

# Increased Glomerular and Tubular Expression of Transforming Growth Factor- $\beta$ 1, Its Type II Receptor, and Activation of the Smad Signaling Pathway in the *db/db* Mouse

Soon Won Hong, Motohide Isono, Sheldon Chen, M. Carmen Iglesias-de la Cruz, Dong Cheol Han, and Fuad N. Ziyadeh

From the Renal-Electrolyte and Hypertension Division of the Department of Medicine and the Penn Center for the Molecular Studies of Kidney Diseases, University of Pennsylvania, Philadelphia, Pennsylvania

**Activation of the renal transforming growth factor- $\beta$  (TGF- $\beta$ ) system likely mediates the excess production of extracellular matrix in the diabetic kidney. To establish the role of the TGF- $\beta$  system in type 2 diabetic nephropathy, we examined the intrarenal localization and expression of the TGF- $\beta$ 1 isoform, the TGF- $\beta$  type II receptor, and the Smad signaling pathway in the 16-week-old *db/db* mouse, a genetic model of type 2 diabetes that exhibits mesangial matrix expansion, glomerular basement membrane thickening, and renal insufficiency that closely resemble the human disease. Compared with its nondiabetic *db/m* littermate, the *db/db* mouse showed significantly increased TGF- $\beta$ 1 mRNA expression by *in situ* hybridization in both glomerular and tubular compartments. Likewise, TGF- $\beta$ 1 protein, by immunohistochemical staining, was increased in both renal compartments, but the fractional expression of TGF- $\beta$ 1 protein was less than that of the mRNA in the glomerulus. *In situ* hybridization and immunohistochemical staining for the TGF- $\beta$  type II receptor revealed concordant and significant increases of both mRNA and protein in the glomerular and tubular compartments of diabetic animals. Finally, immunohistochemistry showed preferential accumulation of Smad3 in the nuclei of glomerular and tubular cells in diabetes. The complementary technique of southwestern histochemistry using a labeled Smad-binding element demonstrated increased binding of nuclear proteins to Smad-binding element, indicating active signaling downstream of the TGF- $\beta$  stimulus. We therefore propose that the TGF- $\beta$  system is up-regulated at the ligand, receptor, and signaling levels throughout the renal cortex in this animal model of type 2 diabetes. Our findings suggest that the profibrotic effects of TGF- $\beta$  may underlie the progression to glo-**

**merulosclerosis and tubulointerstitial fibrosis that characterize diabetic nephropathy. (Am J Pathol 2001, 158:1653-1663)**

An expanding body of *in vitro* and *in vivo* studies implicates the profibrotic cytokine, transforming growth factor- $\beta$  (TGF- $\beta$ ), in the pathogenesis of diabetic kidney disease.<sup>1</sup> Increased amounts of TGF- $\beta$ 1 mRNA and protein have been demonstrated in the glomeruli and tubulointerstitial compartments of diabetic patients with nephropathy.<sup>2,3</sup> In animal models of diabetes, the intrarenal expression of TGF- $\beta$ 1 was similarly elevated at both the mRNA and protein levels in the streptozotocin-induced diabetic rat,<sup>4-6</sup> the streptozotocin diabetic mouse,<sup>7</sup> the spontaneously diabetic BioBreeding rat, and the nonobese diabetic mouse.<sup>8</sup> In cell culture experiments, high glucose has been shown to stimulate the production of TGF- $\beta$ 1 by various renal cell lines derived from the glomerular and tubulointerstitial compartments.<sup>9-14</sup>

In contrast to TGF- $\beta$ , the expression of the TGF- $\beta$  receptors has not been as extensively examined. Only a handful of *in vitro* studies have demonstrated that high ambient glucose stimulates TGF- $\beta$  type II receptor (T $\beta$ RII) expression in cultured renal cells.<sup>15-17</sup> In the streptozotocin-diabetic mouse, we previously showed that T $\beta$ RII mRNA and protein, assessed by Northern and Western blots, were increased in the renal cortex.<sup>7,17</sup>

The *db/db* mouse (C57BL/KsJ), a genetic model of type 2 diabetes, exhibits clinical and histological features

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Address reprint requests to Fuad N. Ziyadeh, M.D., 700 Clinical Research Building, Renal-Electrolyte and Hypertension Division, University of Pennsylvania, 415 Curie Blvd., Philadelphia, PA 19104-6144. E-mail: ziyadeh@mail.med.upenn.edu.

**Table 1.** Characteristics of the Normal and Diabetic Mice

	db/m (n = 6)	db/db (n = 6)
Plasma glucose (mg/dl)	120 ± 7	450 ± 26*
Body weight (g)	23 ± 0.3	50 ± 0.7*
Kidney weight (mg)	130 ± 2	200 ± 6*
Plasma creatinine (mg/dl)	0.3 ± 0.02	0.6 ± 0.06*
Mesangial matrix (% of glomerular tuft area)		
Inner cortex	20.4 ± 2.5	32.7 ± 4.5*
Outer cortex	31.2 ± 4.5	54.6 ± 3.2*
Mesangial cell size (μm <sup>2</sup> )	342.0 ± 13.6	377.4 ± 10.2 <sup>†</sup>
Mesangial cell number (per glomerular cross section)	12.5 ± 1.4	13.3 ± 2.3
GBM thickness (nm)	17.4 ± 0.4	21.9 ± 0.7*

Mice (non-diabetic db/m or diabetic db/db) at 16 weeks of age.

\**P* < 0.05.

<sup>†</sup>*P* = 0.05 versus db/m.

of diabetic nephropathy that track the human disease.<sup>18,19</sup> This animal becomes hyperglycemic by 8 weeks of age and develops overt proteinuria and renal insufficiency by 16 weeks of age. The kidneys show the characteristic histological lesions of diabetic nephropathy including mesangial matrix expansion and glomerular basement membrane thickening. At the molecular level, mRNAs encoding α1(IV) collagen and fibronectin are overexpressed.<sup>20–22</sup> We recently reported that chronic treatment of *db/db* mice with a neutralizing anti-TGF-β antibody successfully prevented mesangial matrix expansion and renal insufficiency,<sup>23</sup> proving that the TGF-β system plays an important role in the development of diabetic nephropathy. However, the precise details of the expression of TGF-β, its type II receptor, and the signaling pathway were not delineated in that study.

The Smad family of proteins has recently been identified as a predominant signaling pathway by which TGF-β activates the transcription of several well-known TGF-β-inducible genes.<sup>24,25</sup> Binding of TGF-β to its type II receptor and subsequent recruitment of the type I receptor result in the phosphorylation and activation of two receptor-regulated Smads (R-Smads), Smad2 and Smad3. After associating with a common-Smad (co-Smad), Smad4, the Smad complex translocates into the nucleus where it cooperates with other transcription factors to coordinate the expression of target genes.<sup>26</sup> Because diabetes results in the activation of the renal TGF-β system, the Smad pathway is probably activated in the diabetic state, but this has not yet been shown either *in vitro* or *in vivo*.

