human TGF-β1 promoter (14) and in glucose-induced regulation of thrombospondin in rat mesangial cells (15). Given that the USF family of transcription factors are ubiquitously expressed and involved in E-box–containing glucose-responsive genes, we asked if the USFs may also

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ChRBP, carbohydrate response element–binding protein; DMEM, Dulbecco's modified essential medium; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal–related kinase; GFX, GF 109203X; L-PK, liver-type pyruvate kinase; MMC, murine mesangial cell line; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TGF-β1, transforming growth factor-β1; USF, upstream stimulatory factor.

be involved in glucose-regulated murine TGF-β1 gene expression in renal cells and in the kidney.

RESEARCH DESIGN AND METHODS

Cell culture. A murine mesangial cell line (MMC) was used in cell culture studies. Cells were originally isolated from kidneys of SJL/J(H-2) normal mice and transformed with non–capsid-forming Simian Virus 40 to establish a permanent cell line (16). Cells were maintained at 37°C in a humidified incubator with 5% CO_2/95% air and propagated in Dulbecco's modified essential medium (DMEM; GIBCO BRL, Gaithersburg, MD) containing 10 mmol/l D-glucose, 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mmol/l supplemental glutamine. Cells were passaged every 72 h by light trypsinization. Experiments were initiated by resting cells overnight in 0% FCS with DMEM containing 5.5 mmol/l D-glucose. Fresh DMEM was then added containing 0% FCS and varying D-glucose concentrations for 24 h, and nuclear protein was isolated, as previously described (17), for Western and electrophoretic mobility shift assays (EMSA) studies.

In experiments with kinase inhibitors, the inhibitors were added at the same time the glucose concentration was modulated. The inhibitors added were *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) (Biomol, Plymouth Meeting, PA), myristoylated protein kinase inhibitor (14–22) inhibitory peptide for cAMP-dependent protein kinase (PKA) (Biomol), p38 inhibitor SB202190 (GlaxoSmithKline, Philadelphia, PA), extracellular signal–related kinase (ERK) inhibitor PD98059 (Biomol), and the protein kinase C (PKC) inhibitor GF 109203X (GFX) (Biomol).

293 (human embryonic kidney) cells were obtained from the American Type Culture Collection and cultured in DMEMG450/10% FCS medium. For transient transfection studies, 293 cells were transfected with wild-type USF1, wild-type USF2, or empty vector control using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Cells were also cotransfected with the glucose-responsive TGF-β1 promoter-reporter construct pA835-luciferase (kindly provided by Fuad Ziyadeh, University of Pennsylvania) and a plasmid for β-galactosidase (pLENXZ; provided by Dr. Pamela Norton) to standardize for transfection efficiency. After transfection, cells were cultured in normal glucose for an additional 24 h in serum-free conditions. Cells were then harvested, and luciferase and β-galactosidase activity was measured, as previously described (13,18).

Protein analysis. Western blot analyses were performed, as previously described (17), using USF1- and USF2-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear proteins were isolated, as previously described (17). Fifteen micrograms of nuclear protein was loaded on 10% SDS-PAGE gel and immunoblotted with USF1 or USF2 antibodies. The signal was developed by Supersignal West chemiluminesence substrate (Pierce, Rockford, IL). Bands were quantitated using National Institutes of Health Image software for PC (ImageJ) and reported as percent control.

EMSA. For EMSA, the DNA-binding reaction was performed as previously described (13) in the presence of nuclear proteins. An oligonucleotide probe for USFs was obtained commercially (Santa Cruz Biotechnology) and encodes the sequence 5′-CACCCGGTCACGTGGCCTACACC-3′. In separate experiments, an oligonucleotide probe that encoded a putative glucose-responsive element (CACGTG) identified at position −641 to −636 of the murine TGF-β1 gene was used. The sense strand was a 20-mer oligonucleotide (5′-AAGCAG G**CACGTG**GACTCTG-3′) that was chemically synthesized (Bioserve Biotech, Laurel, MD) and annealed to its complementary strand. The probe was end labeled with ^{32}P-ATP (Amersham) using T4 polynucleotide kinase (Promega) (19). Nuclear proteins (10 μg) were incubated with 10^5 cpm of probe for 30 min at room temperature in the buffer containing 12 mmol/l HEPES, pH 7.4; 4 mmol/l Tris-HCl, pH 7.5; 60 mmol/l KCl; 5 mmol/l $MgCl_2$; 1 μg poly dI:C; 5 μg BSA; and 2 μl glycerol. The protein-DNA complex was resolved on a 4% nondenaturing polyacrylamide gel and dried, followed by autoradiography. For supershift experiments, 2 μg USF1 or USF2 antibodies were preincubated with nuclear proteins and the binding buffer for 20 min at room temperature before the probe was added. For antibody controls, antibody to c-myc (Santa Cruz) was also used.

Chromatin immunoprecipitation assay. MMC cells (2×10^6) were cultured in DMEM/10% fetal bovine serum medium. The cells were cross-linked by adding formaldehyde directly to culture medium to a final concentration of 0.1% and incubated for 10 min at 37°C and then stopped by the addition of glycine. The cells were washed with cold PBS twice. Cell pellets were resuspended in cell lysis buffer (5 mmol/l KOH, pH 8.0/85 mmol/l KCl/0.5% NP-40) containing protease inhibitors, and nuclei were pelleted by centrifugation at 5,000 rpm for 5 min. The nuclear protein was lysed with 0.1% SDS buffer and incubated on ice for 10 min. The cell lysate was then sonicated with a power setting of 6.90% (Sonifier 450; VWR Scientific) for 20 pulses then kept on ice for 1 min and repeated three times. The supernatant was collected by centrifugation at 15,000 rpm for 10 min at 4°C and then diluted fivefold with dilution buffer. A total of 100 μl of the sample was used as the input control. The remainder of the samples were precleaned with salmon sperm DNA/protein A agarose slurry. A total of 3 μg of USF1, USF2, or control IgG antibody was incubated overnight with the supernatant fraction at 4°C with rotation. The immune complex with salmon sperm DNA/protein A agarose slurry was incubated at 4°C with rotation for 1 h. Beads were then washed. The immune complex was eluted with 0.1% SDS/0.1M $NaHCO_3$ and treated with proteinase K and reverse cross-linked by heating at 65°C for 4 h. The DNA was purified by QiaQuick spin columns. Five microliters of sample was used as template for PCR, with primer pairs spanning 388 bp of the murine TGF-β1promoterbetween−828and−440(primers:5′CACGCAGATACCATCTA CAGC 3′ and 5′ ACCCATGAGAAATACACGCTT 3′). As a positive control for USF1 and USF2, immunoprecipitation, and DNA binding, primers for Hox4B were synthesized and PCR performed based on a prior report (20).

