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Identification of Glucose-Regulated Genes in Human Mesangial Cells by mRNA Differential Display[1]

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Diabetic nephropathy is characterised by an accumulation of extracellular matrix proteins in the glomerular mesangium. Hyperglycaemia is a major factor promoting this progressive expansion of the mesangial matrix. We have used the technique of mRNA differential display to investigate changes in gene expression in cultured human mesangial cells following long-term (21 days) exposure to either physiologic (4 mM) or pathologic (30 mM) D-glucose concentrations. Approximately 12,000 mRNA species were screened for evidence of altered expression and several hundred candidate cDNA fragments were obtained. Northern blot and RT-PCR analysis of ten randomly chosen candidate cDNA fragments revealed three exhibiting increased mRNA expression under elevated D-glucose levels. Nucleotide sequence analysis identified two of the cDNA fragments as encoding prolyl 4-hydroxylase α-subunit and thrombospondin-1. The third cDNA fragment represents a novel glucose-regulated gene, encoding a putative zinc finger protein. Upregulated expression of these genes in response to high levels of D-glucose may contribute significantly to the disease process. mRNA differential display is a useful tool to investigate the mechanism of diabetic nephropathy.

Diabetic nephropathy is a major cause of renal failure in patients with both type I (insulin-dependent) and type II (non-insulin-dependent) diabetes (1). The progression of nephropathy correlates closely with expansion of the glomerular mesangium, primarily due to the accumulation of extracellular matrix (ECM) proteins (2, 3). Recent studies have demonstrated that rigorous control of the blood glucose level can effectively delay the onset and slow the progression of nephropathy in patients with type I diabetes (4-6). This suggests that exposure of the glomerulus to high concentrations of blood glucose is an important factor in inducing ECM accumulation within the mesangium. However, the precise mechanism by which this occurs remains uncertain.

The vast majority of ECM found within the mesangium is secreted by mesangial cells. Consequently, these cells play a pivotal role in the changes observed within the mesangium during the development of diabetic nephropathy. Previously, we used a candidate gene approach to investigate the effect of prolonged hyperglycaemic culture on human mesangial cells (7, 8). These studies demonstrated that elevated D-glucose levels increased synthesis of several ECM components, including collagen types I and III, fibronectin, laminin and decorin. Additionally, hyperglycaemic culture was found to reduce the major neutral protease activity, which may contribute to a reduction in ECM degradation. These results indicate that hyperglycaemic conditions cause a net accumulation of mesangial ECM *in vitro;* a situation akin to that observed in diabetic nephropathy *in vivo.*

mRNA differential display, reported first by Liang and Pardee (9), was developed as a tool to detect and characterise altered gene expression in eukaryotic cells. Unlike the candidate gene approach, this method allows the simultaneous isolation of multiple differentially expressed genes and is not limited solely to known molecules. This technique has been successfully used to identify glucose-regulated genes in bovine aortic smooth muscle cells (10), bovine retinal pericytes (11), rat cardiac ventricles (12) and rat kidneys (13). In this study, we have used mRNA differential display to identify glucose-regulated genes in human mesangial cells. We report that hyperglycaemic conditions increase mRNA levels of prolyl 4-hydroxylase α-subunit, thrombospondin-1 and a novel gene, encoding a putative zinc finger protein. The altered expression of these genes in response to elevated D-glucose levels suggests that they may play a role in the development of diabetic nephropathy.

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MATERIALS AND METHODS

Materials. Normal adult human mesangial cells were obtained from Clonetics. A second set of adult human mesangial cells used to reconfirm the differential expression of Clone D mRNA was obtained from Dr. David Wheeler (Department of Nephrology, Queen Elizabeth Hospital, Edgbaston, Birmingham, U.K.). All cells were prepared and characterised as described previously (7). Special RPMI 1640 medium without D-glucose, fetal bovine serum and antibiotics were from GIBCO BRL. Insulin, transferrin, sodium selenite (ITS) was from Sigma Chemical Co. RNAzol B was purchased from TEL-TEST Inc. and MessageClean Kits were obtained from GenHunter Corporation. RNAguard ribonuclease inhibitor and Oligolabelling Kits were purchased from Pharmacia Biotech. [^{35}S]dATPaS and [a^{32}P]dCTP were obtained from Amersham. Oligonucleotide primers were from Oswel (Southampton, U.K.).

