Kidney International, Vol. 54 (1998), pp. 1107–1116

Transcriptional activation of transforming growth factor-β1 in mesangial cell culture by high glucose concentration

BRENDA B. HOFFMAN, KUMAR SHARMA, YANQING ZHU, and FUAD N. ZIYADEH

Penn Center for Molecular Studies of Kidney Diseases, Renal-Electrolyte and Hypertension Division, Department of Medicine, University of Pennsylvania, and Division of Nephrology, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, USA

Transcriptional activation of transforming growth factor-β1 in mesangial cell culture by high glucose concentration.

Background. Transforming growth factor-β (TGF-β) is an important hypertrophic and prosclerotic cytokine in the pathogenesis of diabetic nephropathy. The mechanisms of regulation of the TGF-β system by high ambient glucose in kidney cells are incompletely defined. This study examined the mechanisms of regulation of TGF-β1 expression by high glucose in murine mesangial cells (MMCs) in culture.

Methods. MMCs were cultured in either normal (100 mg/dl) or high (450 mg/dl) D-glucose concentration. Total TGF-β1 protein secretion and bioactivity, mRNA expression and stability, and gene transcription rate were measured; promoter-reporter chloramphenicol acetyltransferase (CAT) assays and electrophoretic mobility shift assay (EMSA) were performed to investigate the presence of putative glucose-response elements.

Results. Raising the ambient D-glucose concentration for 72 hours increased TGF-β1 bioactivity in cell culture medium by 47% and total TGF-β1 secretion by approximately 90%. Northern analysis demonstrated that the steady-state TGF-β1 mRNA level was increased nearly twofold after 48 hours of growth in high glucose. This increase was not due to increased stability, as the half-life of the message was approximately five hours in both normal and high glucose conditions. Transcriptional activity of the TGF-β1 gene (nuclear run-on assay) was increased by 73% in cells grown in high glucose for 24 hours. Transiently transfected MMCs with CAT constructs containing varying lengths of the murine TGF-β1 promoter demonstrated that high glucose selectively increased the expression of only one of the constructs, pA835. Sequence inspection revealed the presence of a putative glucose responsive element, CACGTG, within this construct. High glucose in MMC culture for 24 hours increased nuclear protein binding to a probe containing this element when analyzed using EMSA.

Conclusions. High glucose stimulates total TGF-β1 protein production and bioactivity as well as the steady-state level of TGF-β1 mRNA. The latter effect is due primarily to stimulation of gene transcription rate rather than message stability. Transcriptional activation by high glucose may involve a region in the TGF-β1 promoter containing a putative glucose-response element.

Diabetic nephropathy is the major cause of kidney failure in the industrialized world. Although genetic [1] and hemodynamic factors [2] play important roles, the mechanisms initiating kidney damage undoubtedly stem from chronic hyperglycemia and its metabolic consequences. Clinical trials have revealed that poor glycemic control predicts the incidence and progression of diabetic retinopathy, neuropathy, and renal disease [3]. Previous studies of the effects of high glucose concentration on renal cells in tissue culture have demonstrated 'direct' alterations in cellular parameters which are highly relevant to diabetic renal disease, such as cellular hypertrophy, enhanced extracellular matrix production, and altered production or action of growth factors [reviewed in 4]. In particular, the multifunctional cytokine TGF-β has received paramount attention as a likely etiologic agent in the pathogenesis of diabetic nephropathy [5]. TGF-β plays a pivotal role in cellular growth and extracellular matrix production [6–8]. Many of the actions of TGF-β on renal cells resemble those of high ambient glucose and are reminiscent of the diabetic changes that are seen in the kidney [9, 10].

It now appears likely that the hypertrophic and prosclerotic effects of high glucose concentration on cultured glomerular mesangial cells [11, 12], glomerular epithelial cells [13], and renal proximal tubular cells [14, 15] are mediated by autocrine production and activation of TGF-β. For instance, we have shown that expression of TGF-β1 mRNA and bioactivity are significantly increased within 48 hours of exposure to high ambient glucose in murine proximal tubular cells and glomerular mesangial cells [11, 12, 16]. Inhibiting TGF-β activity with specific neutralizing antibodies [11–13, 16, 17] or by application of TGF-β1 antisense oligonucleotides [18] attenuates the glucose-induced inhibition of cell proliferation and the stimulated

[1] Dr. Hoffman and Dr. Sharma contributed equivalent work to this study.

Key words: diabetic nephropathy, TGF-β, glomerulus, protein kinase, hyperglycemia, neuropathy, cell growth, extracellular matrix.

Received for publication November 21, 1997
and in revised form May 7, 1998
Accepted for publication May 11, 1998

synthesis of matrix molecules such as types I and IV collagen, fibronectin, and mesangial proteoglycans.

The mechanisms of regulation of the TGF-β system by high glucose in kidney cells are incompletely defined. Modulation of bioactivity may occur through pre-translational or post-translational mechanisms. The latter may involve changes in ligand processing and activation or alterations in receptor number and dimerization. Regions within the untranslated portions of the TGF-β1 gene are involved in post-transcriptional processing of the message in some cell types [19]. Previous studies have also shown that one likely point of regulation of TGF-β1 expression by high glucose media in mesangial cells is at the mRNA level [11, 18], but it remains unclear whether increased steady-state levels of TGF-β1 mRNA by high glucose are due to increased rate of gene transcription or a result of transcript stability. The promoter regions of the TGF-β1 gene in mouse and humans contain several consensus binding sites for known transcription factors including SP1, AP1 and AP2 [20–22]. High ambient glucose in mesangial cell culture has been shown to increase the expression of c-*jun* and c-*fos*, proteins that dimerize to form the AP1 transcription factor [23]. SP1 may function as a glucose-responsive element in the promoter region of a gene involved in glucose metabolism [24]. In addition, inspection of the murine promoter region of TGF-β1 between −641 and −636 5′ to the A transcription start-site [21] reveals a sequence that has previously been described in the S14 and pyruvate kinase genes to be glucose-responsive [25, 26]. This 5′-CACGTG-3′ element is a consensus sequence recognized by a family of transcription factors containing a conserved basic region, helix loop-helix, leucine zipper domain, which includes the c-*myc* family. It is currently not known if any of the elements mentioned above functions as a glucose-response element that can mediate TGF-β1 transcriptional activation by high ambient glucose in mesangial cells.

In the present study we set out to better understand the mechanisms of regulation of TGF-β1 expression by high ambient glucose in murine mesangial cells in culture. We demonstrate that high glucose stimulates total TGF-β1 protein production and bioactivity as well as the steady-state level of TGF-β1 mRNA. The latter effect is due primarily to stimulation of gene transcription rate rather than to message stability. Promoter-reporter assays and DNA-nuclear protein analysis suggest that transcriptional activation by high glucose involves a region in the TGF-β1 promoter that contains a sequence that may serve as a glucose-response element.

METHODS

Cell culture

A murine mesangial cell line (MMC) was used. Cells were originally isolated from kidneys of SJL/J(H-2) normal mice and transformed with noncapsid forming SV-40 virus to establish a permanent cell line [27]. The cells exhibit many phenotypic features of differentiated mesangial cells including positive staining for desmin, vimentin, and collagen types I and IV. 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, USA) containing 100 mg/dl D-glucose, 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM supplemental glutamine. Cells were passaged every 72 hours by light trypsinization. Experiments were initiated by resting cells for 24 hours in 0.5% FCS. Fresh DMEM was then added containing 0.5% FCS and 100 mg/dl D-glucose (unless specified otherwise) and the D-glucose concentration was increased in some plates to specified concentrations (up to 600 mg/dl) by the addition of a small volume of 1.0 M D-glucose in sterile water. D-mannitol was used as an osmotic control. In some experiments cells were treated with recombinant human TGF-β1 (R & D Systems, Minneapolis, MN, USA) at 1 ng/ml for 24 hours or with phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA) at 100 nM for six hours. Media and cells were harvested after a period of 24, 48, 72, or 96 hours as specifically indicated.

