Regulation of gene expression by alternative polyadenylation and mRNA instability in hyperglycaemic mesangial cells

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We have used mRNA differential display to identify a novel high-glucose-regulated gene (*HGRG-14*) in human mesangial cells cultured for up to 21 days in 30 mM D-glucose. The mRNA of HGRG-14 seems to be regulated post-transcriptionally and encodes a small polypeptide of molecular mass 13 kDa. The native protein occurs as a dimer. The recombinant protein is a substrate for casein kinase II kinase. At high glucose concentrations, HGRG-14 protein levels decrease. This correlates with the appearance of a long form of HGRG-14 mRNA under high-glucose conditions. This form has a long 3′ untranslated region containing several ATTTA RNA-destabilizing sequences and has a short half-life. A truncated, more stable mRNA that lacks the long 3′ untranslated region is produced at 4 mM D-glucose. The switch from the truncated to the long-form transcript is detected within 2 h of exposure to 30 mM D-glucose, indicating that hyperglycaemic conditions have an acute effect on HGRG-14 mRNA processing.

INTRODUCTION

Diabetic nephropathy is a fatal complication of both type I and II diabetes mellitus. The major histological characteristic of this disease is the enlargement of the glomerular mesangium due to the accumulation of extracellular matrix (ECM) proteins synthesized by the mesangial cells. The degree of mesangial expansion is correlated with the loss of renal function [1]. Hyperglycaemia is strongly implicated in the pathogenesis of nephropathy [2]. Previously we have demonstrated that cultures of human mesangial cells (HMC) mimic the situation *in vivo* because they produce excessive amounts of particular ECM proteins when exposed to prolonged high glucose concentrations [3]. In contrast, exposure to high glucose decreases the level and the activity of their secreted neutral ECM-degrading proteases [4]. The combined effect would favour the accumulation of mesangial ECM molecules, as seen in diabetic nephropathy. However, the molecular mechanism by which this occurs remains to be elucidated.

Detailed knowledge of the genes differentially expressed in hyperglycaemic conditions might provide important information about the abnormal biochemical events in the pathogenesis of diabetic nephropathy. We have employed the mRNA differential display technique [5] to identify novel high-glucose-regulated genes (HGRGs) in HMC. Here we report the isolation and characterization of one such gene whose expression we found to be regulated in an unexpected way at high glucose concentrations.

MATERIALS AND METHODS

Materials

RPMI 1640 medium without glucose, foetal-calf serum and antibiotics were from Gibco (Grand Island, New York, NY, U.S.A.). Insulin, transferrin and sodium selenite were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Glucose was added to the RPMI medium to the required concentrations.

Isolation and culture of HMC

Normal HMC were established and cultured as described previously [3]. The growth medium consisted of RPMI 1640 supplemented with 20 % (v/v) heat-inactivated foetal-calf serum, 50 i.u./ml penicillin, 50 μg/ml streptomycin, 300 μg/ml glutamine and 4 mM D-glucose. Insulin, transferrin and sodium selenite (final concentrations 5 μg/ml, 5 μg/ml and 5 ng/ml) were added routinely to all culture media. For experiments, confluent post-exponential-phase cultures of HMC (passage 8–10) were maintained in growth medium containing 10 % (v/v) foetal-calf serum and either 4 mM (normoglycaemic) or 30 mM (hyperglycaemic) D-glucose. The medium was changed every 48 h. After 21 days, cultures were washed extensively with PBS and used for the extraction of RNA and protein.

RNA extraction and differential display of mRNA

Total RNA was extracted from 6×10^6 mesangial cells by using the RNAzol B method (AMS). Samples were treated with DNase I using the Message Clean Kit of Bio Gene Ltd. (Bolnhurst, Beds., U.K.). RNA was dissolved in diethyl pyrocarbonate/deionized water, quantified and stored at -70 °C until used. Differential display was performed essentially as described by Liang et al. [6]. In brief, 200 ng of total RNA was reverse-transcribed in 12 independent reactions by using dT_{12} dVdN-anchored oligo(dT) primers and subsequently amplified by PCR with 10-mer arbitrary primers, which were selected from a list of random sequences referenced by Bauer et al. [7]. Differentially displayed bands were recovered from the dried DNA sequencing gel and cDNA fragments were amplified.

Cloning, sequencing and sequence analysis

Reamplified cDNA species were cloned into the TA-vector PCR II (InVitrogen, San Diego, CA, U.S.A.). Both strands of the cDNA were sequenced by using the T7 and SP6 promoter primers and an ABI 373 A automated sequencer. The BLAST N

Abbreviations used: CKII, casein kinase II; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *HGRG-14*, high-glucose-regulated gene 14; HMC, human mesangial cells; MBP, maltose-binding protein; PKC, protein kinase C; RT–PCR, reverse transcriptase-mediated PCR; UTR, untranslated region.