The current study seeks to more fully characterize the intrarenal TGF-β system in type 2 diabetes. The glomerular and tubular distribution of TGF-β1 and its type II receptor were assessed at the mRNA and protein levels by *in situ* hybridization and immunohistochemistry, respectively. Translocation of Smad3 into the nucleus and nuclear binding activity to Smad-binding element (SBE) were ascertained by immunohistochemistry and Southwestern histochemistry, respectively. Our findings present a more complete picture of how the ligand, receptor, and signaling components of the renal TGF-β system may interact to promote the progression of diabetic kidney disease.

## Materials and Methods

### Experimental Animals and Design

The diabetic *db/db* and nondiabetic *db/m* mice were purchased from Jackson Laboratory (Bar Harbor, ME). Six animals from each group were killed at 16 weeks of age when the *db/db* mice have been overtly hyperglycemic and were likely to have developed diabetic nephropathy. Blood glucose was measured by the glucose oxidase technique (Sigma, St. Louis, MO), and serum creatinine was measured by a colorimetric assay (Sigma). The left kidney from each mouse was excised, bisected, and processed in fixative solutions as described below.

Each kidney was processed into frozen sections for *in situ* hybridization and into paraffin sections for periodic acid-Schiff staining, immunohistochemistry, and Southwestern histochemistry. Selected kidneys from each group were processed for ultrastructural examination as described below.

### Histological and Ultrastructural Examination

Formalin-fixed kidneys were embedded in paraffin, sectioned at 5 μm, and stained with periodic acid-Schiff. Thirty glomeruli were randomly selected from each animal, and the extent of extracellular mesangial matrix was identified by periodic acid-Schiff-positive material in the mesangium and factored by the glomerular tuft area.<sup>21</sup>

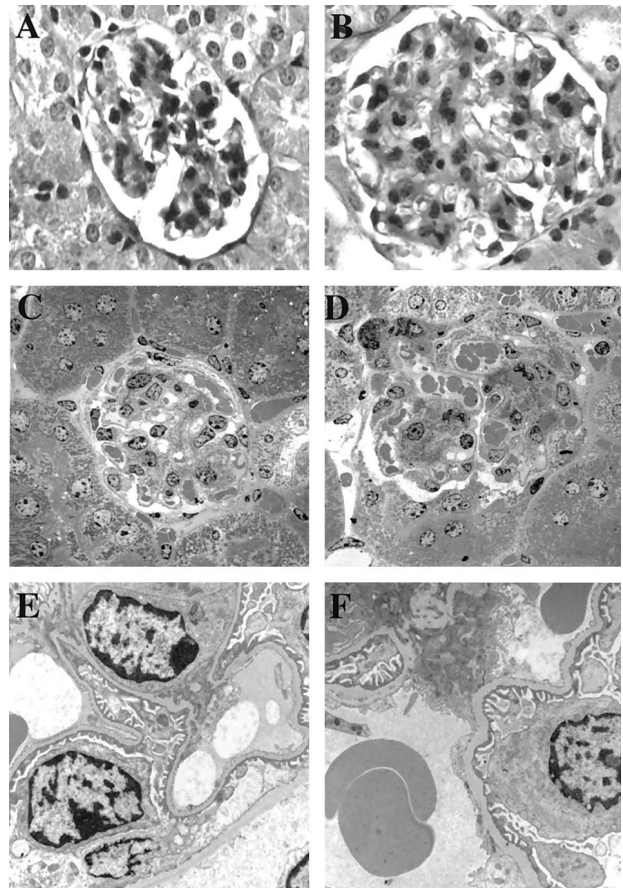
For ultrastructural evaluation, tissue was fixed in 3% glutaraldehyde, postfixed in osmium tetroxide, and stained with uranyl acetate and lead citrate. The specimen was thin-sectioned and examined under a transmission electron microscope. Electron microscopic pictures were randomly taken at ×1,000, ×2,000, and ×10,000 magnification in each group. With the computer program, Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD), the glomerular basement membrane thickness was measured on high magnification. Mesangial cell size and number were determined on low magnification.

### Immunohistochemical Staining of TGF- $\beta$ 1, T $\beta$ RII, and Smad3

Immunohistochemical stains for TGF- $\beta$ 1, T $\beta$ RII, and Smad3 were performed using a streptavidin biotin-staining method (Vector Laboratories, Burlingame, CA). After removal of paraffin, endogenous peroxidase activity was quenched by a 30-minute incubation with 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol. The slides were placed in 10 mmol/L citrate buffer (pH 6.0), microwaved for 10 minutes, and cooled down to room temperature. Slides were then placed in phosphate-buffered saline (PBS) for 15 minutes followed by protein-blocking solution (Immunotech, Cedex, France) for 30 minutes. Sections were incubated overnight at 4°C with one of three primary antibodies as follows: a polyclonal rabbit antibody against mouse TGF- $\beta$ 1, a polyclonal rabbit antibody against mouse T $\beta$ RII (both Santa-Cruz Biotechnology, Santa Cruz, CA), and a polyclonal rabbit antibody against mouse Smad3 (Zymed Laboratory, South San Francisco, CA), all at 1:100 dilution. The same concentration of isotype-matched antibody of irrelevant specificity was used as a negative control. After three 5-minute washes in PBS, a secondary biotinylated anti-rabbit antibody (Vector Laboratories) was added for 45 minutes at room temperature. After three 5-minute washes in PBS, streptavidin-biotin peroxidase (Vector Laboratories) was added for 45 minutes at room temperature. The color reaction was developed with the diaminobenzidine detection kit (Vector Laboratories) and counterstained with hematoxylin.

### TGF- $\beta$ 1 and T $\beta$ RII Riboprobe Preparation and *In Situ* Hybridization

The cDNA probes encoding mouse TGF- $\beta$ 1 and T $\beta$ RII were synthesized by polymerase chain reaction using murine kidney cDNA as template and specific oligonucleotide primers based on the published cDNA sequences as previously described.<sup>7,9,27</sup> The polymerase chain reaction products were subcloned into pCRII TA cloning vector (Invitrogen, Carlsbad, CA). The mouse TGF- $\beta$ 1 cDNA was linearized with *NotI* for antisense and *HindIII* for sense orientation, and the T $\beta$ RII cDNA was linearized with *HindIII* for antisense and *NotI* for sense orientation. The transcription reaction using digoxigenin-labeling of riboprobe was performed according to the manufacturer's instructions (Roche, Indianapolis, IN). The labeling mixture consisted of 1  $\mu$ g of template cDNA (in 13  $\mu$ l of water), 2  $\mu$ l of NTP labeling mixture, 2  $\mu$ l of transcription buffer, 1  $\mu$ l of RNase inhibitor, and 2  $\mu$ l of T7 or SP6 polymerase. Transcription was performed for 2 hours at 37°C, followed by digestion with 2  $\mu$ l of RNase-free DNase for 15 minutes at 37°C and purification by spin columns. For *in situ* hybridization, frozen kidney sections (5  $\mu$ m) were overlaid with 30  $\mu$ l of hybridization buffer containing the labeled RNA probe and incubated at 58°C overnight in a humid chamber. After hybridization, sections were washed once in 2 $\times$  standard saline citrate (SSC) at room temperature, once in 2 $\times$  SSC at