TGF-β1 ELISA

Conditioned media were frozen at −20°C until assayed by a sandwich ELISA kit according to the manufacturer's specifications (Genzyme, Boston, MA, USA). Acid activation of the samples was required in order to convert latent into active TGF-β1 and to record detectable levels of total (latent + active) TGF-β1. Samples were thawed and activated with 1 N HCl for 60 minutes at 4°C followed by neutralization with 1 N NaOH. Samples were plated on anti-TGF-β1-coated microtiter plates and incubated at 37°C for 60 minutes. After vigorous washing, wells were incubated with a second biotin-conjugated anti-TGF-β1 antibody and the peroxidase reaction was initiated. A standard curve was constructed using serial dilutions of ultrapure human TGF-β1 (Genzyme). TGF-β1 levels in samples were compared to known standards and read as ng/ml, and final concentrations were reported per cell number or total cell protein content (Bio-Rad, Richmond, CA, USA).

TGF-β1 bioassay

A highly sensitive bioassay for TGF-β1 was conducted using a reporter mink-lung epithelial cell line that had been stably transfected with a plasminogen activator-inhibitor-1 (PAI-1) promoter linked to luciferase reporter gene [28]. The PAI-1 promoter is very sensitive to stimulation by TGF-β1 and can measure levels as low as 2 pg/ml. The mink cells were plated onto 96-well microtiter plates in DMEM containing 1% FCS. After allowing cells to adhere

to the wells for three hours, serial dilutions of TGF-β1 standard or conditioned media from mesangial cells (1:1 dilution) were added to the medium of the reporter cells and incubated for 24 hours, at which time cells were harvested and the cell lysate was assayed for luciferase activity in a luminometer [29]. Protein in the cell lysates was measured (Bio-Rad), and bioactivity was reported per cell protein content. To assure specificity, samples were pretreated with neutralizing anti-TGF-β antibodies (pan-specific against TGF-β1, β2, and β3; Genzyme) at a concentration of 10 μg/ml prior to addition to wells. We found that more than 90% of bioactivity was neutralized with this concentration of antibody.

Northern analysis

At the end of the incubation period (24, 48, and 72 hr), cells were washed in RNAse-free PBS (pH 7.2), and directly lysed and denatured in 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Total RNA was isolated by repeated phenol-chloroform extractions as previously described [12]. RNA was separated (20 μg) on a 1.2% agarose gel containing 0.67 M formaldehyde, transferred to Gene-Screen nylon membrane (NEN Research Products, Boston, MA, USA) by capillary blotting and UV-crosslinked. Integrity and equal loading of RNA samples were assessed by methylene blue staining of the transferred RNA [30]. All cDNA inserts were separated from their plasmids in low-melt agarose and labeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL, USA) using a random-priming kit (Boehringer Mannheim, Indianapolis, IN, USA). The cDNA probes encoding murine TGF-β1 and 18S were as previously described [30]. The labeled cDNAs were separated from unincorporated nucleotides by passage over a NENSORB purification cartridge (NEN Research Products) and membranes were hybridized with 10^6 cpm/ml probe. Details of the hybridization, washing, and autoradiography were performed as previously reported [12]. Exposed X-ray films (18 hr to 4 days) were scanned with a laser-densitometer, and TGF-β1 mRNA level was calculated relative to that of 18S. Measurements of this ratio in MMCs cultured in 100 mg/dl glucose were assigned a relative value of one.

Message stability assay

Rested MMCs (10^7 cells) were cultured for 48 hours in DMEM + 0.5% FCS containing either 100 mg/dl or 450 mg/dl glucose concentration. Cells were then treated with 5 μg/ml Actinomycin D (Boehringer Mannheim) to inhibit RNA synthesis and total RNA was extracted after specified time periods (between 0 and 16 hr). Northern analysis and hybridization for TGF-β1 and 18S were performed as outlined above to measure the rate of decay of TGF-β1 mRNA and the half-life. Measurement of the ratio of TGF-β1/18S at time = 0 (from Actinomycin D treatment) in cells cultured in 100 mg/dl glucose was assigned a relative value of one, or 100%.

Nuclear run-on assay

Isolation of nuclei and *in vitro* transcription were performed according to established methods [31] with some modification. Briefly, rested MMCs were grown in 100 mm plates in DMEM + 0.5% FCS in the presence of either 100 mg/dl or 450 mg/dl glucose for 24 hours. Cells were rinsed in ice cold PBS and nuclei were isolated [31] and stored at −70°C for future use. For *in vitro* transcription, the nuclei were thawed in an equal volume of reaction buffer (10 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl) containing 1 mM each of adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), cytidine 5′-triphosphate (CTP), and 5 mM dithiothreitol (DTT). One hundred μCi of α-^{32}P-uridine triphosphate (UTP; Amersham) and 2 μl RNAsin (Promega, Madison, WI, USA) were added to each reaction and then incubated at 30°C for 30 minutes. ^{32}P-labeled RNA was then isolated using Trisolv reagent (Biotecx Lab., Inc., Houston, TX), ethanol-precipitated and dissolved in 500 μl TES buffer (10 mM Tris-Cl pH 7.4; 10 mM EDTA; 0.2% SDS, 0.6 M NaCl; 6× Denhardt's). Equal counts were then hybridized to linearized DNA (18S, TGF-β1) between 1 to 2 μg, that had been cut from plasmid and immobilized on nylon membranes using a slot-blot apparatus. The nonspecific vector plasmid pBsks was used as a negative control. Hybridization was carried out for 30 hours at 60°C in a solution containing 1 × Northern prehybridization buffer (5 Prime-3 Prime, Inc., Boulder, CO, USA), 5% SDS, 5% dextran sulfate, 10% Blocking Reagent (Biotecx Lab), yeast tRNA and sheared salmon sperm DNA. Membranes were then washed in 2 × SSC for one hour at 60°C, incubated at 37°C for 30 minutes in 10 μg/ml ribonuclease A (Sigma) washed again for one hour in 2 × SSC at 37°C, air-dried, and exposed to film (XAR-5; Eastman Kodak Co, Rochester NY, USA) with intensifying screens at −70°C. Exposed films were scanned with a laser-densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA), and TGF-β1 levels were calculated relative to those of 18S. Measurements of this ratio in cells cultured in 100 mg/dl glucose were assigned a relative value of one, or 100%.