Cell culture. Human mesangial cells were grown at 37°C with 5% CO_2 in RPMI 1640 medium (4 mM D-glucose, final concentration) supplemented with 10% (v / v) heat-inactivated fetal bovine serum, 100 U / ml penicillin, 100 μg / ml streptomycin and ITS (5 μg / ml, 5 μg / ml and 5 ng / ml, respectively) until confluent. Cells (passage 12) were then maintained in medium containing either 4 mM or 30 mM D-glucose for 21 days with replacement of medium every day in order to maintain consistent glucose levels.

RNA isolation. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (14) using RNAzol B according to the manufacturer's instructions. Residual chromosomal DNA was removed from total RNA samples by treatment with 10 U RNase-free DNase I (MessageClean Kit) in the presence of RNAguard. After phenol / chloroform (3 / 1) extraction and ethanol precipitation, RNA was resuspended in DEPC-treated H_2O.

mRNA differential display. Differential display was performed essentially as previously described (9, 15), with some minor modifications. Four reverse transcription reactions were performed for each RNA sample. Reactions (20 μl, final volume) contained 0.2 μg DNA-free total RNA, 1 × First Strand Buffer (GIBCO BRL), 10 mM DTT, 20 μM dNTPs, 1 μl RNAguard and 1 μM of either T_{12}MG, T_{12}MA, T_{12}MT or T_{12}MC oligonucleotide, where M is threefold degenerate for G, A and C (16). The mixture was heated to 65°C for 5 min, cooled to 37°C for 10 min and then 200 U of Superscript II RNase H$^-$ reverse transcriptase (GIBCO BRL) added. After incubation at 37°C for 50 min, the mixture was heated to 95°C for 5 min before PCR amplification or storage at −20°C. PCR was performed on a Hybaid OmniGene thermal cycler. Reaction mixtures (20 μl, final volume) contained 0.1 vol of reverse transcription reaction mixture, 1 × PCR buffer, 2 μM dNTPs, 10 μCi [^{35}S]dATPαS (1,200 Ci / mmol), 1 μM of the respective T_{12}MN oligonucleotide, 1 μM of one of 21 different specific arbitrary 10-mer oligonucleotides (17) and 1 U *Taq* DNA polymerase (GIBCO BRL). The cycling parameters were as follows: 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles, followed by 72°C for 5 min. Amplification products (4 μl) were denatured by heating for 3 min at 80°C in an equal vol of DNA sequencing stop buffer (U.S. Biochemical Inc.) and electrophoresed at 60 W constant power on a 6% denaturing polyacrylamide gel until the xylene cyanol dye reached the bottom of the gel. After electrophoresis, gels were dried without fixation onto Whatman 3MM filter paper and exposed directly to Kodak BioMax MR-2 film overnight at room temperature.

Recovery and reamplification of cDNAs. Bands reproducibly showing differential expression were excised from the dried gel and the DNA eluted by boiling in 10 mM Tris-HCl / 1 mM EDTA (pH 8.0) for 20 min. After ethanol precipitation, the DNA (0.4 vol) was reamplified in a 40 μl reaction volume using the same primer set and PCR conditions as used in the differential display except for dNTP concentrations of 20 μM and no radioisotope. Siliconised microcentrifuge tubes were used throughout the recovery and reamplification process to minimise DNA losses (18). PCR products were electrophoresed on 1.5% (w / v) agarose gels and visualised by ethidium bromide staining to confirm that reamplified cDNAs were of a size consistent with that observed by differential display.

Cloning and sequencing of cDNAs. The reamplified cDNAs (approximately 200-400 bp) were cloned into the vector pCR 2.1 using the TA Cloning Kit (Invitrogen Corporation). Plasmid DNA was purified using a Wizard Plus Miniprep Kit (Promega Corporation) and the cDNA inserts sequenced by the dideoxynucleotide chain-termination method (19) using an Applied Biosystems Model 373A automated DNA sequencer. Gene database searches were performed through the National Centre for Biotechnology using the BLAST network service (20).