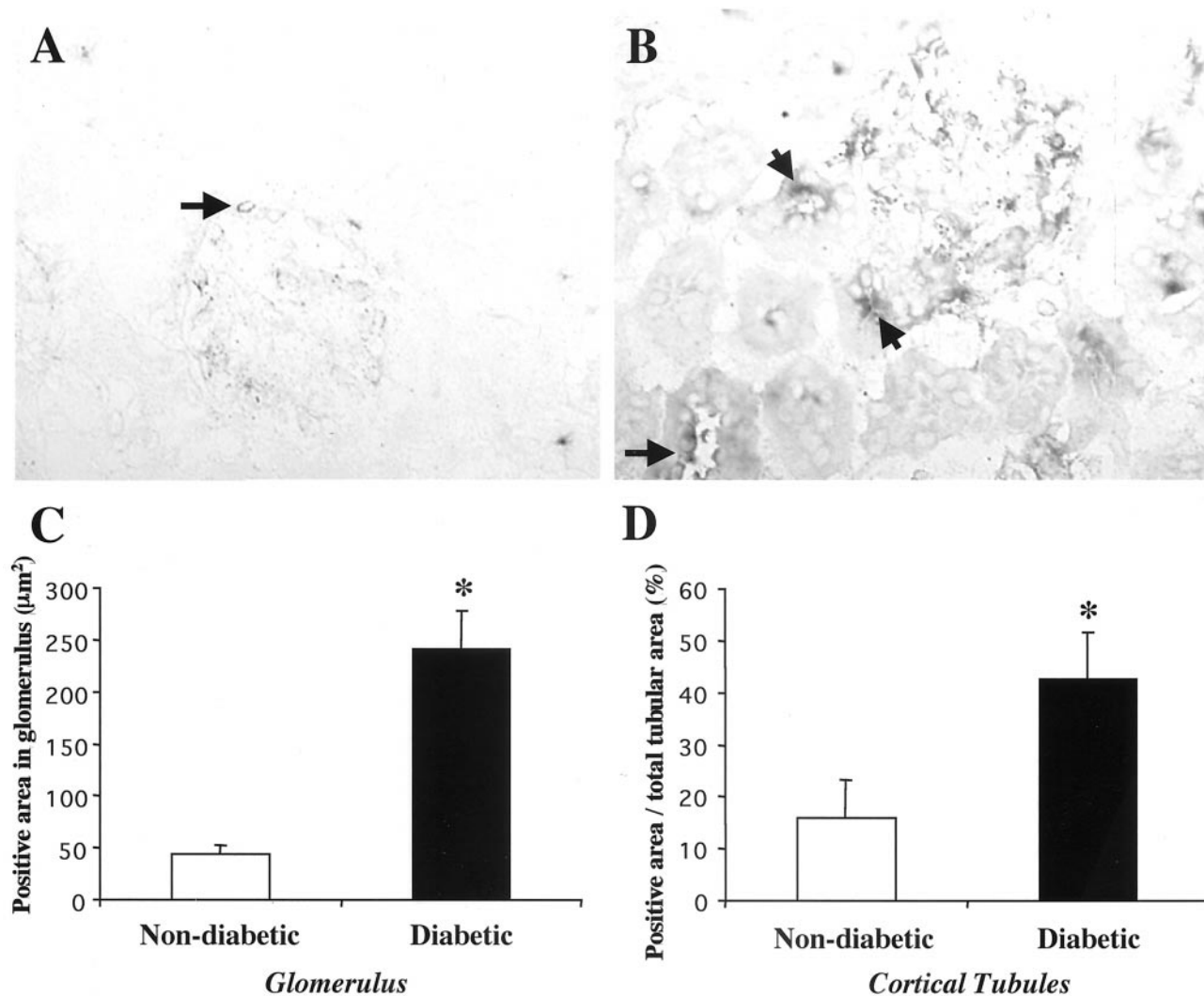
**Figure 1.** Histological and ultrastructural characteristics in diabetic *db/db* mice. Periodic acid-Schiff stain (original magnification,  $\times 400$ ) shows normal appearance in outer cortex of 16-week-old control *db/m* mouse (A) and increase in mesangial matrix and glomerular hypertrophy in diabetic *db/db* mouse (B). Electron-microscopic image (original magnification,  $\times 1,000$ ) shows normal appearance of glomerulus in control *db/m* mouse (C) and increased extracellular mesangial matrix without a change in cellularity in the diabetic *db/db* mouse (D). The higher magnification ( $\times 10,000$ ) shows normal thickness of glomerular basement membrane of control *db/m* mouse (E) and irregular thickening of glomerular basement membrane in diabetic *db/db* mouse (F).

65°C, and once in 0.1 $\times$  SSC at 65°C. Slides were equilibrated with buffer I (100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) for 5 minutes. Anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5,000) was applied to the slides, which were incubated in a humid chamber for 2 hours at room temperature. After two washes in buffer I for 15 minutes each, the sections were equilibrated with developing buffer (100 mmol/L Tris, 100 mmol/L NaCl, 50 mmol/L MgCl<sub>2</sub>, pH 9.5) for 5 minutes. The color reaction was developed using nitro blue tetrazolium (NBT/BCIP, Roche) according to the manufacturer's instructions.

### Southwestern Histochemistry

Southwestern histochemistry was performed according to the literature with slight modifications.<sup>28</sup> Complementary oligonucleotides containing a Smad-binding consensus sequence<sup>29</sup> were synthesized as follows: 5'-GGAG-





**Figure 2.** Renal expression of TGF- $\beta$ 1 mRNA by *in situ* hybridization in diabetic *db/db* mice. *In situ* hybridization of kidney sections with antisense TGF- $\beta$ 1 riboprobe (original magnification,  $\times 400$ ) shows minimal signal (arrow) in control *db/m* mouse (A) and increased hybridization signals in the mesangial area in diabetic *db/db* mouse (B). Quantification of TGF- $\beta$ 1 mRNA expression shows higher glomerular TGF- $\beta$ 1 expression (C) and higher cortical tubular TGF- $\beta$ 1 expression (D) in diabetic *db/db* mice compared to *db/m* mice. Data are mean  $\pm$  SE; \*,  $P < 0.05$ .