Transfections, CAT constructs and CAT assay

Murine TGF-β1 promoter-reporter chimeric constructs were kindly provided by Dr. Andrew Geiser (NCI/NIH, Bethesda, MD, USA). The chloramphenicol acetyltransferase (CAT) constructs employed were pA1079, pA835 and pAB406 [21] containing −1079, −835, or −406 bp 5′ from the A transcription start-site of the murine TGF-β1 gene (the pAB406 also contained the B start site, which lies 290 bp 3′ to the A site). The pAoCAT construct, which contains the CAT gene without regulatory elements, and the pSV_2CAT construct, which contains the CAT gene linked to the SV40 promoter/enhancer, were used as

negative and positive controls, respectively. Transient transfections were performed on cells grown to 60% confluence in DMEM/10% FCS with a glucose concentration of 180 mg/dl. The growth medium was removed, cells were washed twice with PBS, and 2 μg of test plasmid and 1 μg of control β-galactosidase plasmid (PLENX2; kindly provided by Dr. Pamela Norton) were added to cells with Superfect reagent (Qiagen, Chatsworth, CA, USA) in a total volume of 600 μl with DMEM/10% FCS for three hours. The medium was then removed, cells washed once with PBS and fresh medium (DMEM/10% FCS) was added for 16 hours. Cells were then washed twice with PBS and serum-free DMEM was added containing either 100 mg/dl or 450 mg/dl glucose for 24 hours. Additionally, in several experiments TGF-β1 (1 ng/ml) or PMA (100 nM) was added as positive controls for stimulation of TGF-β1 promoter activity. Cells were harvested with TEN buffer (40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 150 mM NaCl) and then lysed with three cycles of freeze-thaw, spun down for 15 minutes and the supernatant assayed for β-galactosidase activity [32]. Based on equivalent amounts of β-galactosidase activity, the cell lysates were added to initiate the CAT enzyme assay using ^{14}C-chloramphenicol (Amersham) as previously described [14, 33, 34]. CAT enzyme activity was expressed as the percent acetylated chloramphenicol compared with total chloramphenicol. Maximal CAT enzymatic activity was taken as that measured with the pSV_2CAT construct, and relative CAT activities of the TGF-β1 promoter constructs were expressed as % of pSV_2CAT.

Electrophoretic mobility shift assay (EMSA)

Nuclei were derived from rested cells grown in fresh DMEM/0.5% FCS containing either 100 mg/dl or 450 mg/dl glucose for 24 hours. Nuclear proteins were isolated as described [35]. The probe used encoded a putative glucose responsive element (CACGTG) identified at position −641 to −636 of the murine TGF-β1 gene. The sense strand was a 20-mer oligonucleotide (5′AAGCAGG**CACGTG**GACTCTG3′) that was chemically synthesized (Bioserve Biotech; Laurel, MD, USA) and annealed to its complementary strand. To prove specificity of nuclear binding to the CACGTG element, a 20-mer probe was synthesized (5′AAGCAGG**CGACGA**GACTCTG3′) containing a mutated CACGTG site. The probe was end-labeled with α-^{32}P-ATP (Amersham) using T4 polynucleotide kinase (Promega) [36]. Nuclear proteins (10 μg) were incubated with 10^5 cpm of probe for 30 minutes at 4°C in buffer containing 12 mM HEPES, pH 7.4, 4 mM Tris-HCl, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$; 1 μg of poly dI:C, 5 μg bovine serum albumin, and 2 μl glycerol. The protein:DNA complex was resolved on a 6% non-denaturing polyacrylamide gel and dried, followed by autoradiography.

Statistics

The data are presented as means (± SE) with N indicating the number of different experiments. Statistical analysis between two groups was performed by a paired or unpaired t-test, as appropriate. $P < 0.05$ was considered a significant difference.

RESULTS

High glucose stimulates TGF-β1 bioactivity and protein production

Murine mesangial cells (MMCs) cultured in media containing normal (100 mg/dl) or high glucose (450 mg/dl) exhibited a sustained, time-dependent increase in the absolute amount of total (latent + active) TGF-β1 protein secretion (measured by ELISA and expressed per total cell protein; Fig. 1A). Supernatants of cells cultured in high glucose for 24 hours displayed a 32% increase of total TGF-β1 protein secretion as compared with cells grown in normal glucose. At 48 hours there was a 48% increase, at 72 hours there was a 45% increase and at 96 hours a 26% increase. The iso-osmotic addition of mannitol did not cause a significant change in total TGF-β1 protein secretion (Fig. 1A). The magnitude of increase in high glucose may have been blunted by the concomitant hypertrophy of the cells. To correct for the latter effect, we also measured total TGF-β1 protein secretion per cell number (Fig. 1B). After 72 hours of culture in high glucose, MMCs displayed a 90% increase of total TGF-β1 protein secretion per cell number (2.73 ± 0.21 in high glucose vs. 1.44 ± 0.11 ng/10^6 cells in normal glucose; mean ± SE, $N = 8$; $P < 0.05$). MMCs cultured in 450 mg/dl glucose also demonstrated an increase in TGF-β1 bioactivity (measured by the mink-lung epithelial cell bioassay) compared with cells cultured in 100 mg/dl glucose (Fig. 1B). At 72 hours MMCs cultured in high glucose demonstrated a 47% increase in TGF-β1 bioactivity compared with normal glucose. Bioactive TGF-β1 production did not exceed 10% of the amount of total TGF-β1 protein in these cells.

High glucose increases steady-state TGF-β1 mRNA level but does not alter TGF-β1 message stability

The increase in the TGF-β1 mRNA steady-state level in MMC by high glucose concentration was time-dependent. Cells cultured for 24 hours in 450 mg/dl glucose displayed a slight increase in the TGF-β1 mRNA level; however, after 48 hours of culture in high glucose, the mRNA level increased by almost twofold compared with cells grown in 100 mg/dl glucose (Fig. 2 A, B). The magnitude of this effect persisted after 72 hours (not shown). The increase in TGF-β1 mRNA level by high glucose at 48 hours was also dose-dependent (Fig. 2C), with maximal effect seen at 450 mg/dl glucose, and no further increase at 600 mg/dl.

To examine if high glucose affected transcript stability, cells that had been grown in either 100 or 450 mg/dl glucose