Nutritional treatment of USF knockout mice. The development and characterization of the USF1- and USF2-deficient mice have been previously described (2,3). For metabolic studies, animals were fed a high-carbohydrate diet for 18 h after a 24-h fasting period. Mice were allowed access to water at all periods. After weighing, mice were killed between 10 and 12 A.M., blood was obtained for glucose testing, and kidneys were snap frozen in liquid nitrogen and stored at −80°C.

RNA analysis. Total RNA was extracted from frozen kidneys by Tri-reagent (MRC, Cincinnati, OH). Northern blot analysis was conducted as previously described for TGF-β1 (21). The probes for TGF-β1 mRNA and 18S have been described in prior studies (21,22). Quantitation was performed via phosphoimager. USF1 and USF2 mRNA levels in mouse kidneys were measured by quantitative real-time PCR using primers specific for mouse USF1 and USF2 and normalized by 18S. The primers used were as follows: for USF1, forward 5′AAG TCA GAG GCT CCC AGG A 3′, reverse 5′CGG CGC TCC ACT TCG TTA T 3′, and probe 5′Fam TT GAG CCC TCC GTT TCT CAT CTC G Tamra 3′; for USF2, forward 5′ AGA CCA ACC AGC GTA TGC AG 3′, reverse 5′ GCT CCT CGA TCT GCT GCC T 3′, and probe 5′ Fam TG CAG CCG CTC TGC CTC CTT GAA G Tamra 3′; for 18S, forward 5′ AGA AAC GGC TAC CAC ATC CA 3′, reverse 5′ CTC GAA AGA GTC CTG TAT TGT 3′, and probe 5′ Fam AG GCA GCA GGC GCG CAA ATT AC Tamra 3′. Quantitative real-time PCR was performed as previously described (23).

Analysis of USF1 and USF2 protein levels in rat kidneys. Streptozotocin-induced (65 mg/kg i.p.) diabetic Sprague Dawley rats and rats with blood glucose level of >300 mg/dl were used. The diabetic rats underwent implantation of insulin pellet (Linplant) to maintain blood glucose levels between 300 and 500 mg/dl and avoid ketosis and weight loss. After 2 weeks of diabetes, rats were weighed, kidneys were isolated, and the kidney cortex was immediately frozen in liquid nitrogen. Kidney cortical protein was isolated as previously described (24), and resolved proteins were immunoblotted with antibodies for USF1, USF2, and β-actin. Quantitation of USF1 and USF2 protein was standardized for β-actin and performed with National Insitutes of Health image software for PC.

Statistics. ANOVA with Bonferroni correction was used for comparison for multiple groups. Statistical analysis between two groups was performed by unpaired *t* test. $P < 0.05$ was considered a significant difference.

RESULTS

Increase in USF1 and USF2 nuclear protein in mesangial cells cultured with varying glucose concentrations. To evaluate whether USFs may increase in response to high glucose, murine mesangial cells were cultured in varying concentrations of glucose in serum-free conditions for 24 h. Western analysis was performed with nuclear protein from MMC cultured in low (2.7 mmol/l), normal (5.5 mmol/l), and high glucose (13.8 mmol/l, 18.3 mmol/l, 25 mmol/l) (Fig. 1). There is an increase of USF1 protein in MMC cultured with high glucose (18.3 and 25 mmol/l) compared with low (2.7 mmol/l) or normal (5.5 mmol/l) glucose (Fig. 1*A* and *B*). USF2 levels did not increase with high glucose (>5.5 mmol/l) compared with normal glucose (5.5 mmol/l) (Fig. 1*C* and *D*). Interestingly, a change in the glucose concentration from 2.7 to 5.5 mmol/l D-glucose resulted in an increase in both USF1 and USF2 levels.

EMSA analysis of nuclear proteins from MMCs cultured in high glucose. To determine whether there was