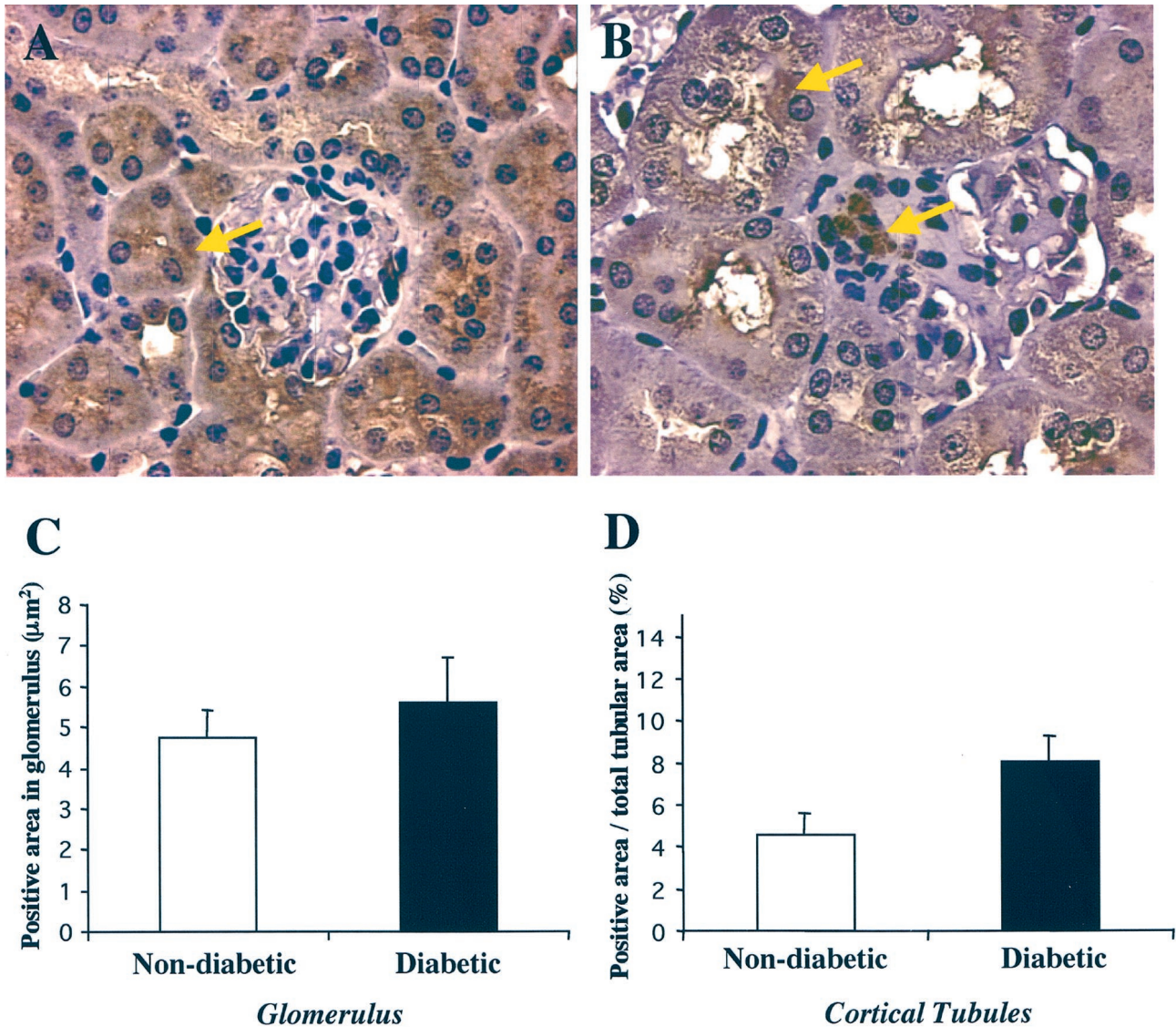
TATGTCTAGACTGACAATGTAC-3'. Paraffin-embedded kidney sections were dewaxed and rehydrated. The preparations were incubated with levamisole (Sigma Chemical Co., St. Louis, MO) to inhibit endogenous alkaline phosphatase activity and then fixed with 0.2% paraformaldehyde for 30 minutes at 28°C. Sections were subsequently digested with pepsin A (433 U/mg, Sigma) and washed twice in HEPES-bovine serum albumin. The sections were incubated with 0.1 mg/ml DNase I in HEPES-bovine serum albumin for 30 minutes at 30°C.

The labeled SBE probe, diluted to 100 pmol/L in HEPES-bovine serum albumin containing 0.5  $\mu$ g/ml poly (dI-dC) (Amersham Pharmacia, Piscataway, NJ), was applied to each slide overnight at 37°C. A 100-fold unlabeled SBE was used as competitive inhibitor. After incubation in blocking solution (0.01 $\times$  SSC, 0.01% sodium dodecyl sulfate, 0.03% Tween 20, 0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5), anti-digoxigenin antibody conjugated with alkaline phosphatase (1:250 in blocking so-

lution, Roche) was added overnight at 4°C. The color reaction was developed using nitro blue tetrazolium (NBT/BCIP, Roche) according to the manufacturer's instructions.

#### Quantification of Immunohistochemistry, *In Situ* Hybridization, and Southwestern Histochemistry Data

Twenty glomeruli and 10 tubular areas, at  $\times 400$  or  $\times 600$  magnification, were randomly selected for analysis in each of the experimental groups. The mRNA expression and protein production of TGF- $\beta$ 1 and T $\beta$ RII were measured by the density of the *in situ* hybridization signal and immunohistochemical-positive staining, respectively, in the glomerular and tubular compartments using the computer program, Image-Pro Plus 3.0. The nuclear accumulation of Smad3 protein and nuclear protein binding to



**Figure 3.** Renal expression of TGF- $\beta$ 1 protein by immunohistochemistry in diabetic *db/db* mice. Immunohistochemical stain of kidney sections (original magnification,  $\times 400$ ) shows that TGF- $\beta$ 1 protein is stained in glomerular and tubular areas (arrow) in the *db/m* mouse (A) and that TGF- $\beta$ 1 protein is slightly increased in glomerular and tubular areas with strong juxtaglomerular signal (arrow) in the diabetic *db/db* mouse (B). Quantification of TGF- $\beta$ 1 protein expression in glomerular (C) and in tubular areas (D) in normal *db/m* and diabetic *db/db* mice. Data are mean  $\pm$  SE.

SBE were assessed by manual tag counting using Image-Pro Plus 3.0.