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and BLAST P programs [8] were used to search the computer databases for sequence similarities. The MacPattern program (Rainer Fuchs, EMBL data library, Heidelberg, Germany) was used to analyse the protein sequence for biologically significant motifs.

Reverse transcriptase-mediated PCR (RT–PCR) analysis

Equal amounts (2 μg) of total RNA from each sample were reverse-transcribed into cDNA species with SuperScript II RNase H^+ reverse transcriptase (Gibco BRL) and random primers. Equal amounts (1 μl) of the reverse transcription reaction (20 μl) were subjected to PCR amplification in a 100 μl volume containing 10 μl of 10 × PCR buffer, 16 μl of dNTPs (1.25 mM each), 2 mM $MgCl_2$, each specific primer at 20 μM and 1.25 units of Amplitaq DNA polymerase. Amplification was started with 5 min of denaturation at 94 °C followed by 30 PCR cycles. Each cycle consisted of 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C. The final extension lasted for 10 min at 72 °C in all instances. To quantify PCR products comparatively and to confirm the use of equal amounts of the RNA species we co-amplified a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin. The amount of reverse transcription reaction used for the amplification (1 μl) was selected as being non-saturating for the PCR product of both GAPDH and β-actin after 30 cycles of amplification.

The sequences of GAPDH and β-actin primers were designed from the published sequences of the human genes: GAPDH sense, 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH anti-sense, 5′-TCCACCACCCTGTTGCTGTA-3′ (450 bp) [9]; β-actin sense, 5′-ATCTGGCACCACACCTTCTACAATGAGC-TGCG-3′; β-actin anti-sense, 5′-CGTCATACTCCTGCTTGC-TGATCCACATCTGC-3′ (838 bp) [10]. Designed primers for the partial *HGRG-14* clone were P1 (sense), 5′-AAGGAGTT-TAATGCAGTGATCTTTGTTTTTGG-3′; P2 (anti-sense), 5′-CTAGAAACAGAGCAGATAGCAAGTTCACCAGG-3′ (208 bp). Designed primers for the open reading frame of *HGRG-14* clone were: P3 (sense plus *Eco*RI site), 5′-CGGGAATT-CATGGAGGAGTACCATCGCCACTGC-3′; P4 (anti-sense plus *Pst*I site), 5′-CTCCTGCAGCTATTAAAGAACAATAG-CAATGGCA-3′ (400 bp). After amplification, 10 μl of each PCR reaction mix was subjected to electrophoresis through a 2 % (w/v) agarose gel containing ethidium bromide (0.5 μg/ml). Gels were scanned with an Epson GT-8000 scanner and Adobe PhotoShop software. The pixel intensity for each band (average pixel intensity × area) was analysed with the Image software (NIH Shareware). The units of peak areas are arbitrary. To minimize error in the scanning procedure, the mean of three scans was used.

Cloning of different forms of HGRG-14 transcripts

Total RNA was extracted from mesangial cells. Poly(A)$^+$ mRNA was isolated with the mini Message Maker kit (R & D Systems Europe, Abingdon, Oxon., U.K.). Poly(A)$^+$ mRNA (1 μg) was reverse-transcribed into cDNA species with RNase H^+ reverse transcriptase as above, except that an oligo(dT) primer was used. The cDNA was then used as a template for PCR reactions in the presence of an oligo(dT) primer and the HGRG-14 5′-end-specific P3 primer (see above). PCR products were cloned into the TA-vector as above. Positive transformants were amplified, and plasmid DNA was prepared, checked by digestion and sequenced.

Expression of recombinant protein and production of antibodies

DNA sequences of the full-length open reading frame of *HGRG-14* were amplified by RT–PCR with the introduction of *Eco*R1 and *Pst*I sites for in-frame unidirectional cloning. The PCR product was subcloned into the expression plasmid pMAL-C2 (New England Biolabs, Hitchin, Herts., U.K.). Plasmid DNA of positive transformants was sequenced to confirm subcloning in the correct reading frame. Maltose-binding protein (MBP) fusion protein was induced and purified on amylose resin as described by the manufacturer. Rabbit antibodies were raised against the fusion protein by Cymbus Bioscience Ltd. (Chilworth Research Centre, Southampton, Hants., U.K.). The fusion protein was cleaved by digestion with factor Xa enzyme (New England Biolabs) to release HGRG-14p from the MBP and the digest was subjected to SDS/PAGE and immunoblotting.