Northern blot analysis. Northern blot analysis was performed according to standard protocols (21). Twenty μg of each total RNA sample was denatured and separated by electrophoresis on 1% (w / v) agarose gels containing 2.2 M formaldehyde. RNA was transferred to Magna nylon membranes (Micro Separations Inc.) by capillary action and immobilised by UV irradiation using a Stratalinker (Stratagene). The cloned cDNA inserts used as probes were released from pCR 2.1 by *Eco*R I digestion, gel-purified using the GeneClean procedure (Bio 101 Inc.) and labelled to high specific activity with [α^{32}P]-dCTP (3,000 Ci / mmol) by the random hexamer method (22) using an Oligolabelling Kit. Prehybridisation was performed in Rapid-Hyb buffer (Amersham) for 15 min at 65°C. Hybridisation was performed in the same solution with 2-5 × 10^7 cpm labelled probe for 2 hours at 65°C. Membranes were subsequently washed in 2 × SSC / 0.1% SDS for 30 min at room temperature, followed by 0.1 × SSC / 0.1% SDS for 30 min at 65°C and exposed to Hyperfilm-MP (Amersham) at −80°C with intensifying screens for 1-3 days. Membranes were stripped and rehybridised with the housekeeping gene GAPDH as a control for RNA loading and transfer. Autoradiographs were scanned on a Chromoscan 3 densitometer (Joyce-Loeble) and the hybridisation signal for each cDNA probe normalised against the signal for GAPDH.

RT-PCR analysis. Two μg of each DNA-free total RNA sample, prepared as above, was converted into cDNA with Superscript II RNase H$^-$ reverse transcriptase and oligo(dT)$_{12-18}$ primer (GIBCO BRL) according to the manufacturer's instructions. Equal amounts of cDNA were subsequently amplified by PCR in a 100 μl reaction volume containing 1 × PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.2 μM of each specific primer and 2.5 U *Taq* DNA polymerase. Conditions for amplification were 94°C for 4 min (denaturation step), followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension of 72°C for 10 min was included in all instances. Control amplifications were performed with GAPDH to confirm the use of equal amounts of RNA and to allow PCR products to be quantitated comparatively. The amount of cDNA used for PCR was selected as being non-saturating for the GAPDH PCR product after 30 cycles of amplification. The sequences of the primers for clone D mRNA were designed from the sequence of the cloned differential display product: (sense) 5′-CTGACTTAGGTCTCATCCAG-3′ and (antisense) 5′-GGACACATATAGGGACGAAG-3′. The GAPDH primers were designed from the published sequence of the human gene: (sense) 5′-CCATGGAGAAGGCTGGGG-3′ and (antisense) 5′-CAAAGTTGTCATGGATGACC-3′. Amplification products were electrophoresed on 2% (w / v) agarose gels and visualised by ethidium bromide staining.

RESULTS

Confluent cultures of human mesangial cells were maintained in media containing physiologic (4 mM) or pathologic (30 mM) D-glucose concentrations for a period of 21 days. Subsequently, total RNA was extracted and mRNA differential display performed to identify genes whose expression was altered in response to ele-

vated levels of D-glucose. A total of 21 arbitrary 10-mer primers were used in combination with all four $T_{12}MN$ primers. Each primer set displayed 150-200 discrete bands, allowing over 12,000 mRNA species to be screened in this study. Comparison of the amplified cDNA products between cells exposed to 4 mM and 30 mM D-glucose revealed the profiles to be largely identical (data not shown). This provided a uniform background over which specific differences could be observed. Approximately 2% of the mRNAs screened appeared to be differentially expressed, yielding several hundred candidate cDNA fragments. This relatively high percentage of differentially expressed genes is in close agreement with the percentage of hyperglycaemia-regulated genes observed in a similar study using diabetic rat kidneys (13).

From the pool of candidate cDNA fragments obtained, ten were randomly selected for detailed characterisation. All ten excised bands were successfully recovered from the dried gel and reamplified, each producing a single band of the expected size (data not shown). Reamplified cDNAs were cloned into the vector pCR 2.1 and the cDNA inserts sequenced in both directions. All clones had flanking primer sequences identical to those used in the differential display. The ten subcloned cDNA inserts were then excised from the vector and used as probes for Northern blot analysis. Signals for eight of the clones were detected on Northern blots containing total RNA. Two of the clones, identified as encoding prolyl 4-hydroxylase α-subunit (P4-Hα) and thrombospondin-1 (TSP-1), showed significant changes in expression (Figure 1). mRNA levels for P4-Hα and TSP-1 were upregulated 7.9-fold and 2.3-fold respectively, in 30 mM D-glucose as compared to 4 mM D-glucose. The other six clones showed equal expression in both 4 mM and 30 mM D-glucose. The remaining two candidate clones, presumably due to their low abundance, were only detected by RT-PCR analysis using gene specific primers designed from the sequences of the cloned differential display products. Only one of these, Clone D, showed a significant change in expression. As shown in Figure 2, a predicted 352 bp PCR product for Clone D mRNA was observed predominantly in 30 mM D-glucose, with only low level expression in 4 mM D-glucose. RT-PCR analysis was performed on RNA extracted from two further sets of mesangial cell cultures derived from a different patient. In both cases, Clone D mRNA expression was increased in 30 mM D-glucose, with only low level expression in 4 mM D-glucose (data not shown). This suggests that the gene corresponding to Clone D is constitutively expressed in physiologic glucose concentrations, although at such a low level that it is difficult to detect. However, the increased expression of Clone D mRNA in 30 mM D-glucose in all three sets of mesangial cell cultures confirms that it is reproducibly upregulated by elevated levels of D-glucose.