### Statistical Analysis

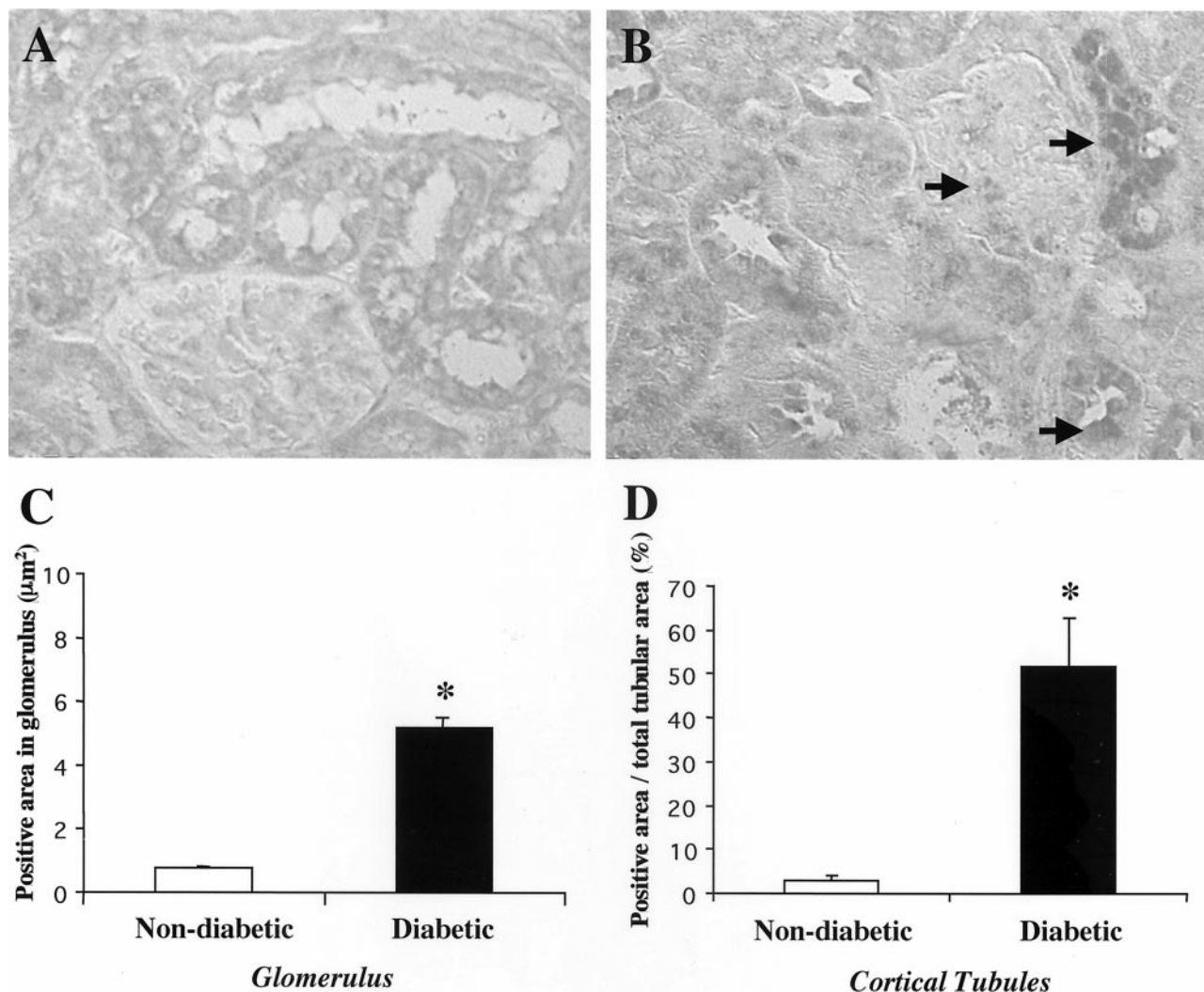
Data are presented as mean  $\pm$  SE. Groups were compared by unpaired Student's *t*-test.  $P < 0.05$  was considered significant.

### Results

#### Clinical Characteristics, Glomerular Histology, and Ultrastructural Findings

Clinical characteristics of the experimental mice are shown in Table 1. The diabetic mice were frankly hyper-

glycemic by the end of the experimental period. At age 16 weeks, the body weights of the *db/db* mice were significantly greater than those of the *db/m* mice. Kidney weights were also significantly greater in the *db/db* mice. However, average kidney-to-body weight ratios were predictably lower in the *db/db* group because the diabetic animals were much heavier. The *db/db* mice developed renal insufficiency as evidenced by the elevated plasma creatinine concentration. To assess glomerulosclerosis, we measured the glomerular fraction occupied by mesangial matrix and divided the analysis by location in the inner or outer cortex, because glomeruli are normally larger in the inner cortex. The mesangial matrix fraction was significantly increased in both inner and outer cortices in *db/db* compared with *db/m* mice (Figure 1, A and B; Table 1). Ultrastructural examination of the kidney of *db/db* mice revealed increased mesangial extracellular



**Figure 4.** Renal expression of T $\beta$ RII mRNA by *in situ* hybridization in diabetic *db/db* mice. *In situ* hybridization of kidney sections with antisense T $\beta$ RII riboprobe (original magnification,  $\times 400$ ) in control *db/m* mouse (**A**) and in diabetic *db/db* mouse (**B**) shows increased hybridization signal (**arrow**) in the glomerular and tubular areas in the diabetic *db/db* mouse. Quantification of T $\beta$ RII mRNA expression shows higher glomerular T $\beta$ RII expression (**C**) and higher tubular T $\beta$ RII expression (**D**) in diabetic *db/db* mice compared to *db/m* mice. Data are mean  $\pm$  SE; \*,  $P < 0.05$ .

matrix (Figure 1, C and D). The size of the mesangial cell was slightly increased in *db/db* mice compared with *db/m* mice. However, the number of mesangial cells in the glomerulus was not different between the two groups (Table 1). In *db/db* mice, the glomerular basement membrane was thickened with irregular distortions and multifocal foot process effacement of the podocytes (Figure 1, E and F; Table 1). No electron dense deposits were seen.

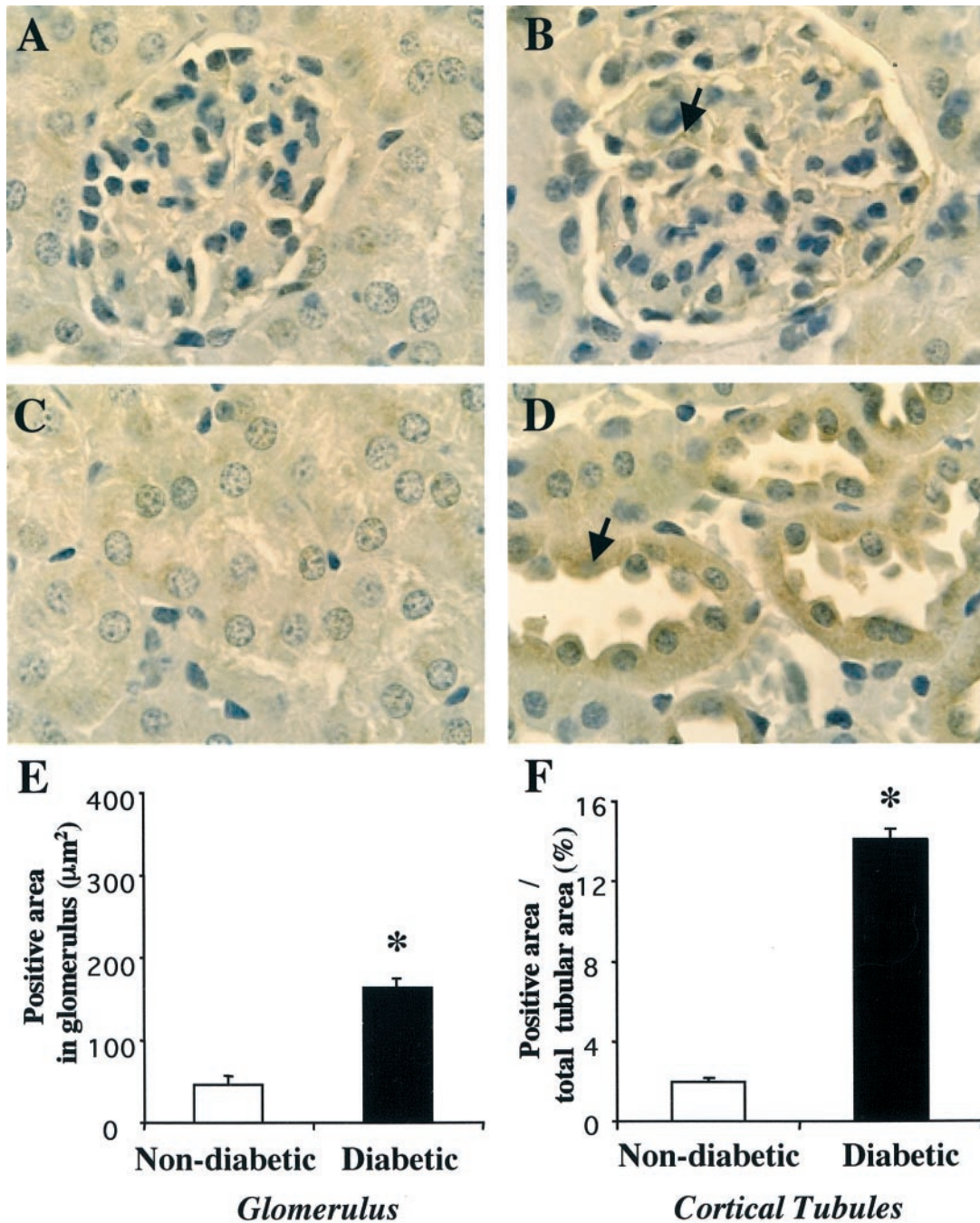
#### *Intrarenal TGF- $\beta$ 1 mRNA and Protein Localization*