Immunoblotting

Samples were solubilized in SDS/PAGE loading buffer containing a cocktail of protease inhibitors [1 mM EDTA, 1 mM EGTA, 0.2 mM 7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 1 mM *N*-ethylmalemide, 0.1 mM 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one and 2 mM PMSF (Sigma Chemical Co., Poole, Dorset, U.K.)]. Samples were resolved by SDS/PAGE [15 % (w/v) gel] [11] under both reducing and non-reducing conditions. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, Beds., U.K.) with a Bio-Rad transfer apparatus. Immunodetection was performed essentially as described by Towbin et al. [12]. Bound antibodies were detected with peroxidase-conjugated IgGs and 4-chloro-1-naphthol (Boehringer Mannheim Biochemicals, Lewes, East Sussex, U.K.).

Northern blot analysis

Total RNA (20 μg) was subjected to electrophoresis through a 1 % agarose formamide gel and transferred to a Hybond-N^+ membrane (Amersham International) by the method of Sambrook et al. [13]. Probes were synthesized by PCR and labelled by random priming in the presence of [α-^{32}P]dCTP with the Rediprime DNA labelling system (Amersham International). The membranes were prehybridized for 15 min then hybridized at 65 °C for 1 h with the Amersham Rapid-hyp buffer. Filters were washed at high stringency [0.1 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS at 55 °C] and autoradiographed with an intensifying screen. For rehybridization, the membranes were stripped by being washed in 0.1 × SSC/0.1 % SDS at 90 °C for 15 min and verified for residual bound probe by exposure to X-ray film for 3 days.

Transcription stability

The stability of HGRG-14 and β-actin mRNA species under normoglycaemic and hyperglycaemic conditions was assessed by incubating cells in actinomysin D (5 μg/ml). After incubation for various times, total RNA was isolated as above and assayed by slot-blot analysis as described previously [3]. A 400 bp cDNA corresponding to the coding region of *HGRG-14* was synthesized by PCR and used as the probe (see above). Autoradiograms were scanned with an Epson GT-8000 scanner and Adobe PhotoShop software as described above.

Protein phosphorylation *in vitro*

The recombinant fusion and MBP proteins were buffer-exchanged into the casein kinase II (CKII) kinase buffer [80 mM

KCl/5 mM $MgCl_2$/10 % (v/v) glycerol/20 mM Tris/HCl (pH 7.9)/0.1 % (v/v) Nonidet P40/10 mM dithiothreitol/0.1 mM PMSF] or protein kinase C (PKC) buffer [150 mM NaCl/10 % (v/v) glycerol/20 mM Hepes (pH 7.5)/0.1 % (v/v) Nonidet P40/ 10 mM dithiothreitol/0.1 mM PMSF] on PD-10 columns (Pharmacia). For PKC phosphorylation reactions, 20 μg of protein in 200 μl of kinase buffer was mixed with 10 μl of 40 mM $CaCl_2$, 1 μl of 400 mM $MnCl_2$, 175 ng of PKC (purified from rat brain; Biomol Research Laboratories) and 1 μl of [γ-^{32}P]ATP. The reactions were incubated at room temperature for 30 min after which 40 μl of amylose resin (1:1 solution equilibrated in kinase buffer) was added and the mixture incubated on ice for 15 min. The beads were then washed five times with 1 ml of cold kinase buffer and mixed with 60 μl of Laemmli sample buffer; the mixture was then analysed by SDS/PAGE [15 % (w/v) gel] and autoradiography. The same procedure was applied for CKII phosphorylation, except 1.4 μg of recombinant CKII (Biomol Research Laboratories) was used.

RESULTS

Confluent cultures of HMC at passage 8–10 were maintained in medium containing either 4 mM (normoglycaemic) or 30 mM (hyperglycaemic) D-glucose for 21 days. Total RNA was extracted and the mRNA differential display technique was applied. With this technique we have cloned many partial cDNA species, one of which, clone *HGRG-14*, we report here. This clone was 320 bp long. With the use of RT–PCR, this partial cDNA seemed to be expressed only in hyperglycaemic cells, whereas cells under both conditions expressed equal levels of the housekeeping genes GAPDH (Figure 1) or β-actin (results not shown). This partial clone was identical to the 3′ untranslated region (UTR) of a complete cDNA in the computer databases that was previously investigated as a candidate disease gene in retinitis pigmentosa. It was cloned from the RP3 locus on the X chromosome (p21), which is believed to be involved in the disease. However, although a single base-pair polymorphism was found in the coding reading frame, it was not associated with the disease [14]. The authors identified additional processed copies of the gene and pseudogenes on autosomes in the human genome. The complete cDNA is 2.1 kb in length with a small open reading frame of 116 residues encoding a protein of molecular mass approx. 13 kDa. Although Roux et al. [14] could not identify any putative protein motifs in the deduced amino acid sequence, we identified several motifs for potential post-translational modifications. These included a putative myristylation site, two putative CKII kinase phosphorylation sites, one putative PKC phosphorylation site and one N-linked glycosylation site (Figure 2). The 3′ UTR is approx. 1.75 kb in length and contains six putative polyadenylation signals and seven potential RNA-destabilizing signals (ATTTA). The latter are present in the mRNA of many transiently expressed genes and are associated with the rapid turnover of mRNA [15]. A conserved AU sequence from the 3′ UTR of granulocyte/macrophage colony-stimulating factor mRNA mediates selective mRNA degradation.