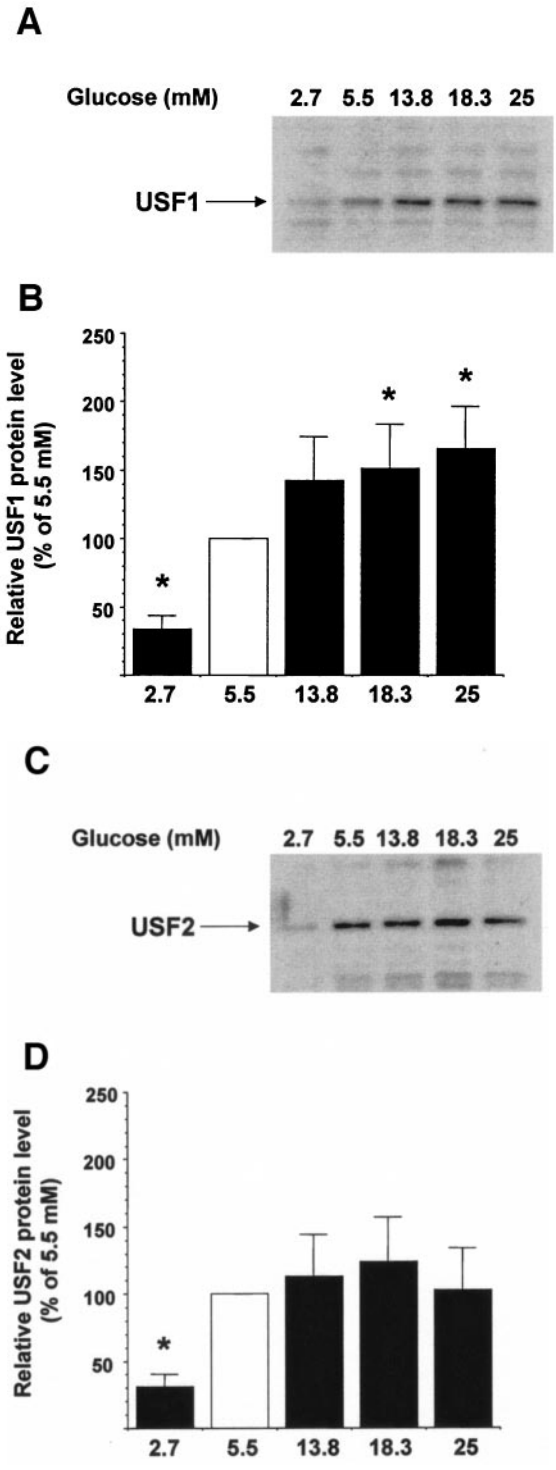

FIG. 1. USF protein in mesangial cells cultured under varying glucose concentrations. Mesangial cells were cultured in DMEM without serum for 24 h with 2.7, 5.5, 13.8, 18.3, or 25 mmol/l D-glucose and harvested for nuclear protein. Nuclear proteins were resolved on SDS-PAGE and immunoblotted with antibody for USF1 (*A*), and the same membrane was stripped and immunoblotted with antibody to USF2 (*C*). The USF1 and USF2 protein levels were quantitated and reported as percent control with 5.5 mmol/l D-gluocose set at 100% (*B* and *D*). Data are presented as means ± SE of three separate experiments. *$P < 0.05$ vs. 5.5 mmol/l D-glucose.

alteration in binding of nuclear proteins from mesangial cells cultured in varying concentrations of glucose, EMSA was performed with a USF consensus element (Fig. 2).

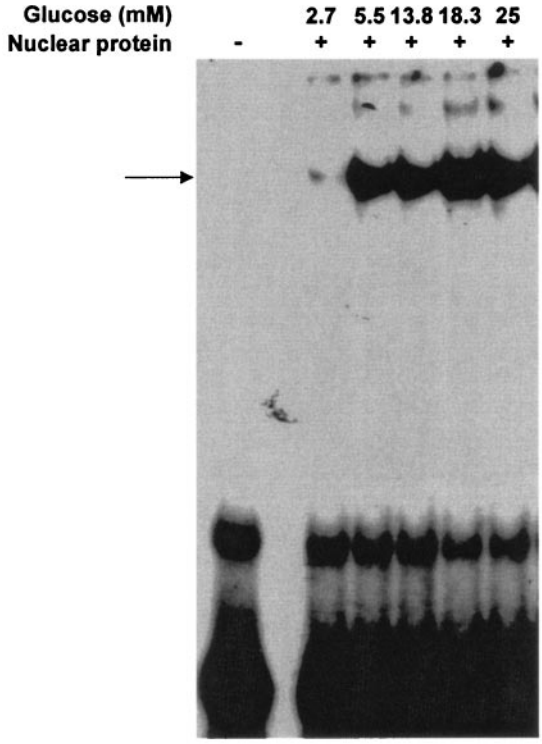

FIG. 2. Nuclear protein binding to USF consensus probe with varying concentrations of glucose. Mesangial cells were cultured in indicated concentrations of D-glucose for 24 h and nuclear protein isolated. EMSA was performed as described in RESEARCH DESIGN AND METHODS with a consensus probe for USF.

There was a marked increase in binding to the USF consensus element as the glucose concentration was increased from 2.7 to 5.5 mmol/l (Fig. 2). There was a further increase with higher concentrations of glucose with maximal binding occurring at concentrations between 18.3 and 25 mmol/l. Specificity of binding of the nuclear proteins to the USF probe is shown in Fig. 3. Excess cold probe for USF completely inhibits all binding of nuclear protein to the USF probe. In addition, excess cold probe containing the E-box motif from the murine TGF-β1 promoter (GRE) markedly reduced binding, whereas excess cold probe for nuclear factor-κB had no effect.

To establish the identity of the nuclear protein complex binding to the USF probe, supershift analysis was performed with antibodies to USF1, USF2, and c-myc (Fig. 3). Addition of antibody for USF1 results in complete supershift of the band to the top of the gel. Antibody to USF2 results in supershift of part of the complex, and antibody to c-myc had no effect. We interpret this result as evidence that the entire complex contains USF1 as USF1/USF2 heterodimers and USF1 homodimers. There appear to be no USF2 homodimers in this complex, as the USF1 antibody caused complete supershifting of the complex. In addition, c-myc did not bind to the USF consensus element in mesangial cells.

USF1 binding is regulated by PKC. It has been previously demonstrated that many effects of high glucose occur via a PKC-dependent pathway (25), including regulation of TGF-β1 expression (26). Other pathways involved in mediating high-glucose–induced gene transcription in-