To evaluate the renal TGF- $\beta$  system in the *db/db* mouse, we first examined the expression of TGF- $\beta$ 1 mRNA by *in situ* hybridization. As shown in Figure 2, A and B, the expression of TGF- $\beta$ 1 mRNA was significantly increased in the glomerular tuft, in a mesangio-capillary pattern, in

*db/db* mice compared with controls (Figure 2C). Although the staining of TGF- $\beta$ 1 mRNA was weaker in the cortical tubules of both nondiabetic and diabetic mice, formal quantitative analysis revealed a significant increase in the tubular TGF- $\beta$ 1 mRNA in *db/db* versus *db/m* mice as well (Figure 2D).

Immunohistochemistry localized the TGF- $\beta$ 1 protein predominantly to the proximal and distal tubules rather than the glomeruli in nondiabetic mice (Figure 3A). In diabetic mice, the intensity of staining in the tubules was slightly but not significantly increased (Figure 3, A and B). In addition, the glomerular staining was particularly prominent in the juxtaglomerular areas of diabetic mice (Figure 3, B versus A). Although the *db/db* group displayed increased TGF- $\beta$ 1 protein in both glomerular and tubular compartments, these differences did not reach statistical significance when compared with the *db/m* group (Figure 3, C and D). Thus, the relative changes in the quantity of





**Figure 5.** Renal expression of T $\beta$ RII protein by immunohistochemistry in diabetic *db/db* mice. Immunohistochemical stain of kidney sections for T $\beta$ RII protein (original magnification,  $\times 600$ ) in control *db/m* mouse (A and C) and in diabetic *db/db* mouse (B and D) shows increased staining (arrow) in the glomerular and tubular cytoplasm and in the plasma membrane of the diabetic *db/db* mouse. Quantification of T $\beta$ RII protein expression shows increased glomerular T $\beta$ RII expression (E) and increased tubular T $\beta$ RII expression (F) in diabetic *db/db* mice compared to *db/m* mice. Data are mean  $\pm$  SE; \*,  $P < 0.05$ .

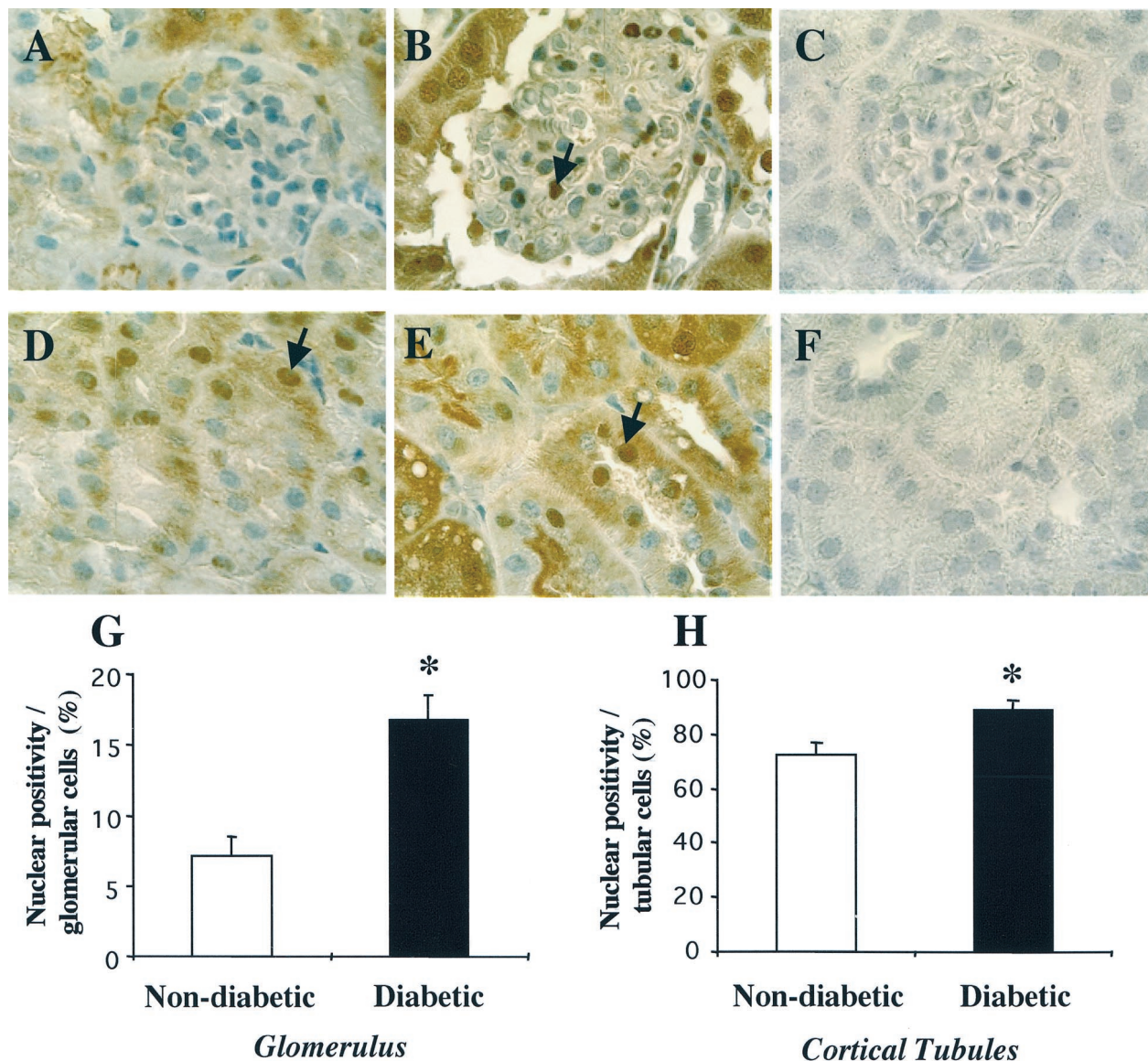
TGF- $\beta$ 1 protein in the diabetic mouse were less pronounced than the changes in the TGF- $\beta$ 1 mRNA discussed above.