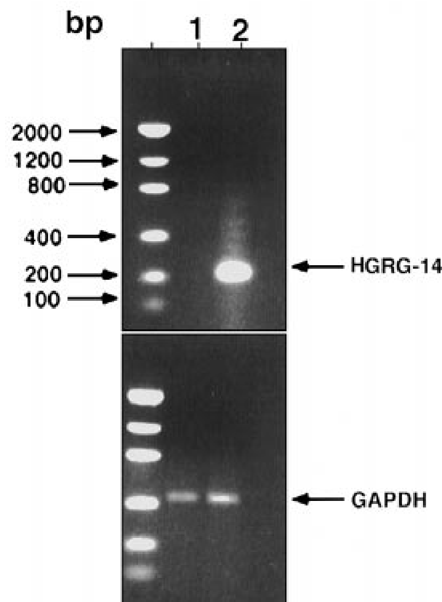

Figure 1 RT–PCR amplification of the partial *HGRG-14* cDNA and GAPDH

Cultures were maintained in medium containing 4 or 30 mM D-glucose for a period of 21 days. RT–PCR was performed with the P1 and P2 primers and GAPDH primers as described in the Materials and methods section. A 10 μl sample of each PCR reaction was subjected to electrophoresis through a 2% (w/v) agarose gel in the presence of ethidium bromide. Lane 1, PCR product from normoglycaemic cells; lane 2, PCR product from hyperglycaemic cells. The left-hand lane contained a DNA mass ladder, for which lengths are shown at the left.

Because the partial *HGRG-14* clone represents only 320 bp of the extreme 3′ UTR of the published sequence (Figure 2), we used RT–PCR to amplify sequences for the full open reading frame. The amplified product was subcloned into the expression vector pMAL C2. When cells (DH5 α) transformed with this vector were induced with isopropyl β-D-thiogalactoside, they expressed HGRG-14 protein (HGRG-14p) fused to MBP, which was purified on an amylose affinity column (results not shown). We used the recombinant fusion protein to raise polyclonal antibodies. Figure 3(a) shows that the antibody recognized both the fusion protein and the cleaved products. Western blot analysis showed that the same antibody cross-reacts with a native protein present in mesangial cell lysate from cultures maintained in either 4 or 30 mM glucose conditions for 21 days (Figure 3b). The molecular mass of this cross-reacting protein is approx. 26 kDa. It can be detected only under non-reducing conditions (Figure 3b) because no bands were detected if 5 % (v/v) 2-mercaptoethanol was added to the samples and boiled for 5 min before the gel was loaded (results not shown). The level of the detected protein also seems, unexpectedly, to be reduced under hyperglycaemic conditions. This change in protein expression is supported by the results of a second RT–PCR analysis with primers specific for the full open reading frame of *HGRG-14*. The oligonucleotide primers (P3 and P4) generated a 400 bp product from a template of RNA from either normoglycaemic or hyperglycaemic mesangial cells (Figure 4). However, the cDNA derived from the hyperglycaemic cells is less than that derived from normoglycaemic cultures. Thus it is likely that the lower level of HGRG-14p in hyperglycaemic cells is due to lower levels of mRNA coding for it in these conditions.

The recombinant protein was also used as a substrate in phosphorylation studies *in vitro*. No protein phosphorylation was detected by PKC (results not shown). In contrast, both the fusion protein and the cleaved HGRG-14p showed a low but appreciable level of phosphorylation by CKII kinase. Figure 5(A) shows the autophosphorylation of the α and β subunits of CKII at approx. 45 and 30 kDa respectively (lanes 1, 2 and 3), the phosphorylation of MBP–HGRG-14p fusion protein (lane 2) and the phosphorylation of cleaved HGRG-14p (lane 3). A Coomassie Blue-stained SDS/PAGE gel of the MBP–HGRG-14p fusion protein before and after cleavage with factor Xa is shown for comparison in Figure 5(B). No phosphorylation was