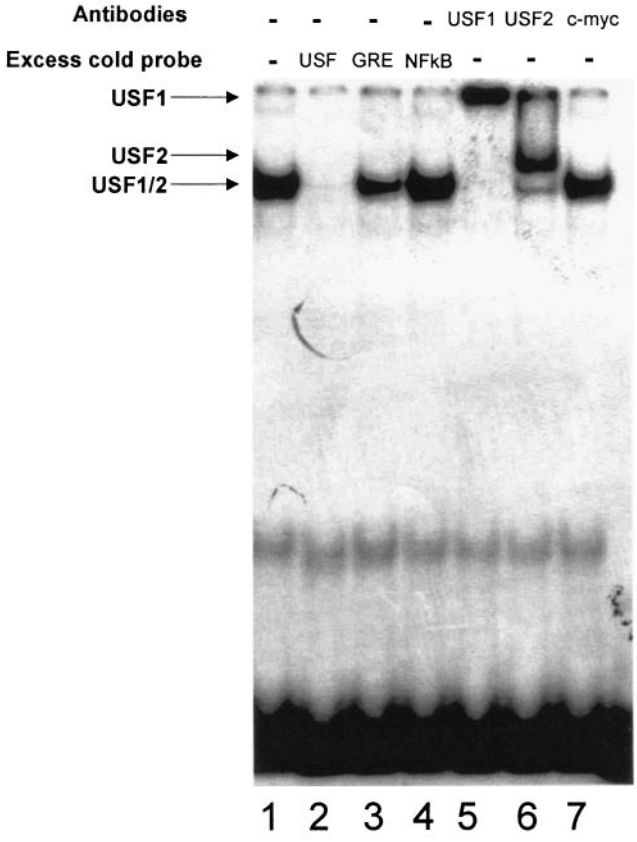

FIG. 3. Identification of USF1 and USF2 in high-glucose–stimulated nuclear protein bound to USF consensus probe. EMSA was performed with nuclear protein from mesangial cells cultured in high glucose (25 mmol/l) and labeled USF consensus probe. One hundred–fold excess cold probe for USF (*lane 2*), TGF-β1 E-box motif (−648 to −629) (*lane 3*), and nuclear factor-κB (NF-κB) (*lane 4*) shows specificity of binding. Supershifting with antibodies to USF1 (*lane 5*), USF2 (*lane 6*), and c-myc (*lane 7*) identifies presence of USF1 and USF2 in nuclear protein extract bound to USF probe. Supershifted USF1 and supershifted USF2 are indicated by respective arrows. USF1/2 complex is indicated by arrow.

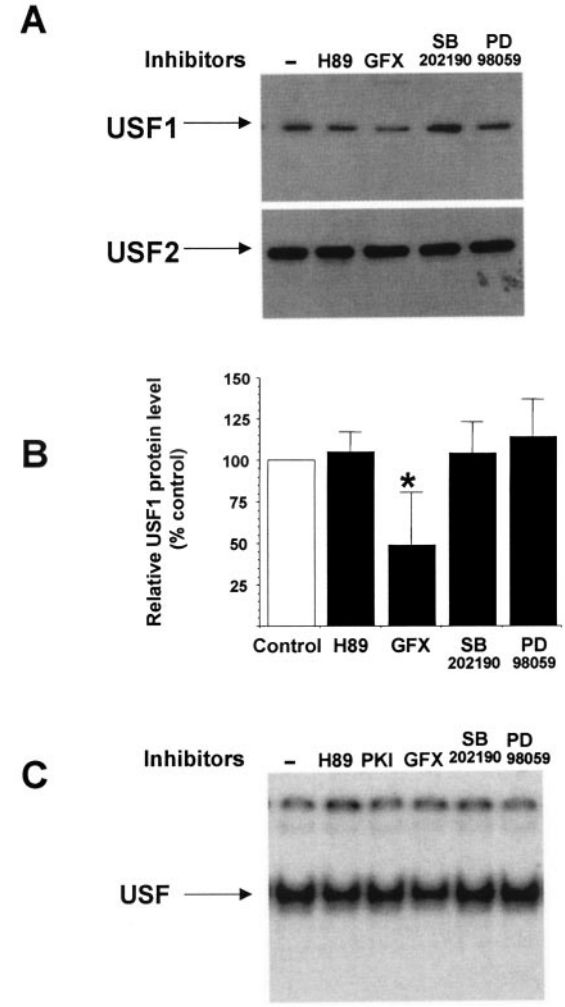

FIG. 4. Regulation of USF protein by kinase inhibitors. *A*: Mesangial cells cultured in high glucose (25 mmol/l) were exposed to vehicle, inhibitors for PKA (H-89 3 μmol/l), PKC (GFX 10 μmol/l), p38 (SB202190 1 μmol/l), and ERK (PD98059 10 μmol/l) for 24 h. Nuclear proteins were isolated and immunoblotted with antibody to USF1 (*upper panel*) and USF2 (*lower panel*). *B*: Nuclear protein USF1 levels were quantitated and reported as percent control with 25 mmol/l D-gluocose set at 100%. Data are presented as means ± SE of three separate experiments. *$P < 0.05$ vs. vehicle control. *C*: EMSA was performed with aliquots of nuclear proteins isolated as indicated above and a radiolabeled probe for USF. PKI was added at a concentration of 10 μmol/l.

clude PKA (27), p38 (28), and mitogen-activated protein kinase–ERK (29,30). To determine which pathway may play a role in high-glucose–induced USF1 upregulation, cells were cultured in the presence of inhibitors for PKA, PKC, p38, or ERK in the high-glucose condition. As noted in Fig. 4, by Western analysis, there was an inhibition of USF1 by the PKC inhibitor GFX (Fig. 4*A* and *B*); however, there was no inhibition by the PKA, p38, or the ERK inhibitors (Fig. 4*B*). Inhibition of USF2 was not found with any of the inhibitors (Fig. 4*A*). By EMSA, there was a reduction of USF binding with the PKC inhibitor GFX but not with the other inhibitors tested (Fig. 4*C*).

The E-box motif of the murine TGF-β1 promoter bind USFs. As we had previously found that nuclear proteins from MMCs grown in high glucose have increased nuclear protein binding to an E-box element in the murine TGF-β1 promoter (13), we determined if this complex also contains USF1 (Fig. 5). High glucose (25 mmol/l) stimulated binding of two major complexes: one corresponding in size to USF and another complex that migrates lower. Adding antibodies to USF1 results in a complete supershifting of the upper band, indicating presence of USF1 in this complex (Fig. 5, *lanes 2 and 5*). Interestingly, the lower band increased in intensity with antibody to USF1, suggesting that USF1 competitively inhibits binding of another transcription factor with predilection for this site. The identity of the lower band remains unknown. Addition of USF2 antibody also resulted in loss of the upper band (data not shown).