*Intrarenal T $\beta$ RII mRNA and Protein Localization*

Whereas TGF- $\beta$ 1 mRNA staining was stronger in the glomeruli than in the tubules, T $\beta$ RII mRNA staining was stronger in the tubules than in the glomeruli of both groups of mice (Figure 4, A and B). Quantitatively, the

expression of T $\beta$ RII mRNA within the tubules was clearly and significantly increased in *db/db* mice (Figure 4D), but the expression of T $\beta$ RII mRNA within the glomeruli was also significantly increased in *db/db* compared with control *db/m* mice (Figure 4C).

By immunohistochemistry, T $\beta$ RII protein was primarily distributed in the distal tubules (Figure 5, C and D), and overall expression was markedly enhanced in diabetic versus nondiabetic mice (Figure 5, A–D). Like its mRNA counterpart, the T $\beta$ RII protein stained more heavily in the



**Figure 6.** Nuclear expression of Smad3 protein by immunohistochemistry in diabetic *db/db* mice. Immunohistochemical stain for Smad3 protein (original magnification,  $\times 600$ ) shows patchy glomerular (A) and tubular (D) cytoplasmic staining with scanty nuclear accumulation in control *db/m* mouse, and strong diffuse glomerular (B) and tubular (E) cytoplasmic staining with intense nuclear accumulation (arrow) in diabetic *db/db* mouse. The incubation with substituted isotype-matched antibody shows negative staining in glomerulus and tubule (C and F). Quantification of Smad3 protein expression in glomeruli (G) and in tubules (H) shows significantly increased nuclear signal in diabetic *db/db* mice compared to *db/m* mice. Data are mean  $\pm$  SE; \*,  $P < 0.05$ .

tubular than in the glomerular compartments (Figure 5, D versus B). Compared with the nondiabetic controls, the diabetic mice displayed significantly greater staining densities for T $\beta$ RII protein in the tubules as expected (Figure 5F) but also in the glomeruli (Figure 5E).

#### Nuclear Accumulation of Smad3

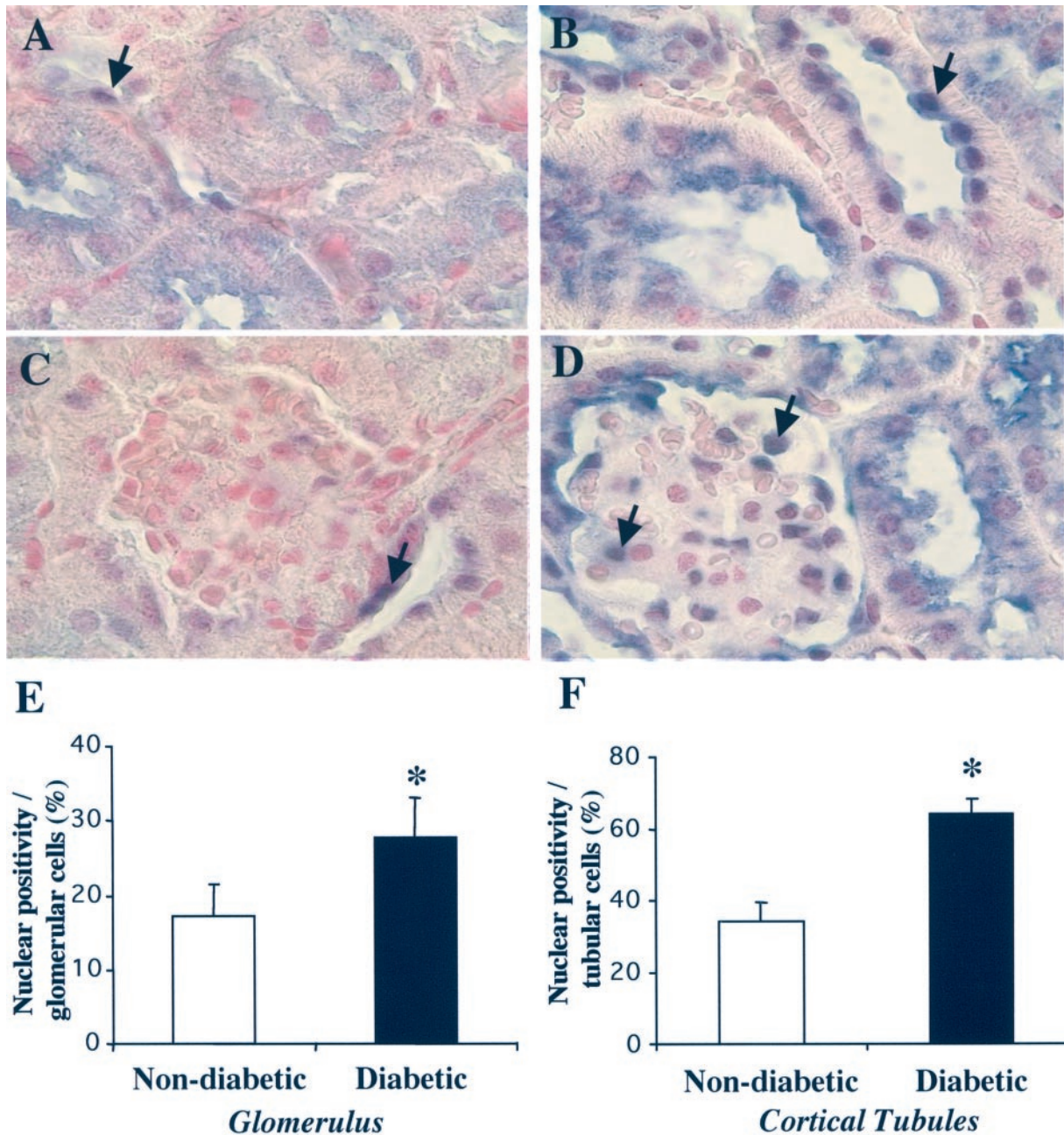
Recent pieces of evidence have demonstrated that Smad proteins are important intracellular transducers of TGF- $\beta$  signaling.<sup>26</sup> Therefore, we hypothesized that the Smad pathway is activated in the diabetic kidney, and Smad proteins would accumulate in renal cell nuclei. Immunohistochemical staining for Smad3 protein revealed diffusely increased cytoplasmic and nuclear staining in *db/db* mice compared with controls. By visual inspection,

nuclear staining of Smad3 protein was noticeably more prominent in the *db/db* mice (Figure 6, B and E) compared with *db/m* mice (Figure 6, A and D). By manual tag counting, Smad3 nuclear staining was confirmed to be significantly increased in both glomeruli and tubules in *db/db* mice (Figure 6, G and H).