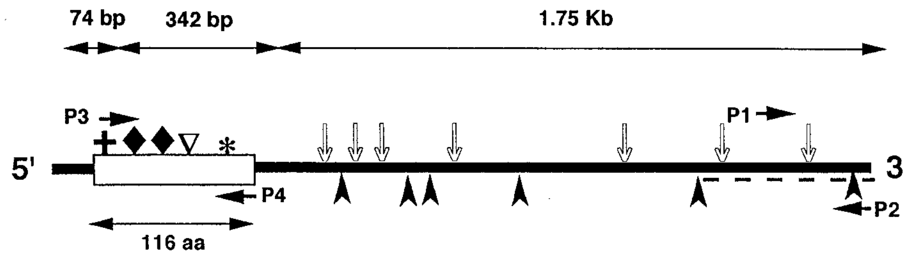

Figure 2 ***HGRG-14*** **cDNA and its identified putative motifs**

Symbols: +, putative myristylation site; ◆, putative CKII phosphorylation site; ▽, putative PKC phosphorylation site; *, putative N-linked glycosylation site; upwards arrowhead, putative polyadenylation signals, AATAAA; downwards open arrow, potential RNA-destabilizing sequences, ATTTA. P1 and P2 are designed sense and anti-sense primers for the partial *HGRG-14* clone; P3 and P4 are designed sense and anti-sense primers for the open reading frame of the *HGRG-14* clone. The broken line represents sequences identified by the mRNA differential display technique.

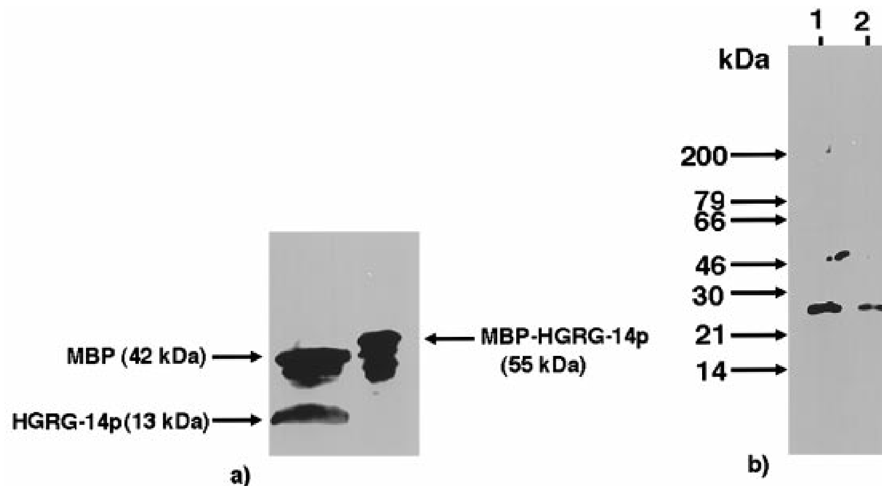

Figure 3 Identification of the recombinant and native HGRG-14 products by immunoblotting

Samples were solubilized in SDS/PAGE loading buffer in the presence of protease inhibitors and subjected to SDS/PAGE [15% (w/v) gel]. Proteins were electroblotted to membrane filters and probed for HGRG-14p. (**a**) Immunoblot of recombinant HGRG-14p after and before cleavage with factor Xa. (**b**) Immunoblot of mesangial cell lysates from cultures maintained in 4 mM (lane 1) or 30 mM (lane 2) D-glucose for 21 days. Each lysate was from 1.2×10^4 cells.

detected for MBP alone or MBP after cleavage from HGRG-14p. Three bands of low molecular mass are present in lanes 2 and 3 in addition to HGRG-14p. The identity of these is unclear but they might represent breakdown products of the CKII kinase or the fusion protein.

To clarify our results, we used Northern blot analysis to reassess the expression level of HGRG-14 in mesangial cells exposed to normoglycaemic and hyperglycaemic conditions for 21 days. Figure 6 shows Northern blot analyses of total RNA extracted from cells grown under both conditions. When the blot was hybridized with the ^{32}P-labelled 200 bp PCR product (Figure 1) that represents nucleotide sequences at the extreme 3′ UTR of *HGRG-14*, a single band of approx. 2.0 kb was detected only among RNA transcripts extracted from hyperglycaemic cells (Figure 6a, lane 1). The same blot was stripped and the ^{32}P-labelled 400 bp PCR product (Figure 4) that represents the nucleotide sequence of the full open reading frame was used as a probe. One hybridization band of approx. 700 bp was detected among RNA transcripts extracted from normoglycaemic cells, whereas two bands of approx. 2000 and 700 bp were detected among RNA transcripts extracted from hyperglycaemic cells. When the blot was restripped and reprobed once more for β-actin, one band of equal intensity and of the size expected (approx. 2 kb) for the housekeeping gene was detected among RNA transcripts extracted from cells maintained under both conditions. These results indicate that hyperglycaemic conditions regulate HGRG-14 mRNA processing.