In vivo binding of USF1 to glucose-responsive region of the murine TGF-β1 promoter. To determine whether mesangial cells have in vivo binding of USFs to the glucose-responsive region of the murine TGF-β1 promoter, a chromatin immunoprecipitation assay was performed. As shown in Fig. 6*A*, immunoprecipitates with USF1 bound DNA encoding the region −828 to −440 from the TGF-β1 promoter. This region of DNA was not identified with nonspecific IgG or with immunoprecipitates with antibody to USF2. The ability of the antibodies to immunoprecipitate USF1 and USF2 with retention of its DNA-binding properties was demonstrated using primers en-

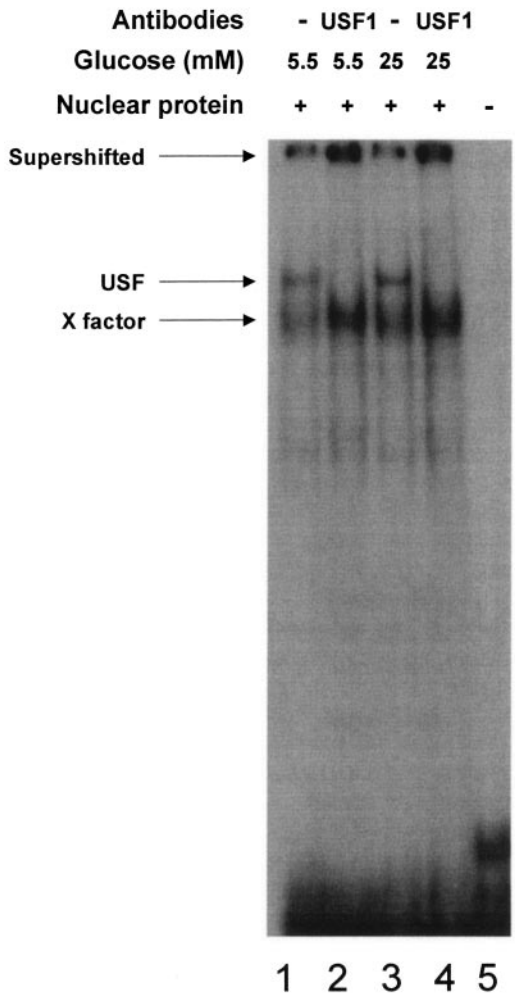

FIG. 5. EMSA with TGF-β1 promoter E-box motif. Nuclear protein from mesangial cells cultured in normal glucose concentration (5.5 mmol/l; *lanes 1 and 2*) or high glucose (25 mmol/l; *lanes 3 and 4*) were exposed to radiolabeled TGF-β1 E-box motif (−648 to −629). *Lane 5* is probe alone without nuclear protein. Supershifting with antibody for USF1 was performed as shown in *lanes 2 and 4*. Supershifted USF is indicated by top arrow. The two major complexes binding the TGF-β1 promoter element are indicated by middle and lower arrows, with upper band indicating USFs (USF arrow) and lower band indicating unknown factor (X factor arrow).

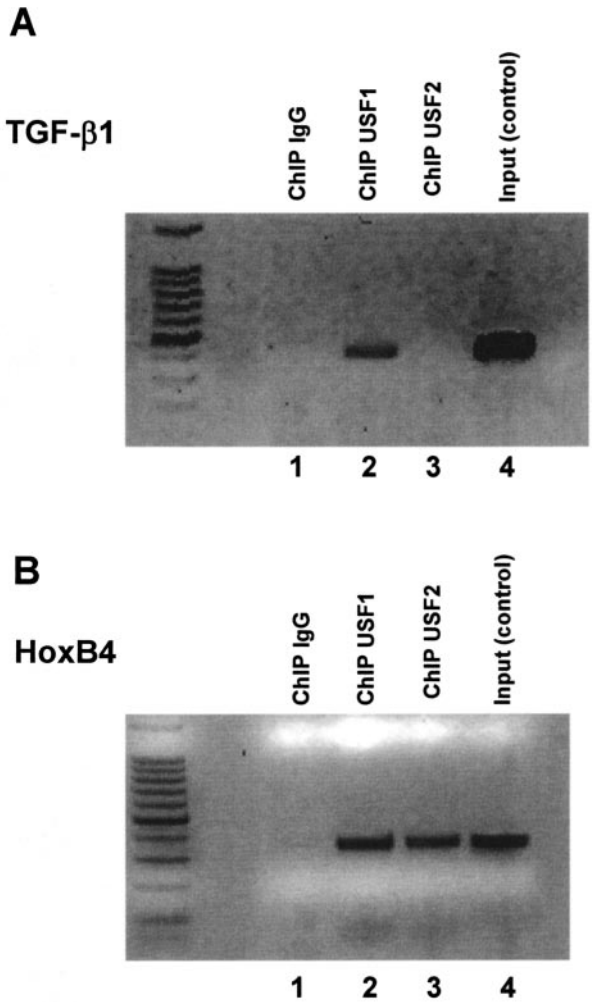

FIG. 6. Chromatin immunoprecipitation analysis showing interaction of USF1 with TGF-β1 promoter. *A*: Immunoprecipitation of mesangial cells cross-linked with DNA was performed with antibody to control IgG (*lane 1*), USF1 (*lane 2*), USF2 (*lane 3*), or input DNA (*lane 4*). Immunoprecipitated DNA-protein complex was subjected to PCR as described in RESEARCH DESIGN AND METHODS with primers specific for TGF-β1 promoter. The expected 388 bp band for TGF-β1 promoter from USF1-immunoprecipitated samples is noted (*lane 2*) but not with USF2-immunoprecipitated samples (*lane 3*). *B*: Immunoprecipitated DNA-protein complex was subjected to PCR with primers specific for HoxB4 promoter. There is a distinct band for HoxB4 (363 bp) with USF1- and USF2-immunoprecipitated samples. The positive control for PCR is shown with input DNA (*lane 4*), and specificity control for antibody is shown in *lane 1*. The molecular weight marker is shown on left.

coding a region of the HoxB4 promoter (Fig. 6*B*) that had previously been identified to bind both USF1 and USF2 (31). The data thus provide evidence that mouse mesangial cells exhibit preferential binding of USF1, versus USF2, to the TGF-β1 promoter in vivo.