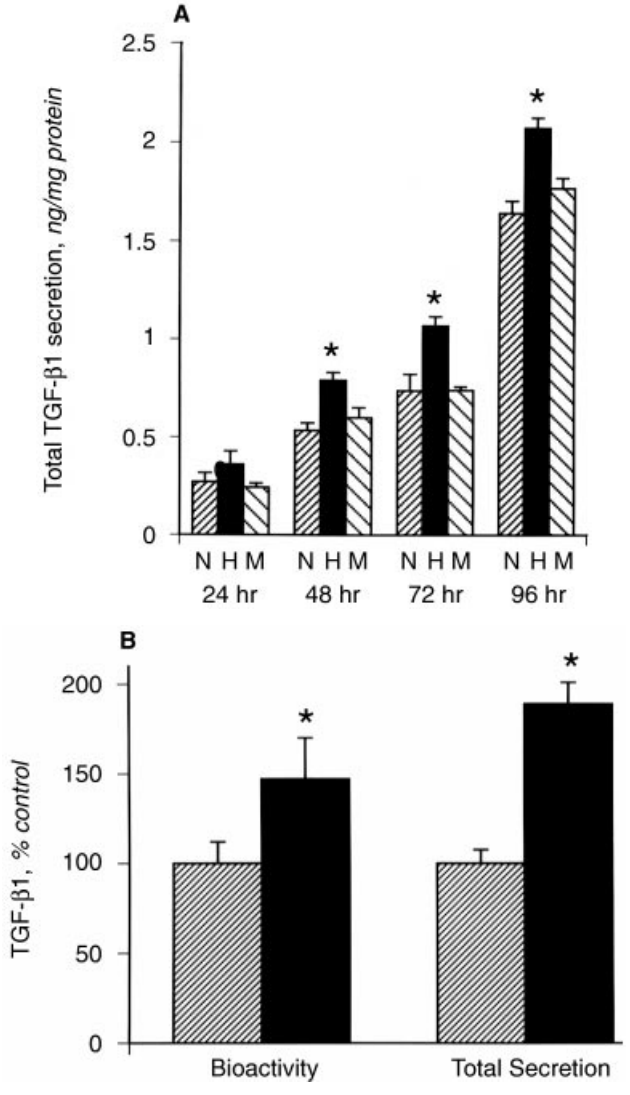

Fig. 1. Effect of glucose concentration on bioactivity and total secretion of transforming growth factor-β1 (TGF-β1) in murine mesangial cells (MMCs). *(A)* Conditioned media from MMCs grown for 24 to 96 hours in DMEM/0.5% FCS containing either normal (N; 100 mg/dl) or high (H; 450 mg/dl) D-glucose concentration or iso-osmotic D-mannitol (M; + 350 mg/dl) were assayed by ELISA for total TGF-β1 protein secretion (latent + active) per total cell protein content. The conditioned media from cells grown in high glucose demonstrate a persistent increase in total TGF-β1 secretion compared with cells grown in normal glucose ($N = 6$; $^*P < 0.05$ vs. H). Mannitol did not cause a significant change in total TGF- β1 secretion. *(B)* Conditioned media from MMCs grown in DMEM/0.5% FCS containing either normal (▨) or high (■) glucose concentration were assayed for either TGF-β1 bioactivity by the mink lung epithelial cell reporter assay or total TGF-β1 secretion (latent + active) by ELISA as outlined in the **Methods** section. Data are mean ± SE. Freshly harvested conditioned media from MMCs grown in high glucose for 72 hours demonstrate a 47% increase in TGF-β1 bioactivity ($N = 8$; $^*P < 0.05$) when compared with normal glucose. These conditioned media demonstrate approximately 90% increase in total TGF-β1 protein secretion compared with cells grown in normal glucose ($N = 8$; $^*P < 0.05$).

for 48 hours were treated with actinomycin D for an additional period up to 16 hours to inhibit gene transcription. Actinomycin treatment resulted in a progressive decay in message level over time; the rate of decay was of equivalent magnitude in cells grown in either normal or high glucose (Fig. 3). After five hours of actinomycin treatment the TGF-β1 mRNA level was 49% of the level without actinomycin in cells grown in normal glucose. Similarly, the TGF-β1 mRNA level was 48% of the level without actinomycin in cells grown in high glucose (Fig. 3B). At each time point, the ratio of TGF-β1 mRNA relative to 18S was virtually unchanged when comparing cells grown in high versus normal glucose. Therefore, the increase in the steady-state level of TGF-β1 mRNA induced by high ambient glucose in MMCs is not due to increased mRNA stability.

High glucose stimulates TGF-β1 gene transcription rate in murine mesangial cells

To confirm that the increase in the steady-state level of TGF-β1 mRNA by high glucose is due to increased gene transcription rate, MMCs were grown in 100 or 450 mg/dl glucose for 24 hours and *in vitro* gene transcription rate was measured by the nuclear run-on assay as described in the **Methods** section. Figure 4 demonstrates that TGF-β1 gene transcription rate (when controlled for gene expression of ribosomal 18S RNA) is stimulated by 73 ± 6% (mean ± SE; $N = 3$; $P < 0.05$) in cells grown in high versus normal glucose for 24 hours. As would be expected, the nonspecific vector DNA did not hybridize with nuclear RNA from either normal or high glucose (Fig. 4). Note that the magnitude of stimulation of gene transcription rate by high glucose after 24 hours shown in Figure 4 is comparable to the observed increase in steady-state TGF-β1 mRNA level after 48 hours depicted in Figure 2. These results are consistent with the conclusion that high glucose stimulates TGF-β1 mRNA level predominantly by a transcriptional mechanism.

High glucose stimulates TGF-β1 promoter activity

Murine mesangial cells were transiently transfected with varying lengths of the murine promoter of TGF-β1 linked to the CAT reporter gene. The media were then changed to normal or high glucose concentration. As noted in Figure 5, there was a consistent and significant stimulation in CAT reporter activity with the pA835 construct (corrected for transfection efficiency using β-galactosidase) when cells were grown for 24 hours in high glucose media. A shorter construct (pAB406) did not demonstrate stimulation in high glucose media; this suggests that an element may exist in the region between −406 and −835 of the murine TGF-β1 gene that could be glucose-responsive. There was low basal CAT activity and no significant stimulation with a longer construct (pA1079), possibly because of the presence of negative regulatory elements that are known to exist in the upstream promoter region [21]. To evaluate whether the pA835 construct also confers stimulation by other known activators of the TGF-β1 promoter, additional

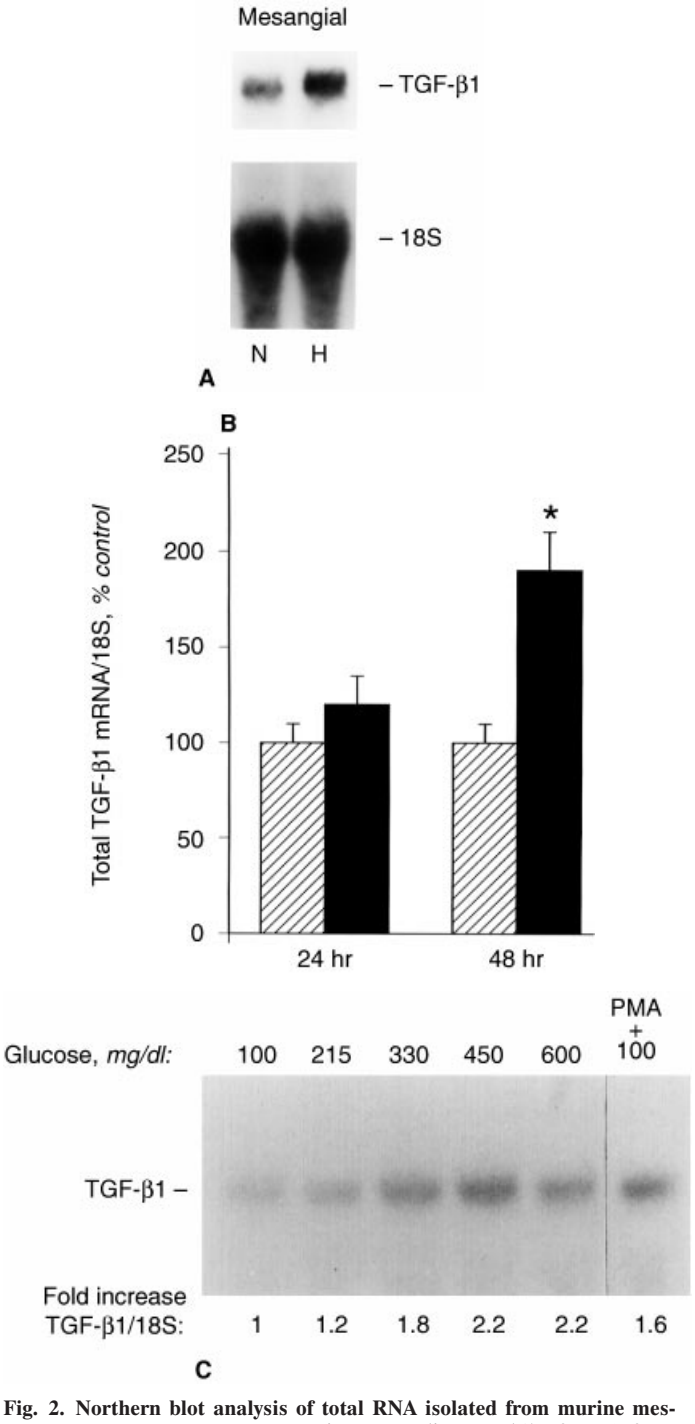

Fig. 2. Northern blot analysis of total RNA isolated from murine mesangial cells (MMCs). Representative autoradiogram *(A)* of RNA from cells grown in high glucose (H; 450 mg/dl) for 48 hours demonstrating increased level of the 2.5-kb TGF-β1 mRNA (relative to 18S) as compared with cells grown in normal glucose (N; 100 mg/dl). Values in *(B)* are mean ± SE, $N = 5$, showing significant stimulation of TGF-β1 mRNA at 48 hours but not 24 hours in high glucose (■) as compared with normal glucose (▨) ($^*P < 0.05$). *(C)* Representative autoradiogram of RNA from cells grown for 48 hours demonstrating a progressive increase in TGF-β1 mRNA (relative to 18S) with increasing glucose concentrations (215, 330, 450 and 600 mg/dl) compared with cells grown in 100 mg/dl. The peak effect was observed at glucose concentrations of 450 to 600 mg/dl. The addition of the phorbol ester PMA (100 nM) to media containing 100 mg/dl glucose for the last six hours of culture also caused an increase in TGF-β1 mRNA.