To confirm this we cloned and sequenced the cDNA species from two clones derived from the short form of the HGRG-14 transcript. Both cDNA species were 600 bp long and were identical to the coding sequence of the gene mapped to the RP3 region of chromosome Xp21 [14]. Both cDNA species had a short poly(A) tail occurring 20 nt downstream from the first polyadenylation site. These results confirm that the truncated transcript of HGRG-14 is present in HMC maintained in 4 mM D-glucose conditions.

Cultures of HMC were transferred from medium containing 4 mM D-glucose to medium containing 30 mM D-glucose for 2 h, 2 days and 1 week to establish how rapidly changes in HGRG-14 transcript processing occurred after exposure to hyperglycaemic conditions. RNA was extracted and analysed by RT–PCR. Primers 3 and 4 were used to detect the coding region, whereas primers 1 and 2 only amplified cDNA from longer-form transcripts containing the full 3′ UTR. The results confirm that

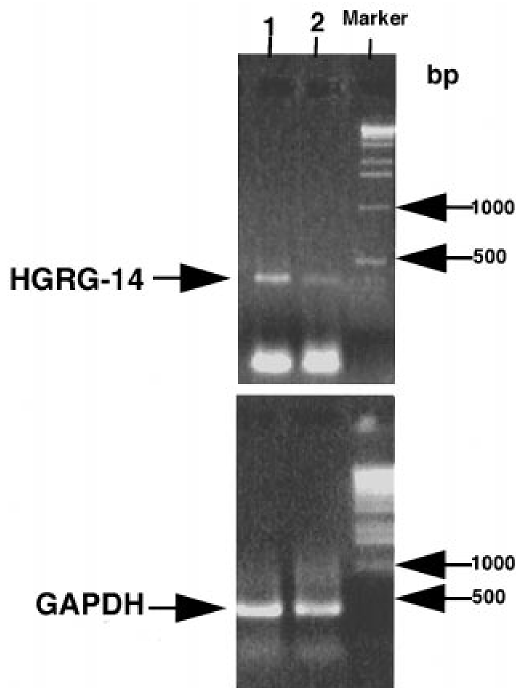

Figure 4 RT–PCR amplification of the open reading frame sequence of the HGRG-14 clone and GAPDH

RT–PCR was performed with the P3 and P4 primers and GAPDH primers as described in the Materials and methods section. A 10 μl sample of each PCR reaction was subjected to electrophoresis through a 2% (w/v) agarose gel in the presence of ethidium bromide. Lane 1, PCR product from normoglycaemic cells; lane 2 PCR product from hyperglycaemic mesangial cells. The experiment was repeated twice and RT–PCR was performed in triplicate for each experiment. Gels were photographed and the bands were quantified as described in the Materials and methods section. The mean pixel intensity units for HGRG-14 per equivalent GAPDH loading were 806 ± 52 (mean $\pm$ S.E.M.) for normoglycaemic cells and 238 ± 59 (mean $\pm$ S.E.M.) for hyperglycaemic cells.

the latter are not detectable in normoglycaemic conditions but appear within the first 2 h after exposure to high-glucose conditions (Figure 7).

To investigate whether the differential processing of HGRG-14 mRNA affects its stability and, in turn, the level of its translated protein, mesangial cells were maintained under normoglycaemic and hyperglycaemic conditions for 21 days and then incubated with actinomycin D to block transcription initiation. Total RNA was extracted at intervals after the block and analysed by slot-blotting for HGRG-14 and β-actin transcripts. Levels of mRNA were plotted as a semilogarithmic plot against time. The results in Figure 8 indicate that under hyperglycaemic conditions, HGRG-14 mRNA decays rapidly with a half-life of approx. 1.4 h, whereas under normoglycaemic conditions HGRG-14 mRNA remains relatively stable over the 4 h period. β-Actin mRNA seems to be stable under both conditions.