Transfection with USFs exhibit increased TGF-β1 promoter activity and TGF-β1 protein secretion. To determine whether USF1 may regulate gene expression of TGF-β1, 293 cells were transiently cotransfected with wild-type USF1, USF2, or an empty vector control along with pA835-luciferaseTGF–β1 and β-galactosidase. The pA835 construct has previously been shown to exhibit glucose responsiveness (13). Cotransfection studies were performed in 293 cells, as the degree of cotransfection in mesangial cells were of low efficiency. As noted in Fig. 7*A*, transfection with USF1 resulted in a twofold increase in the pA835-luciferase promoter activity compared with empty vector. Cotransfection with USF2 also stimulated the murine TGF-β1 promoter but less so than with USF1. USF1-transfected cells also exhibited increased secretion of TGF-β1 protein, whereas USF2-transfected cells did not (Fig. 7*B*).

USF1 knockout mice have reduced carbohydrate-induced renal TGF-β1 expression. To determine whether USF allele status contributed to regulation of renal TGF-β1 expression under conditions of fasting and carbohydrate refeeding, USF1 and USF2 knockout mice were studied (2,3). The body weights and blood glucose from the mice after undergoing fasting or fasting followed by a carbohydrate-rich refeeding is shown in Table 1. The initial body weight in the USF2-deficient mice was significantly lower than wild-type or USF1-deficient mice. Further characterization of the USF2-deficient mice has been reported elsewhere (2). There was a significant increase in blood glucose levels in the fasted–high-carbohydrate

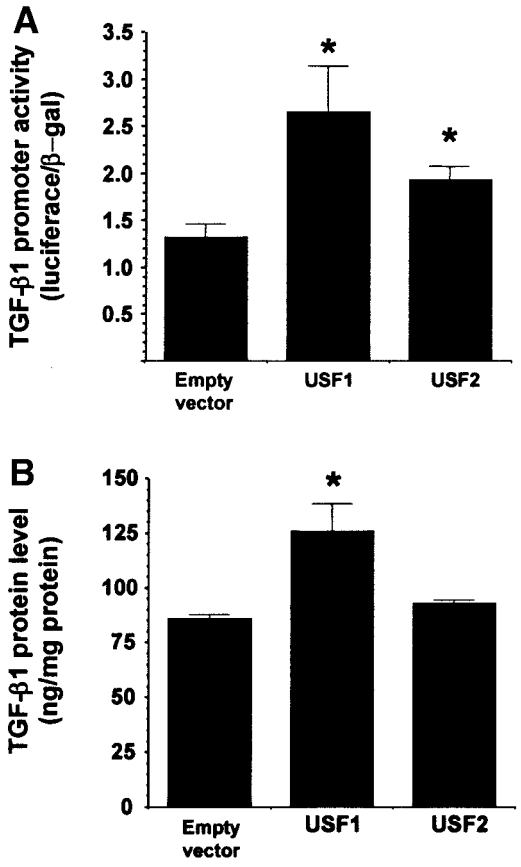

FIG. 7. Regulation of TGF-β1 promoter and TGF-β1 protein secretion by USFs. *A*: 293 cells were cotransfected with pA835-luc (glucose-sensitive promoter construct of TGF-β1 promoter) along with plasmids for empty vector, USF1, or USF2. Cells were additionally cotransfected with plasmid for β-galactosidase to correct for transfection efficiency. *B*: Secreted TGF-β1 protein was measured in conditioned media from transfected 293 cells. Data are expressed as means ± SE from a representative experiment of three separate experiments (n = 6 per condition). *P < 0.05 vs. empty vector.

refeeding groups compared with fasting alone in the various allele groups, but there was no difference between the groups.

Analysis of renal TGF-β1 standardized by 18S for all three groups is presented in Fig. 8. In wild-type mice and USF2 knockout mice, there is an increase of renal TGF-β1 mRNA levels of ~2.5-fold after 18 h of refeeding a high-carbohydrate diet compared with fasted mice. In contrast, mice deficient in USF1 had no increase of renal TGF-β1 expression compared with its fasted counterparts. Renal upregulation of L-PK was significantly increased by threefold in all allele groups with carbohydrate refeeding (data not shown).

Regulation of USFs in kidneys with alterations in blood glucose. To determine whether glucose levels may

TABLE 1
Body weight and blood glucose in mice undergoing fasting-refeeding with high carbohydrate diet

	Body weight (g)		Blood glucose (mmol/l)	
Genotype	Fasting	Refed	Fasting	Refed
Wild type	21.79 ± 3.55	22.58 ± 4.69	4.0 ± 0.17	7.4 ± 0.25*
USF1 KO	22.95 ± 5.48	21.12 ± 4.55	2.3 ± 0.11	7.0 ± 0.05*
USF2 KO	14.30 ± 1.04	14.65 ± 3.28	3.1 ± 0.13	7.3 ± 0.22*

Data are means ± SD. *P < 0.05 vs. fasting blood glucose

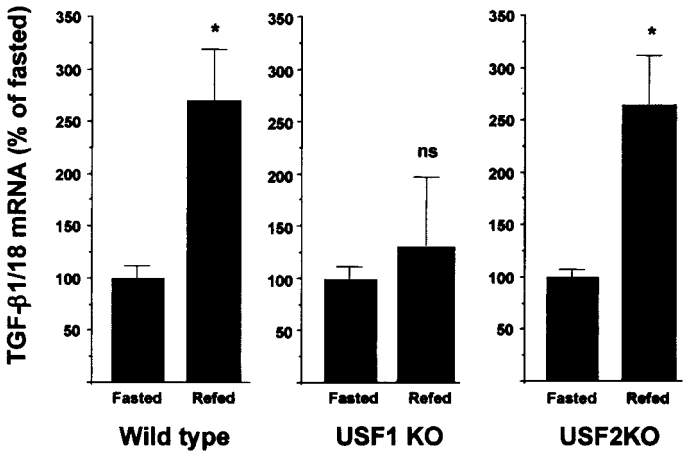

FIG. 8. TGF-β1 mRNA in kidneys of fasted and carbohydrate-refed wild-type mice, USF1$^{-/-}$ mice, and USF2$^{-/-}$ mice. Northern blot analysis with TGF-β1 probe was performed on RNA isolated from kidneys after fasting for 24 h and fasting followed by refeeding a high-carbohydrate diet for 18 h. Quantitative data are shown for wild-type mice, USF1$^{-/-}$ mice, and USF2$^{-/-}$ mice (n = 3 per group). *P < 0.01 vs. fasted.

regulate USFs in vivo, wild-type mouse kidneys that underwent fasting or fasting-refeeding were subjected to real-time quantitative PCR analysis for detection of USF1 and USF2. As shown in Fig. 9*A*, renal USF1 mRNA levels were significantly upregulated with carbohydrate refeeding compared with fasted mice (P = 0.007). Renal USF2 levels were also stimulated, but the data failed to reach statistical significance (P = 0.066).