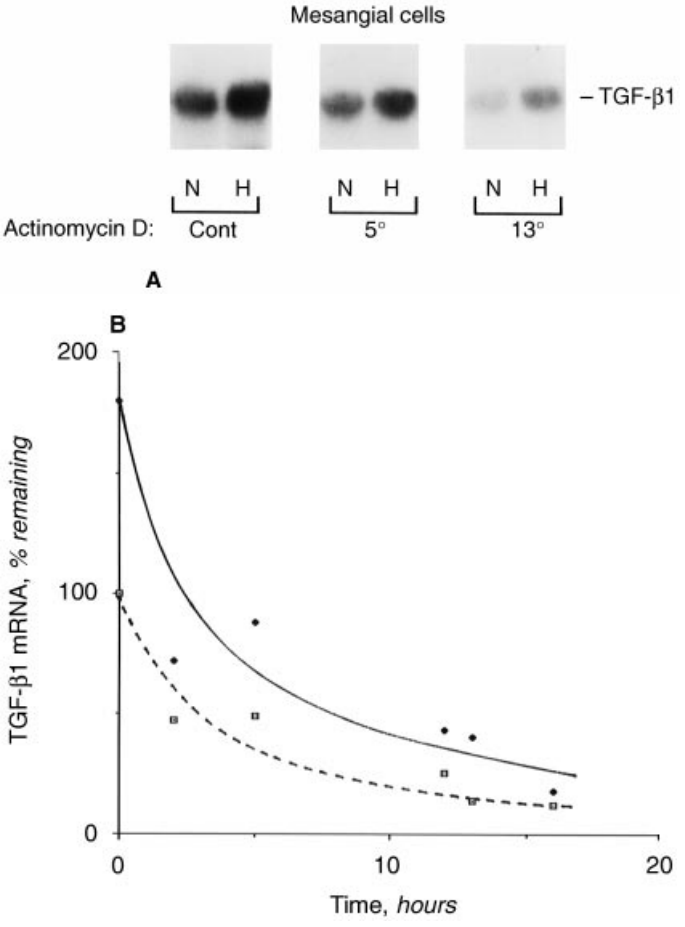

Fig. 3. TGF-β1 message stability in MMCs. Representative Northern blot *(A)* of total RNA isolated from MMCs grown in either normal (N; 100 mg/dl) or high (H; 450 mg/dl) glucose for 48 hours and then treated with Actinomycin D (5 μg/ml) to inhibit gene transcription for the time points indicated in hours. The stimulation of TGF-β1 mRNA level by high glucose persists at all time points following treatment with Actinomycin D. Data in *(B)* represent the profile of TGF-β1 mRNA decay (relative to 18S) following treatment with Actinomycin D for cells grown in either normal (– – –) or high (——) glucose. The estimated half-life of TGF-β1 message is approximately five hours in either media. Data points represent mean values from three different experiments. Curves represent best-fit lines.

experiments were performed and the data are shown in Table 1. The addition of exogenous TGF-β1 (1 ng/ml) led to a similar degree of stimulation of CAT activity in the presence of normal or high glucose. Short-term treatment (6 hr) with the phorbol ester PMA also stimulated pA835 activity. The degree of stimulation of pA835 activity by high glucose alone ranged between 60% (Fig. 5) and nearly twofold (Table 1), and is commensurate with the degree of stimulation observed by high glucose in the nuclear run-on studies shown in Figure 4.

High glucose stimulates nuclear protein binding to a putative glucose-responsive element

Analysis by EMSA was performed on nuclear proteins derived from MMCs grown in various glucose concentrations. Nuclear proteins were mixed with a ^{32}P-labeled

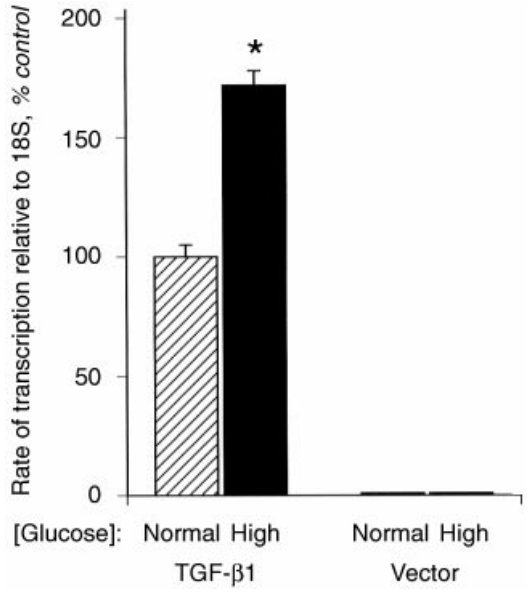

Fig. 4. High glucose stimulates TGF-β1 gene transcription rate in MMCs. *In vitro* gene transcription rates were measured in nuclei isolated from MMCs grown for 24 hours in media containing either normal (100 mg/dl) or high (450 mg/dl) glucose as outlined in the **Methods** section. MMCs grown in high glucose demonstrate a 73 ± 6% increase (mean ± SE; $N = 3$, $P < 0.05$) in TGF-β1 gene transcription rate (relative to 18S) compared with cells grown in normal glucose. Densitometric measurements of TGF-β1 transcription rate were calculated relative to 18S. Note that nuclear RNA did not hybridize with the nonspecific vector pBsks.