#### Activation of Nuclear Protein Binding to SBE

After Smad3 is activated in response to TGF- $\beta$ , it translocates to the nucleus where it binds to certain DNA sites such as the SBE.<sup>29</sup> We next examined whether nuclear protein binding to SBE is enhanced in the diabetic kidney. To evaluate the extent of SBE binding, we performed Southwestern histochemistry.<sup>28,30</sup> As shown in Figure 7, labeled SBE localized more often to the nuclei of both





**Figure 7.** Nuclear binding activity to SBE by Southwestern histochemistry in diabetic *db/db* mice. Southwestern histochemistry with SBE (original magnification,  $\times 600$ ) shows patchy, scanty tubular cytoplasmic staining with sparse nuclear (arrow) accumulation in control *db/m* mouse (A and C) and strong nuclear accumulation (arrow) in tubular and glomerular cells in diabetic *db/db* mouse (B and D). Quantification of nuclear SBE staining in glomerulus (E) and in tubules (F) shows significantly increased binding in diabetic *db/db* mice compared to *db/m* mice. Data are mean  $\pm$  SE; \*,  $P < 0.05$ .

tubular (Figure 7, A and B) and glomerular (Figure 7, C and D) cells in *db/db* mice than in *db/m* controls. Figure 7, E and F, which quantitate the percentage of glomerular or tubular cells that display nuclear SBE binding, showed significant increases in the nuclear localization of SBE in diabetic compared with nondiabetic mice.

### Discussion

In this study, we attempted to provide a more complete description of the components of the renal TGF- $\beta$  system

in the *db/db* mouse, a genetic model of type 2 diabetes. The renal cortical parenchyma exhibited increases in both TGF- $\beta 1$  and T $\beta$ RII in the glomerular and tubular compartments. In addition, we demonstrated that the nuclear accumulation of Smad3 and the nuclear protein binding to SBE were increased in parallel with the mesangial expansion and glomerular hypertrophy that typically develop in the diabetic *db/db* mouse.

It has been well established that the TGF- $\beta$  cytokine is up-regulated in the kidney of animal models of type 1 diabetes. The intrarenal expression of TGF- $\beta$  mRNA, as-

sessed by Northern analysis or reverse transcriptase-polymerase chain reaction of whole kidney RNA, is increased in streptozotocin-induced diabetic animals.<sup>4,5,7</sup> Using immunohistochemistry and *in situ* hybridization, Park and colleagues<sup>6</sup> reported that the TGF- $\beta$  protein and mRNA were increased in the glomerular and interstitial compartments during the early stages of streptozotocin diabetes in rats. Our group has shown with Northern analysis that the expression of renal TGF- $\beta$ 1 mRNA is increased in the BioBreeding rat and the nonobese diabetic mouse, other models of type 1 diabetes.<sup>8</sup> Together, these results strongly support a pathogenic role for this cytokine in type 1 diabetic nephropathy.

The role of TGF- $\beta$  has not been as extensively studied in type 2 diabetic nephropathy. One study found by immunohistochemistry that the TGF- $\beta$ 1 protein was increased in the renal cortex of the Otsuka-Long-Evans-Tokushima-Fatty rat, another model of type 2 diabetes.<sup>31</sup> Another group showed that TGF- $\beta$  was overexpressed in the glomeruli of *db/db* mice in association with increased expression of fibronectin and type IV collagen.<sup>22</sup> Similarly, the present study demonstrated that the expression of TGF- $\beta$ 1 mRNA, assessed by *in situ* hybridization, was markedly enhanced in the glomeruli of *db/db* mice. Although this finding seems to conflict with our previous study in which Northern analysis of whole kidney RNA failed to detect an increase of TGF- $\beta$ 1 mRNA in *db/db* mice versus control,<sup>32</sup> these disparities may be reconciled if one considers that TGF- $\beta$ 1 overexpression occurred predominantly in the glomerular compartment only. Hybridization signals for TGF- $\beta$ 1 mRNA were also increased in the cortical tubular compartment between diabetic and nondiabetic groups, but the staining was patchy and irregular. Furthermore, the renal medulla did not show any increase in TGF- $\beta$ 1 expression by *in situ* hybridization (data not shown). Because glomeruli comprise <10% of the renal parenchyma, the local increase in glomerular TGF- $\beta$ 1 was masked by the lack of increase in tubular and medullary TGF- $\beta$ 1 when whole kidney RNA was examined. In addition, Northern analysis of the whole kidney may not be sensitive enough to resolve small differences in message expression in the various renal compartments.

The marked increase in glomerular TGF- $\beta$ 1 mRNA, however, did not result in a proportional increase in TGF- $\beta$ 1 protein. This contrasts with the situation in type 1 diabetic animals that show significant elevations of both TGF- $\beta$ 1 message and protein in the glomerulus.<sup>7</sup> The answer may lie with insulin, which has metabolic and growth-promoting effects on the kidney in diabetes.<sup>33,34</sup> We speculate that the hyperinsulinemia of type 2 diabetes, absent in type 1, may inhibit the translation of TGF- $\beta$ 1 mRNA to protein. In fact, preliminary work on cultured renal tubular cells has revealed that exogenous insulin may alter the production of TGF- $\beta$ 1 protein through a posttranscriptional mechanism.<sup>35,36</sup>

On the other hand, T $\beta$ RII protein was up-regulated in accord with the mRNA expression in both glomeruli and tubules of the diabetic mice. These findings are consistent with our previous study using Western and Northern analyses of whole kidney tissue.<sup>23,32</sup> In experimental di-

abetic conditions, the up-regulation of T $\beta$ RII by high glucose may sensitize the mesangial cell to the effects of exogenous TGF- $\beta$ 1, which will considerably enhance its type IV collagen production for example.<sup>17</sup> Extrapolated to the *in vivo* situation, these findings suggest that increased T $\beta$ RII expression may enhance the responsiveness to TGF- $\beta$ , thus accentuating the extracellular matrix accumulation in the kidneys of *db/db* mice.

Interestingly, nuclear accumulation of Smad3 and nuclear protein binding to SBE were increased in the kidney of diabetic *db/db* mice. To our knowledge, these *in vivo* observations are the first to prove that the specific downstream signaling pathway of TGF- $\beta$  is activated in the kidney in diabetes. After the binding of the TGF- $\beta$  ligand, Smad3 is phosphorylated and translocates into the nucleus where it participates in the transcriptional regulation of TGF- $\beta$  target genes.<sup>26</sup> Several TGF- $\beta$  target genes relevant to renal fibrosis, such as plasminogen activator inhibitor PAI-1 and type I collagen, have been reported to be regulated by Smad3 through Smad binding sites in their promoters.<sup>37-39</sup> Furthermore, Smad proteins have been shown to interact with other transcription factors, such as activator protein-1 and the family of cAMP-responsive element-binding proteins, which are required for TGF- $\beta$ -induced transcription of other fibrosis-related genes.<sup>26,40,41</sup> Although the precise involvement of the Smad pathway in the regulation of extracellular matrix proteins needs to be clarified, it is possible that the activation of the Smad pathway leads to up-regulation of not only type I collagen but also other extracellular matrix proteins.

Our histological observations and their quantitation prove that the critical components of the TGF- $\beta$  system are up-regulated throughout the renal cortex of the *db/db* mouse model of type 2 diabetes. Although the level of TGF- $\beta$ 1 protein was variably increased, the expression of T $\beta$ RII was particularly pronounced in both glomerular and tubular compartments of the renal cortex, and this translated into the downstream activation of Smad3, an important element of the TGF- $\beta$  signaling system. Given the well-known prosclerotic effects of TGF- $\beta$ , these findings strongly support a central role for the TGF- $\beta$  system in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis in the nephropathy of type 2 diabetes.

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