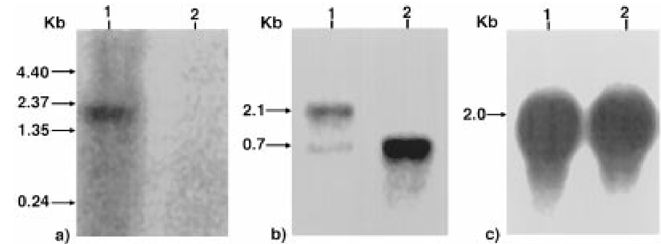

Figure 6 Northern blot analysis of HGRG-14 transcripts

Total RNA (20 μg) extracted from hyperglycaemic (lane 1) or normoglycaemic (lane 2) mesangial cells was subjected to electrophoresis through a 1.0% (w/v) agarose formamide gel and blotted to a filter membrane. (**a**) The filter was hybridized with ^{32}P-labelled 200 bp PCR product represented in Figure 1, corresponding to the 3′ UTR of the transcript. (**b**) The same filter was stripped and rehybridized with labelled 400 bp PCR product represented in Figure 4, corresponding to the coding region of the transcript. (**c**) The filter was stripped and rehybridized once more with labelled β-actin cDNA.

DISCUSSION

Investigation of the molecular mechanisms underlying the response of mesangial cells to high concentrations of glucose has led to the identification and subsequent characterization of a gene that we have called *HGRG-14*. The gene is one of many that seem to regulate their mRNA by using alternative polyadenylation sites at the 3′-end UTR [16], although the present paper is the first to report that changes in the environmental glucose concentration can modulate this mechanism. HGRG-14 uses alternative cleavage/poly(A) addition sites that either include or

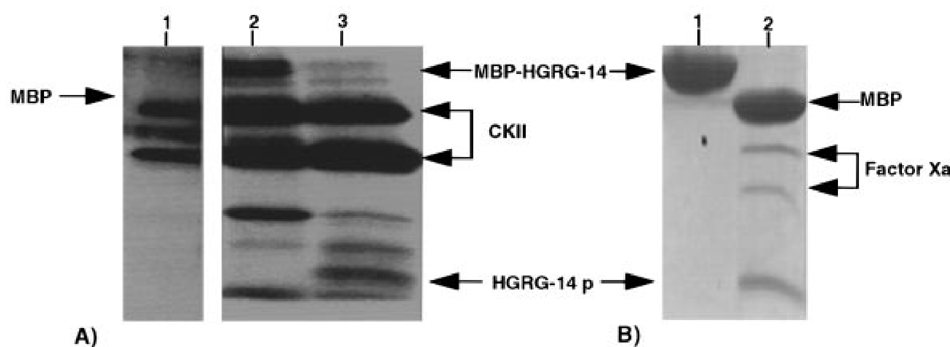

Figure 5 Phosphorylation of recombinant HGRG-14p *in vitro* by CKII

(**A**) Autoradiogram of phosphorylation products separated by SDS/PAGE [15% (w/v) gel]. Phosphorylation reactions were performed as described in the Materials and methods section in the presence of [γ-^{32}P]ATP and either MBP (lane 1), MBP–HGRG-14p fusion protein (lane 2) or MBP–HGRG-14p fusion protein treated with factor Xa (lane 3). (**B**) SDS/PAGE [15% (w/v) gel] stained with Coomassie Blue. Lane 1, MBP–HGRP-14p fusion protein; lane 2, fusion protein after cleavage with factor Xa.

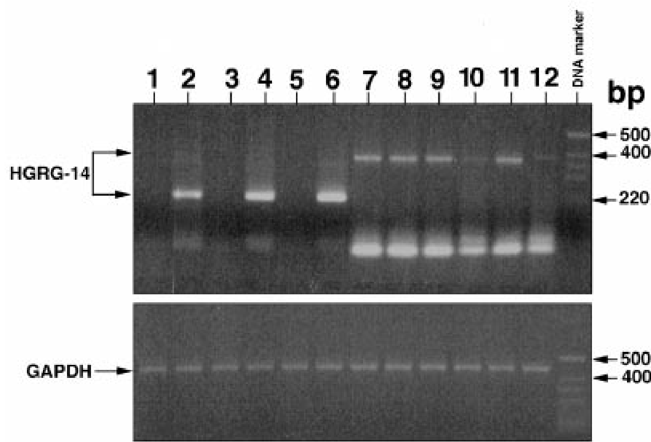

Figure 7 Identification of full-length transcripts of HGRG-14 after short exposure of mesangial cells to high-glucose conditions

Cultures were maintained in medium containing 4 mM D-glucose (lanes 1, 3, 5, 7, 9 and 11) or 30 mM D-glucose (lanes 2, 4, 6, 8, 10 and 12), for 2 h (lanes 1, 2, 7 and 8), 2 days (lanes 3, 4, 9 and 10) or 1 week (lanes 5, 6, 11 and 12). RNA was extracted and analysed by RT–PCR for a terminal sequence in the 3′ UTR of full-length HGRG-14 transcripts (lanes 1–6; 208 bp product, primers P1 and P2) or for the coding region of HGRG-14 transcripts (lanes 7–12; 400 bp product, primers P3 and P4).