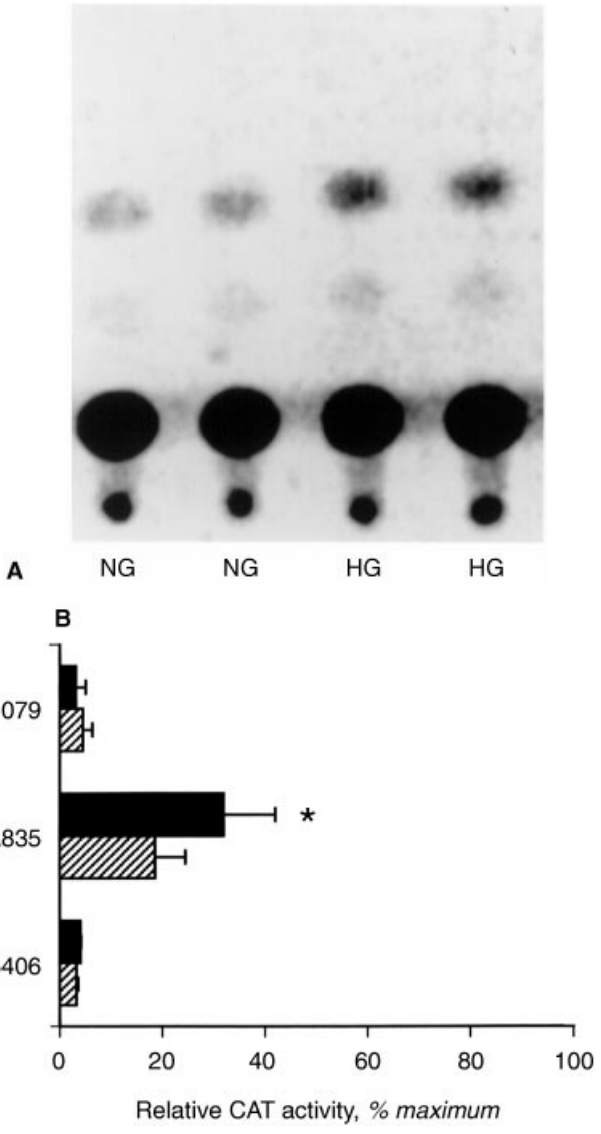

Fig. 5. High glucose increases CAT activity of pA835 construct containing a putative glucose responsive element in the TGF-β1 promoter. Representative thin layer chromatography plate *(A)* after separation of acetylated ^{14}C-chloramphenicol (CAT activity) from unacetylated fraction of extracts of MMCs transiently transfected with the pA835 construct. Lanes 1 and 2 are from MMCs grown in normal glucose (N; 100 mg/dl) and lanes 3 and 4 are from MMCs grown for 24 hours in high glucose (H; 450 mg/dl). A beta-galactosidase-containing plasmid was cotransfected to normalize for differences in transfection efficiency. Data in *(B)* are mean ± SE for relative CAT activities of the pAB406, pA835, and pA1079 constructs transiently transfected into MMCs and grown in normal (▨) or high glucose (■) for 24 hours. High glucose induces a 60% increase in relative CAT activity only in those cells transfected with the pA835 construct. Data represent five different transfection experiments, and CAT activity of each construct is expressed relative to maximal activity seen with the positive control pSV_2CAT construct ($^*P < 0.05$).

synthetic DNA probe encoding −648 to −629 bp of the murine TGF-β1 promoter that contains a putative glucose responsive element, CACGTG [26]. Figure 6 demonstrates enhanced binding of the DNA probe with nuclear protein obtained from cells grown in 450 mg/dl glucose for 24 hours (lane 5) as compared with 100 mg/dl glucose (lane 3). There appeared to be four distinct retarded bands that were all increased in density to various extents by the high glucose concentration. To demonstrate specificity of binding, excess unlabeled ('cold') probe was added to the reaction mix and this caused a marked decrease of binding of protein to the labeled probe (Fig. 6). In addition, a mutated probe lacking the intact CACGTG site failed to bind nuclear proteins from either normal or high glucose-treated MMCs (lanes 8 and 9). Thus, the high glucose condition stimulates specific binding of nuclear proteins to DNA fragments containing the CACGTG element in the murine TGF-β1 promoter.

DISCUSSION

The nature of the factors directly arising as a consequence of hyperglycemia and the steps involved in diabetic complications are not completely understood. Previous studies have demonstrated that many of the actions of high ambient glucose on renal cells resemble those of TGF-β [9, 10]. In fact, the hypertrophic and prosclerotic effects of high glucose concentration on cultured glomerular mesangial cells [11, 12], glomerular epithelial cells [13], and proximal tubular epithelial cells [14, 15] are mediated by autocrine production and activation of TGF-β. The present study demonstrates that high glucose concentration stimulates the transcriptional activation of the TGF-β1 gene in

Table 1. Effects of glucose, TGF-β1, and phorbol ester on CAT activity of the pA835 construct

Glucose concentration *mg/dl*	Addition	CAT activity *% maximal*
100	—	15.8 ± 1.2
450	—	29.6 ± 4.6[a]
100	TGF-β1	39.6 ± 4.3[a]
450	TGF-β1	42.2 ± 1.3[a]
100	PMA	41.1

MMCs were transiently transfected with the pA835 construct. A beta-galactosidase-containing plasmid was cotransfected to normalize for differences in transfection efficiency. Cells were cultured in either normal (100 mg/dl) or high (450 mg/dl) glucose for 24 hours. Cells were also treated with either TGF-β1 (1 ng/ml) or the active phorbol ester PMA (100 nM, 6 hours), known activators of TGF-β1 promoter activity. CAT activity is expressed relative to maximal activity seen with the pSV_2CAT construct. Data are mean ± SE of three different transfection experiments (except for two experiments using PMA). Abbreviations are in the **Appendix.**

[a] $P < 0.05$ vs. 100 mg/dl glucose

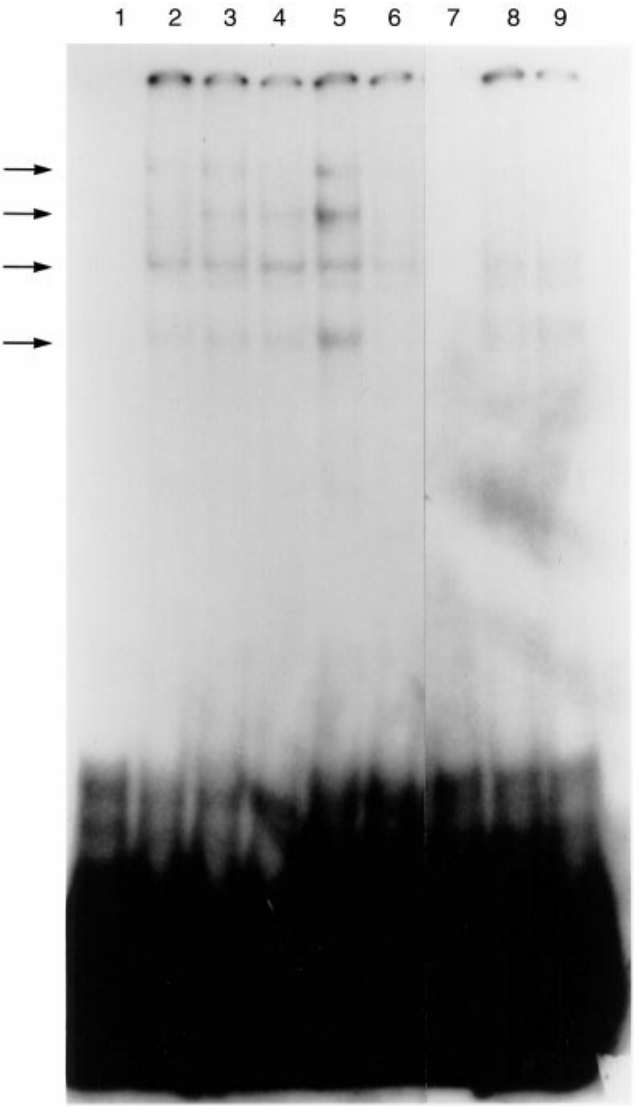

Fig. 6. High glucose stimulates nuclear protein binding to a putative glucose-response element. A 20-bp oligonucleotide probe encoding the sequence between −648 and −629 of the murine TGF-β1 promoter was end-labeled and used in the EMSA as described in the **Methods**. Lane 1 represents probe alone. Probe was incubated with nuclear proteins from MMCs grown in low glucose (50 mg/dl: lane 2), normal glucose (NG; 100 mg/dl; lane 3), intermediate high glucose (300 mg/dl: lane 4) or high glucose (HG; 450 mg/dl; lane 5). Four discrete bands were gel-shifted (arrows) by nuclear proteins and increased in density in 450 mg/dl glucose (lane 5). For specificity control, nuclear proteins from MMCs cultured in 450 mg/dl glucose were incubated in the presence of 100-fold excess 'cold' probe along with labeled probe (lane 6) prior to analysis by EMSA. To further prove specificity of nuclear binding to the CACGTG element, a mutated 20-mer probe was synthesized containing a scrambled CACGTG site, and was added to nuclear proteins from MMCs cultured in 100 or 450 mg/dl glucose (lanes 8 and 9, respectively). Lane 7 represents mutated probe alone, without nuclear proteins.