To further establish relevance to the diabetic state, rats were made diabetic with streptozotocin and studied after 2 weeks of diabetes. Elevated blood glucose levels and weight loss in the diabetic rats were controlled by insulin pellet implantation. Terminal blood glucose values were 8.2 ± 1.5 mmol/l in the normal group and 25.8 ± 3.3 mmol/l in the diabetic group (n = 5 per group). Body weight increased 215 ± 11 g in the normal group and 118 ± 17 g in the diabetic group. Western analysis revealed a 2.3-fold increase in USF1 levels in diabetic rat kidneys, whereas there was no significant change in renal USF2 levels with diabetes (Fig. 9*B* and *C*).

DISCUSSION

In the present study, we demonstrate that USF1 protein is stimulated by glucose in mesangial cells, and USF1 exhibits binding to a glucose-responsive element in the murine TGF-β1 promoter both in vitro and in vivo. USF1 bound to the TGF-β1 promoter element exists as both a homodimer and heterodimer with USF2. Increased expression of USF1 stimulates TGF-β1 promoter activity. Wild-type and USF2 knockout mice exhibit an increase in renal TGF-β1 expression after fasting-refeeding with a high-carbohydrate diet, whereas mice deficient in USF1 have attenuated carbohydrate-induced renal TGF-β1 expression. Renal USF1 levels were increased with carbohydrate refeeding and in diabetic rats. Based on the results of our study, we provide evidence that changes in the glucose concentration, at physiologic and pathologic levels, stimulate a pathway involving USF1 that contributes to an increase in renal TGF-β1 expression.

The USF family of transcription factors has been linked

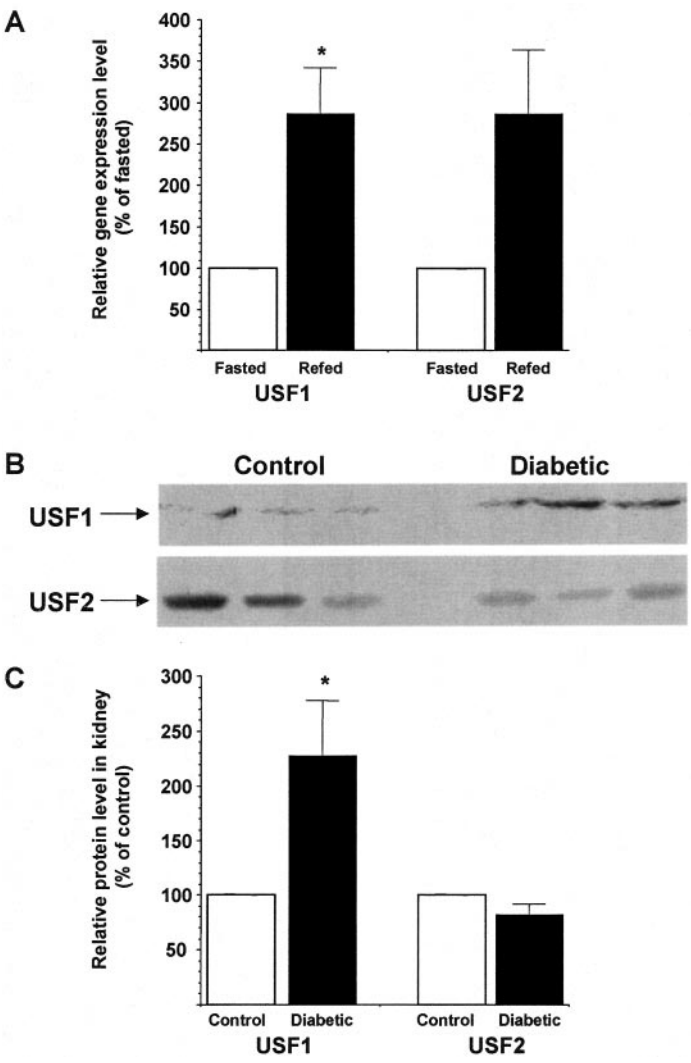

FIG. 9. In vivo regulation of renal USF1 and USF2. *A*: Renal mRNA levels for USF1, USF2, and 18S were analyzed by real-time PCR from kidneys of fasted and carbohydrate-refed wild-type mice. Data are standardized by 18S and expressed as means ± SE from three mice in each group. *$P < 0.05$ vs. fasted. *B*: Representative Western blot of USF1 (*upper panel*) and USF2 (*lower panel*) in kidneys from control and diabetic rats. *C*: Quantitative data of USF1 and USF2 standardized for β-actin are expressed as means ± SE from five rats in each group. *$P < 0.05$ vs. control.

to a variety of glucose-regulated genes in hepatocytes, epithelial cells, and smooth muscle cells. The USFs primarily function as USF1/2 heterodimers and/or USF1 homodimers (32,33). Our data in mesangial cells are consistent with this finding. The relative role of individual USF isoforms is unclear in the context of gene regulation. Mice lacking USF2 have increased embryonic lethality (2), whereas mice deficient in USF1 are phenotypically normal (3,33), possibly due to compensation from USF2 (33). The USF2 knockout mice in the present study had significantly reduced baseline body weight but nevertheless had similar fasting and stimulated blood glucose levels as wild-type mice. In prior studies with fasting–carbohydrate refeeding, USF1 knockout mice had an intact response to carbohydrate refeeding with respect to L-PK and S14 gene stimulation in the liver (3). With respect to the kidney, there appears to be no gross phenotypic abnormalities in the kidneys of USF1 knockout mice (3,33), although prior studies have not evaluated regulation of genes in the kidneys of USF-deficient mice. In the present study, we found that refeeding-induced L-PK gene stimulation in kidneys was not altered in USF1- or USF2-deficient mice. Indeed, a recent study (8) suggested that USFs may not be critical for L-PK gene stimulation.