The nucleotide sequence of Clone D (Figure 3) re-

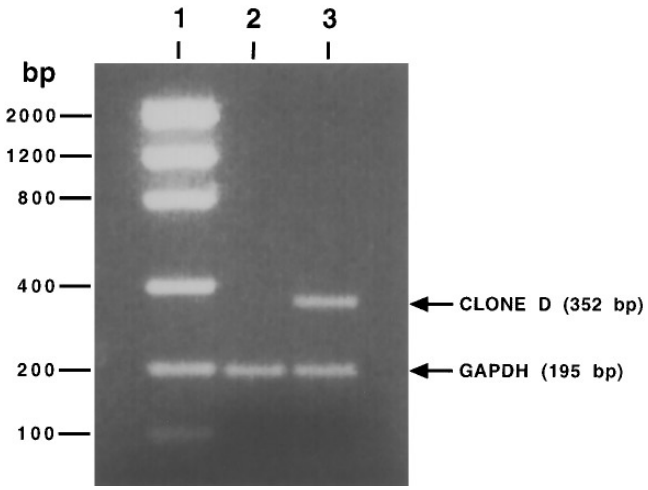

FIG. 2. Effect of elevated D-glucose levels on Clone D mRNA expression in human mesangial cells. RT-PCR analysis was performed as described under Materials and Methods using DNA-free total RNA (2 μg) isolated from cells exposed to 4 mM (*Lane 2*) or 30 mM (*Lane 3*) D-glucose for 21 days. Low DNA Mass Ladder (GIBCO BRL) was used as a molecular size marker (*Lane 1*). A predicted 352 bp PCR product for Clone D mRNA is observed predominantly in 30 mM D-glucose. Control amplifications were performed with GAPDH (195 bp PCR product) to confirm the use of equal amounts of RNA. Clone D and GAPDH amplification products were co-loaded.

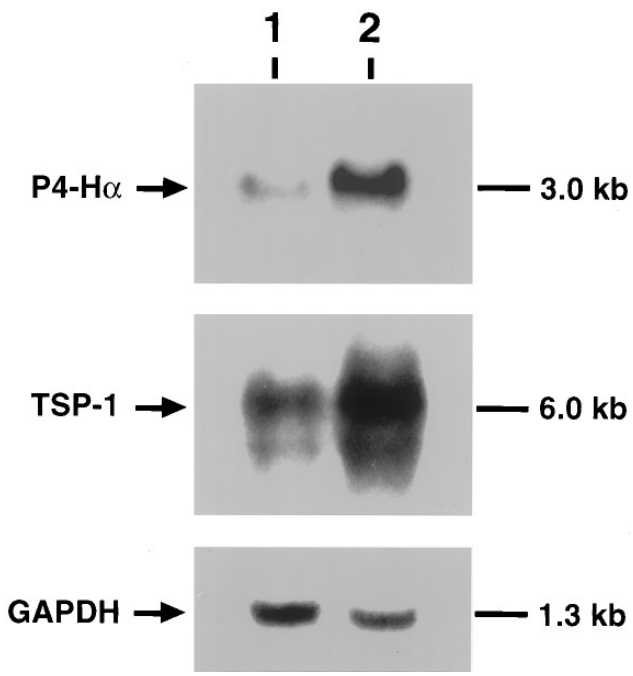

FIG. 1. Effect of elevated D-glucose levels on P4-Hα and TSP-1 mRNA expression in human mesangial cells. Northern blot analysis was performed as described under Materials and Methods using total RNA (20 μg per lane) isolated from cells exposed to 4 mM (*Lane 1*) or 30 mM (*Lane 2*) D-glucose for 21 days. The same blot was probed sequentially for P4-Hα, TSP-1, and GAPDH expression. Approximate mRNA sizes are shown in kb.