murine mesangial cells. The degree of transcriptional activation at 24 hours correlates well with the magnitude of mRNA stimulation at 48 hours and of the protein level at 72 hours. In addition, high glucose did not significantly affect mRNA stability, consistent with the view that the mechanism underlying the stimulation of TGF-β1 by high glucose is primarily due to transcriptional activation. It is conceivable that enhanced TGF-β bioactivity by high glucose is also regulated by the conversion of latent to active TGF-β. Studies in other cell types have demonstrated that the latent moiety may be affected by various conditions [37]. We found a 47% increase in active TGF-β whereas total TGF-β1 protein (per cell number) was increased by almost 90% after 72 hours in culture in 450 mg/dl glucose. Therefore, enhanced conversion of latent TGF-β1 to active TGF-β1 alone does not account for the entire high glucose effect.

Increased mRNA expression in high glucose media is most likely due to enhanced synthesis of TGF-β1 mRNA. This supposition was confirmed by the nuclear run-on studies wherein we found a 73% stimulation of TGF-β1 gene transcription rate. Promoter activity studies also demonstrated a similar degree of stimulation with one of the promoter-reporter constructs (pA835). A shorter construct (pAB406) did not exhibit significant stimulation by high glucose, suggesting that a glucose-responsive element resides in the region between −406 and −835 located 5′ to the A start site. Inspection of this region identified a sequence between −641 and −636 that is similar to a glucose-responsive sequence previously described in the S14 and pyruvate kinase genes [25, 26]. This region contains the CACGTG sequence which is a consensus site for the c-*myc* family of transcription factors. EMSA studies employing the native sequence (−648 to −629) in the murine TGF-β1 promoter as a probe demonstrated enhanced binding of nuclear proteins derived from cells grown in high glucose. A recent report by Shih, Liu and Towle demonstrated that the presence of two CACGTG elements separated by a 5 bp sequence conferred greater degree of glucose-responsiveness than a single CACGTG site [38]. As the native TGF-β1 promoter in the mouse

contains only one CACGTG site, this may explain the relatively modest degree of stimulation of the TGF-β1 promoter by high glucose. However, it should be noted that the degree of stimulation of TGF-β1 by high glucose media was generally of equivalent magnitude at the protein, mRNA, and transcriptional levels. Additionally, our previous studies in diabetic mice and rats have demonstrated that kidney TGF-β1 mRNA level is increased by two- to threefold within the first week of development of diabetes mellitus [9, 30].

Our results demonstrate that high glucose alone is a sufficient stimulus for the enhanced synthesis and secretion of TGF-β1 into the conditioned medium of murine mesangial cells. This stimulation likely accounts for the high glucose-mediated stimulation of collagen biosynthesis by mesangial cells without the need for exogenous addition of other growth factors [12]. We cannot, however, exclude a contribution *in vivo* from autocrine production of other growth factors such as platelet-derived growth factor [17, 39].

High glucose has been demonstrated to affect the formation in mesangial cells of the transcription factors c-*jun* and c-*fos* [23], components of the AP-1 heterodimeric complex. This complex binds with high affinity to TPA (phorbol ester)-responsive elements (TRE). A recent report by Wilmer and Cosio indicates that high glucose increases DNA binding of AP-1 in human mesangial cells, through an effect involving protein kinase C (PKC) activation and post-translational modifications of the transcription factor complex [40]. Previous reports have demonstrated that PKC inhibitors attenuate the high glucose-induced TGF-β1 mRNA stimulation in mesangial cells in culture [41]. In addition, selective inhibition of the PKC-β isoform in diabetic rats attenuates diabetes-induced stimulation of TGF-β1 mRNA in glomeruli [42]. Treatment with phorbol esters, which are known to stimulate the classic PKC isoforms, can stimulate TGF-β1 gene transcription via TRE sites [43]. However, our studies suggest that there are other regions in the murine TGF-β1 promoter (for example, between −406 and −835, which do not contain consensus AP-1 binding sites) that exhibit glucose-responsiveness, and likely are regulated via non-AP1 transcription factors. It has been demonstrated that the combined treatment with high glucose plus TGF-β1 causes phosphorylation of a cAMP responsive element binding (CREB) protein in rat mesangial cells [44], and we have recently demonstrated that TGF-β1-induced CREB phosphorylation can be blocked by inhibition of protein kinase A in murine mesangial cells [45]. Thus, additional studies are required to identify other signaling pathways and transcription factors that may be involved in the transcriptional activation of TGF-β1 by high glucose. It is quite possible that a combination of signaling pathways and transcription factors with cross-talk between them is necessary to generate an optimal stimulation of the TGF-β system by high ambient glucose in specific cell types that are relevant to diabetic renal disease [46].

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health (NIH) grants (DK-44513 and DK-45191 to F.N.Z., and training grant DK-07006), and the DCI-RED Fund. Dr. Brenda Hoffman was supported by an Individual National Research Service Award from the NIH. Dr. Kumar Sharma was supported by the National Kidney Foundation-Young Investigator Grant and a KO8 grant DK-02308 from the NIH.

Reprint requests to Fuad N. Ziyadeh, M.D., Renal-Electrolyte Division, 700 Clinical Research Building, University of Pennsylvania, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104-6144, USA.
E-mail: ziyadeh@mail.med.upenn.edu

APPENDIX

Abbreviations used in this article are: ATP, adenosine 5′-triphosphate; CAT, chloramphenicol acetyltransferase; CREB, cAMP responsive element binding; CTP, cytidine 5′-triphosphate; DMEM, Dulbecco's modified essential medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GTP, guanosine 5′-triphosphate; MMC, murine mesangial cells; PAI-1, plasminogen activator inhibitor-1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TGF-β, transforming growth factor-beta; UTP, uridine triphosphate.

REFERENCES

1. Krolewski AS, Warram JH, Laffel LMB: Genetic susceptibility to diabetic nephropathy. (abstract) *Adv Nephrol* 21:69, 1992
2. Brenner BM, Anderson S: Glomerular function in diabetes mellitus. *Adv Nephrol* 19:135–144, 1990
3. The Diabetes Control, Complications Trial Research Group: The effects of intensive insulin treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
4. Ziyadeh FN: Mediators of hyperglycemia and the pathogenesis of matrix accumulation in diabetic renal disease. *Miner Electrolyte Metab* 21:292–302, 1995
5. Sharma K, Ziyadeh FN: Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. *Diabetes* 44:1139–1146, 1995
6. Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
7. Roberts AB, McCune BK, Sporn MB: TGF-β: Regulation of extracellular matrix. *Kidney Int* 41:557–559, 1992
8. Massague J: Receptors for the TGF-β family. *Cell* 69:1067–1070, 1992
9. Sharma K, Jin Y, Guo J, Ziyadeh FN: Neutralization of TGF-β by anti-TGF-B antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in streptozotocin-induced diabetic mice. *Diabetes* 45:522–530, 1996
10. Sharma K, Ziyadeh FN: Biochemical events and cytokine interactions linking glucose metabolism to the development of diabetic nephropathy. *Semin Nephrol* 17:80–92, 1997
11. Wolf G, Sharma K, Chen Y, Ericksen M, Ziyadeh FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int* 42:647–656, 1992
12. Ziyadeh FN, Sharma K, Ericksen M, Wolf G: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by activation of transforming growth factor-β. *J Clin Invest* 93:536–542, 1994
13. van Det NF, van den Born J, Tamsa JT, Verhagen NAM, Berden JHM, Brujin JA, Daha MR, van der Woude FJ: Effects of high glucose on the production of heparan sulfate proteoglycan by mesangial and epithelial cells. *Kidney Int* 49:1079–1089, 1996
14. Ziyadeh FN, Snipes ER, Watanabe M, Alvarez RJ, Goldfarb S, Haverty TP: High glucose induces cell hypertrophy and stimulates collagen gene transcription in proximal tubule. *Am J Physiol* 259: F704–F714, 1990