USFs have not been found to be altered by glucose concentration in liver cells; however, two recent studies have found regulation of USFs by high glucose in human (14) and rat (15) mesangial cells. Our study provides the first evidence that USFs are regulated by normal levels of glucose in mesangial cells, as mesangial cells exposed to 5.5 mmol/l of glucose have an increase in USF1 and USF2 protein and increased USF binding to DNA compared with 2.7 mmol/l (Figs. 1 and 2). Our data are similar to the finding by Bidder et al. (7), wherein USF1 was stimulated by 5 mmol/l D-glucose in aortic smooth muscle cells, although no increase of USF2 was detected. Stimulation of USF1 was also noted with glucose concentrations >5.5 mmol/l in our study, consistent with recent findings of Weigert et al. (14) in human mesangial cells, although Wang et al. (15) found that high glucose induced stimulation of USF2 but not USF1 in rat mesangial cells. The basis for the various findings may be due to species specificity and specific culturing conditions. Of note, our in vivo findings support our in vitro observations. Renal USF1 was stimulated in the model of fasting and carbohydrate refeeding with accompanying blood glucose levels changing from 4.0 mmol/l in the fasted mice to 7.4 mmol/l with carbohydrate refeeding. In addition, diabetic rat kidneys exhibited increased renal USF1 with blood glucose levels varying from 8.2 mmol/l in the normal rats to 25.8 mmol/l in the diabetic rats. The pathway by which high glucose stimulates USF1 appears to involve the classical PKC pathway but not the p38, ERK, or PKA pathway. Involvement of the PKC pathway, primarily PKC-β, has been previously implicated in regulation of TGF-β1 gene expression by high glucose in vitro and in vivo (25,26,34). The specific PKC isoform contributing to USF1 stimulation by glucose remains to be determined.

The mechanisms of how USFs are involved in regulating TGF-β1 gene transcription are unclear. Based on our findings, there is binding of USF1 to a glucose-responsive region of the TGF-β1 promoter, both in vitro and in vivo. The binding occurs at an E-box site located at −641 to −636 of the murine TGF-β1 promoter (13). The role of additional factors that bind to this region of the murine TGF-β1 promoter remain to be identified but likely interact with USFs to regulate TGF-β1 gene transcription. Transcription factors that have been identified to work in tandem with USFs include cAMP response element–binding protein (CREB) and activating transcription factor 1 (ATF-1) (35), whereas COUP-TFII (chicken ovalbumin upstream promoter–transcription factor II) competes for the same binding sites as USFs and represses USF-dependent transactivation of the L-PK promoter (32). A prior report (36) has also provided strong evidence that coactivators confer cell type and promoter specificity for USFs; however, the coactivators remain to be identified. Of interest is that the promoter for the TGF-β2 isoform is coordinately regulated by USF, CREB, and ATF-1 (35,37). Our study provides the first evidence that USFs stimulate

the murine TGF-β1 gene promoter. Weigert et al. (14) recently provided evidence that USFs are involved in regulation of human TGF-β1 promoter in in vitro studies. The relative role of USF1 versus USF2 in regulating the TGF-β1 promoter is likely to be complex. As we found that only USF1 binds to the TGF-β1 promoter element with chromatin immunoprecipitation assay and conferred a greater degree of stimulation of the TGF-β1 promoter, USF1 may play a preferential role to regulate TGF-β1 gene transcription in murine mesangial cells. It is also likely that lower molecular weight factors binding the TGF-β1 promoter (Fig. 5) may play an interactive role with USFs to regulate TGF-β1 promoter activity. The identity of the factor(s) in the lower molecular weight complex is currently under study. ChRBP is unlikely to be present in this complex, as ChRBP has a higher molecular weight than USFs and migrates higher on EMSA (38).

High-glucose–regulated TGF-β1 gene expression has been demonstrated in both glomerular mesangial cells (13) and in tubular epithelial cells (39). In addition, several studies have found that short periods of hyperglycemia (24–48 h) are sufficient to stimulate renal TGF-β1 gene expression in animal models (21,40). Our study is the first to demonstrate that fluctuations in blood glucose, even in the normal range, are sufficient to stimulate renal TGF-β1 gene expression in vivo. The physiologic functional consequence of early upregulation of TGF-β1 expression in response to transient and modest elevation in glucose concentration is unclear. It is interesting to note that not only is TGF-β1 stimulated by glucose levels, but TGF-β1 has also been shown to stimulate glucose uptake in mesangial cells (41,42). A positive feedback relationship between fluctations in blood glucose, USF1 stimulation leading to TGF-β1 upregulation, and consequent increased glucose uptake may predispose renal cells to the pathologic sequelae of hyperglycemia and diabetic nephropathy. Other well-described features of TGF-β include cell hypertrophy, increased matrix deposition, and altered calcium regulation. All these features are relevant to the effects of diabetes in experimental models of diabetic kidney disease, and TGF-β has been shown to play a critical mediatory role (11,22,24). Of note, several other genes implicated in the pathogenesis of diabetes complications, including TGF-β2 (35,37), thrombospondin (15), osteopontin (7), renin (43), and plasminogen-activating factor 1 (44), have also been shown to be regulated by USFs. The regulatory role of USF in controlling key genes involved in diabetes complications, coupled with the recent association of USF1 polymorphism with familial hyperlipidemia and possibly the insulin resistance syndrome (45), makes it highly likely that USFs play a critical role in the development of disorders linked to carbohydrate metabolism. Further understanding of the regulation of USFs by glucose, as well as the role of USFs in regulation of critical disease-causing genes, will likely be of great relevance to diabetes and its vascular complications.

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