1	**GAA**	**CCA**	**ATC**	CTC	ATA	ACT	GAC	TTA	GGT	CTC	ATC	CAG	CCC	ATT	
	E	P	I	L	I	T	D	L	G	L	I	Q	P	I	14
43	CCA	AAA	AAC	CAG	TTT	TTC	CAA	AGC	TAT	TTC	AAT	AAT	AAT	TTT	
	P	K	N	Q	F	F	Q	S	Y	F	N	N	N	F	28
85	GTC	AAT	GAA	GCA	GAT	AGA	CCA	TAC	AAG	TGT	TTT	TAC	TGT	CAT	
	V	N	E	A	D	R	P	Y	K	*C*	F	Y	*C*	H	42
127	CGT	GCA	TAT	AAA	AAA	TCT	TGC	CAC	CTT	AAA	CAA	CAC	ATC	AGA	
	R	A	Y	K	K	S	C	H	L	K	Q	*H*	I	R	56
169	TCC	CAT	ACA	GGT	GAA	AAA	CCT	TTT	AAA	TGT	TCT	CAG	TGT	GGA	
	S	*H*	T	G	E	K	P	F	K	*C*	S	Q	*C*	G	70
211	AGA	GGC	TTT	GTC	TCT	GCA	GGC	GTC	CTC	AAA	GCA	CAC	ATC	AGA	
	R	G	F	V	S	A	G	V	L	K	A	*H*	I	R	84
253	ACA	CAC	ACA	GGA	CTG	AAA	TCT	TTC	AAG	TGT	CTG	ATA	TGT	AAT	
	T	*H*	T	G	L	K	S	F	K	*C*	L	I	*C*	N	98
295	GGG	GCT	TTC	ACT	ACT	GGT	GGC	AGC	TTA	CGG	CGA	CAC	ATG	GGT	
	G	A	F	T	T	G	G	S	L	R	R	*H*	M	G	112
337	ATC	CAC	AAC	GAC	CTT	CGT	CCC	TAT	ATG	TGT	CCC	TAT	TGC	CAA	
	I	*H*	N	D	L	R	P	Y	M	*C*	P	Y	*C*	Q	126
379	**AAA**	**AAA**	**AAA**	**A**											
	K	K	K												129

FIG. 3. Nucleotide and deduced amino acid sequence of Clone D. Flanking primer sequences (*bold*) and the cysteines and histidines of the putative zinc fingers (*italics* and *underlined*) are noted.

vealed several interesting features. Firstly, the sequence represents putative coding sequence and not 3′ untranslated sequence as is predicted by the differential display method. This situation arose from priming of the anchored primer at an internal poly(A) site, corresponding to a series of lysine residues, and not at the targeted poly(A) tail. Secondly, the deduced amino acid sequence of Clone D is predominantly basic and contains at least three zinc finger motifs of the Cys_2His_2 type. Comparison of the sequence of Clone D with the gene databases revealed significant homology (up to 68% at the nucleotide level) to other zinc finger proteins, but only in the region of the zinc fingers. This suggests that Clone D represents a novel gene, encoding a putative zinc finger protein.

DISCUSSION

In this study, we have identified three genes in cultured human mesangial cells whose expression is significantly increased in response to elevated D-glucose levels. None of these genes, identified by mRNA differential display, have previously been reported to be transcriptionally regulated by glucose. DNA sequencing and database analysis identified two of these genes as P4-Hα and TSP-1. Northern blot analysis confirmed that transcript levels for both P4-Hα and TSP-1 were increased in 30 mM D-glucose as compared to 4 mM D-glucose. The third gene, Clone D, was identified as a previously uncharacterised gene, encoding a putative zinc finger protein. No signal was observed for Clone D mRNA on Northern blots containing total RNA, necessitating the use of an alternative method to verify differential expression. The failure of some differential display products to detect mRNAs on Northern blots containing total RNA has been well documented in the literature (10, 11, 15, 16). It is generally believed that these products perform poorly as probes because typically they are short and AT rich, and often represent mRNAs of very low abundance. More sensitive methods of detection include ribonuclease protection assays and Northern blots containing poly(A) RNA. However, these methods require large quantities of RNA and thus are not suitable when RNA is in limited supply. In these circumstances, RT-PCR analysis is the preferred method, provided that adequate sequence information is available for the design of suitable primers. RT-PCR analysis of Clone D mRNA expression confirmed the increased expression of Clone D mRNA in 30 mM D-glucose.