15. Ziyadeh FN, Simmons DA, Snipes ER, Goldfarb S: Effect of *myo*-inositol on cell proliferation and collagen transcription and secretion in proximal tubule cells cultured in elevated glucose. *J Am Soc Nephrol* 1:1220–1229, 1991
16. Rocco M, Chen Y, Goldfarb S, Ziyadeh FN: Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule. *Kidney Int* 41:107–114, 1992
17. Dipaolo S, Gesualdo L, Ranieri E, Grandaliano G, Schena F: High glucose concentration induces the overexpression of transforming growth factor-β through the activation of a platelet-derived growth factor loop in human mesangial cells. *Am J Pathol* 149:2095–2106, 1996
18. Kolm V, Sauer U, Olgemoller B, Schleicher ED: High glucose-induced TGF-β1 regulates mesangial production of heparan sulfate proteoglycan. *Am J Physiol* 270:F812–F821, 1996
19. Wager RE, Scotto L, Assoian RK: Analysis of transforming growth factor β1 messenger RNA degradation by the transcript-selective, 12-O-tetradecanoylphorbol-13-acetate-regulated ribonuclease system from U937 promonocytes. *Cell Growth Differ* 5:117–124, 1994
20. Kim S-J, Glick A, Sporn MB, Roberts AB: Characterization of the promoter region of the human transforming growth factor-β1 gene. *J Biol Chem* 264:402–408, 1989
21. Geiser AG, Kim S-J, Roberts AB, Sporn MB: Characterization of the mouse transforming growth factor-β1 promoter and activation by the Ha-ras oncogene. *Mol Cell Biol* 11:84–92, 1991
22. Scotto L, Vaduvas PI, Wager RE, Assoian RK: Type β1 transforming growth factor gene expression. *J Biol Chem* 265:2203–2208, 1990
23. Kreisberg JI, Radnik RA, Ayo SH, Garoni J, Saikumar P: High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cell culture. *Kidney Int* 46:105–112, 1994
24. Daniel S, Kim K-H: Sp1 mediates glucose activation of the acetyl-CoA carboxylase promoter. *J Biol Chem* 271:1385–1392, 1996
25. Liu Z, Thompson K, Towle H: Carbohydrate regulation of the rat L-type pyruvate kinase gene requires two nuclear factors: LF-A1 and a member of the c-myc family. *J Biol Chem* 268:12787–12795, 1993
26. Shih H, Towle HC: Definition of the carbohydrate response element of the rat S14 gene. *J Biol Chem* 267:13222–13228, 1992
27. Wolf G, Haberstroh U, Neilson EG: Angiotensin II stimulates the proliferation and biosynthesis of type I collagen in cultured murine mesangial cells. *Am J Pathol* 140:95–107, 1992
28. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB: An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 216:276–284, 1994
29. Fumo P, Kuncio GS, Ziyadeh FN: PKC and high glucose stimulate collagen alpha1(IV) transcriptional activity in a reporter mesangial cell line. *Am J Physiol* 267:F632–F638, 1994
30. Sharma K, Ziyadeh F: Renal hypertrophy is associated with upregulation of TGF-β1 gene expression in diabetic BB rat and NOD mouse. *Am J Physiol* 267:F1094–F1101, 1994
31. Farrell RE: *RNA Methodologies.* New York, Academic Press, Inc., 1993
32. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning* (2nd ed). Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 1989
33. Sharma K, Danoff TM, Depiero A, Ziyadeh FN: Enhanced expression of inducible nitric oxide synthase in murine macrophages and glomerular mesangial cells by elevated glucose levels: possible mediation via protein kinase C. *Biochem Biophys Res Comm* 207:80–88, 1995
34. Wolf G, Ziyadeh FN, Zahner G, Stahl RAK: Angiotensin II-stimulated expression of transforming growth factor-β1 in renal proximal tubular cells: Attenuation after stable transfection with *c-mas* oncogene. *Kidney Int* 48:1818–1827, 1995
35. Schreiber E, Matthias P, Muller MM, Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. (abstract) *Nucleic Acids Res* 1:6419, 1989
36. Scmidt C, Fischer G, Kadner H, Genersch E, Kuhn K, Poschl E: Differential effects of DNA-binding proteins on bidirectional transcription from the common promoter region of human collagen type IV genes COL4A1 and COL4A2. *Biochim Biophys Acta* 1174:1–10, 1993
37. Wakefield L, Kim S-J, Glick A, Winokur T, Coletta A, Sporn M: Regulation of transforming growth factor-β subtypes by members of the steroid hormone superfamily. *J Cell Sci* 13(Suppl):139–148, 1990
38. Shih H-M, Liu, Z, Towle HC: Two CACGTG motifs with proper spacing dictate the carbohydrate regulation of hepatic gene transcription. *J Biol Chem* 37:21991–21997, 1995
39. Phillips AO, Steadman R, Topley N, Williams JD: Elevated D-glucose concentrations modulate TGF-beta 1 synthesis by human cultured renal proximal tubular cells. The permissive role of platelet-derived growth factor. *Am J Pathol* 147:362–374, 1995
40. Wilmer WA, Cosio FG: DNA binding of activator protein-1 is increased in human mesangial cells cultured in high glucose concentrations. *Kidney Int* 53:1172–1181, 1998
41. DeRubertis FR, Craven PA: Activation of protein kinase C in glomerular cells in diabetes. Mechanisms and potential links to the pathogenesis of diabetic glomerulopathy. *Diabetes* 43:1–8, 1994
42. Koya D, Jirousek MR, Lin Y-W, Ishii H, Kuboki K, King GL: Characterization of protein kinase β isoform activation on the gene expression of transforming growth factor-β, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 100:115–126, 1997
43. Kim S, Denhez F, Kim KY, Holt J, Sporn MB, Roberts AB: Activation of the second promoter of the transforming growth factor-β1 gene by transforming growth factor-β1 and phorbol ester occurs through the same target sequences. *J Biol Chem* 264:19373–19378, 1989
44. Kreisberg JI, Radnik RA, Kreisberg SH: Phosphorylation of cAMP responsive element binding protein after treatment of mesangial cells with high glucose plus TGF-β or PMA. *Kidney Int* 50:805–810, 1996
45. Wang L, Zhu Y, Sharma K: Transforming growth factor β-1 stimulates protein kinase A in mesangial cells. *J Biol Chem* 273:8522–8527, 1998
46. Ziyadeh FN, Han DC: Involvement of transforming growth factor-β and its receptors in the pathogenesis of diabetic nephropathy. *Kidney Int* 52(Suppl 60):S7–S11, 1997