Prolyl 4-hydroxylase plays a vital role in collagen synthesis, catalysing the posttranslational formation of 4-hydroxyproline residues in collagens (23). This reaction is essential for the correct folding of newly synthesised procollagen polypeptide chains into triple helical molecules. The active enzyme is an $\alpha_2\beta_2$ tetramer, composed of two distinct types of monomer. The amount of prolyl 4-hydroxylase activity is regulated by the level of synthesis of the α-subunit, which in turn is regulated by the rate of collagen synthesis (23-25). This correlation has been observed both in cultured cells and *in vivo* in many physiological and pathological states. Previously, in our candidate gene studies (7), we demonstrated that hyperglycaemic conditions cause increased synthesis of collagen types I and III in cultured human mesangial cells. Consequently, increased mRNA expression of P4-Hα is likely to reflect the increased collagen synthesis observed in human mesangial cells in response to elevated levels of D-glucose.

TSP-1 is an extracellular matrix glycoprotein that influences many aspects of cell behaviour, including cell adhesion, motility and growth (26). Synthesis of TSP-1 has previously been demonstrated in cultured human mesangial cells (27) and transcription of its mRNA has been shown to be regulated by both PDGF and TGF-β (28). Increased expression of TSP-1 mRNA may contribute to an accumulation of ECM proteins within the mesangium in several ways. Firstly, TSP-1 has been shown to activate latent TGF-β by a protease and cell independent mechanism, possibly by inducing a conformational change in the latent complex (29-31). TGF-β is a major factor stimulating the expression of ECM protein genes (32) and has been proposed to be an important factor in driving fibrosis of the diabetic glomerulus (33). Additionally, we have shown previously that chronic hyperglycaemic culture conditions stimulate TGF-β expression in human mesangial cells (7). Consequently, any increase in the levels of active TGF-β by TSP-1 activation may stimulate increased synthesis of ECM proteins. Secondly, TSP-1 has been shown to inhibit plasmin both directly (34) and indirectly through stimulation of plasminogen activator inhibitor 1 (PAI-1) synthesis (35). Increased levels of PAI-1 have been reported in cultured human mesangial cells in response to elevated levels of D-glucose (8) and in diabetic patients (36, 37). Plasmin plays an important role in regulating the turnover of ECM proteins (38-40) and inhibition of this protease might reduce turnover and thus contribute to an accumulation of ECM proteins.

Clone D represents a previously uncharacterised gene whose product and role have yet to be determined. The deduced amino acid sequence of Clone D is predominantly basic and contains at least three zinc finger motifs of the Cys_2His_2 type, characteristic of TFIIIA-like zinc finger proteins (41). The presence of these sequence elements strongly suggests a role for Clone D in DNA binding. Given the increased expression of Clone D mRNA under elevated levels of D-glucose and the large number of glucose-induced changes in gene expression observed in this study, it is possible that Clone D may play a role in mediating the transcriptional response of some of these genes to glucose. However, verification of this will require isolation of the full length sequence for Clone D and characterisation of the encoded protein.

In summary, we have demonstrated the utility of mRNA differential display by isolating genes that are transcriptionally regulated by glucose in cultured human mesangial cells. Identification of these genes and the mechanism by which their expression is regulated is likely to provide important insights into the overall mechanism responsible for the development of diabetic nephropathy. The precise mechanism by which high D-glucose levels trigger changes in gene expression remains unclear. Several pathways which could be activated in high glucose conditions may contribute to modulation of gene expression. These include activation of protein kinase C and pathways downstream of it, polyol pathway activation and stress (hyperosmolarity)-activated pathways, although most studies published to date have shown that the effects of high glucose on gene expression are not mediated by an osmotic effect (e.g. refs. 10, 11). The three genes described here, P4-Hα, TSP-1 and Clone D, all exhibit increased mRNA expression in response to elevated D-glucose levels. However, further studies on the corresponding protein levels both in culture and *in vivo* are required before these genes can be implicated with a role in diabetic nephropathy.

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