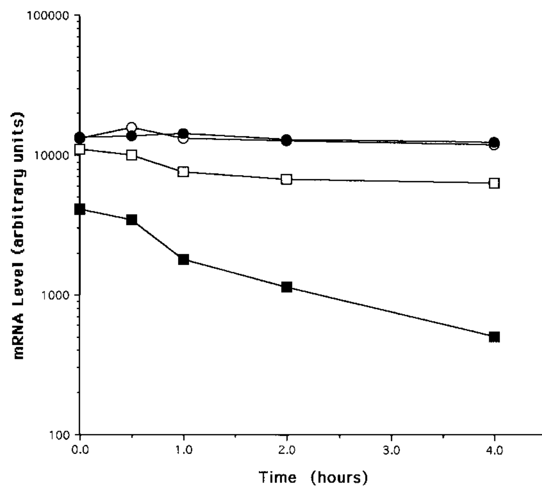

Figure 8 Stability of HGRG-14 and β-actin transcripts in mesangial cells under normoglycaemic and hyperglycaemic culture conditions

Mesangial cells were maintained under normoglycaemic and hyperglycaemic conditions for 21 days. Cells were then incubated with actinomycin D for 4 h. Total RNA was extracted at various times and assayed by slot-blot analysis for both HGRG-14 and β-actin mRNA, as described in the Materials and methods section. Levels of mRNA (pixel intensity, in arbitrary units) were plotted as a semilogarithmic plot against time. The results are means for each time point for two independent experiments ($n = 3$ for each point in each experiment). Individual measurements were within $\pm 7.5\%$ of the mean for each time point. Symbols: □, HGRG-14 in normoglycaemic cells; ■, HGRG-14 in hyperglycaemic cells; ○, β-actin in normoglycaemic cells; ●, β-actin in hyperglycaemic cells.

exclude the presence of five AUUUA motifs in the 3′ UTR. The same regulatory mechanism has been described for the cAMP response element modulator gene (*CREM*) [17]. The level of translated CREM activator isoform is increased in germ cells in response to follicle-stimulating hormone. This is the result of an alternative use of polyadenylation sites in the CREM transcript that truncates the 3′ UTR and results in the omission of ten AUUUA destabilizer elements from it [18]. Thus mRNA stability is increased and the translation of CREM activator protein is elevated. It is noteworthy that AUUUA motifs generally occur in transcripts coding for proteins with a signalling function such as proto-oncogenes (e.g. c-*fos*), growth factors (e.g. granulocyte macrophage colony-stimulating factor) and interleukins (e.g. interleukin 3) [19]. In each case it is likely that the intracellular levels of such transcripts need to be rapidly up-regulated or down-regulated in response to specific physiological circumstances. It is noteworthy that changes in HGRG-14 mRNA processing occur in mesangial cells within 2 h of their exposure to hyperglycaemic conditions. An endoribonuclease analogous to the bacterial RNA-processing enzyme RNase E has been found and purified from human cells [20]. This enzyme specifically cleaves the AUUUA motif in the 3′ UTR of RNA and mediates rapid mRNA decay. The enzyme was also found to cleave RNA containing more than one AUUUA motif more efficiently than RNA with only one or a mutated AUUUA motif.

The exact molecular mechanism regulating the alternative use of polyadenylation sites in CREM and HGRG-14 transcripts remains to be elucidated. However, experiments in other genetic systems have provided evidence for three different mechanisms that could regulate the production of an alternative 3′ UTR in mRNA species based on the cellular environment. These mechanisms are based on either alternative transcription stop sites [21] or the use of alternative endonucleolytic cleavage adjacent to a poly(A) site [22–26]. Yet another mechanism could be by alternative exon splicing [23]. This is unlikely to be the mechanism for *HGRG-14* because we could not identify the consensus sequence for the vertebrate 3′ splice site associated with alternative 3′ splice site selection [27]. Recognition of a poly(A) site by the cleavage/poly(A) complex involves a network of multiple interactions between a number of sequence elements and different components of the 3′-processing apparatus as well as those between the components of the complex themselves [28]. At least five different factors are required to make the processing complex: the cleavage polyadenylation specificity factor (CPSF), the cleavage stimulatory factor (CstF), two cleavage factors (CF I and CF II) and poly(A) polymerase (PAP) [29–31]. Modulating the abundance of any of these factors might regulate the polyadenylation efficiency.

The expression of *HGRG-14* varies between different tissues, being most abundant in brain and adult retina and less abundant in foetal retina, lung, liver, spleen, heart and muscle [14]. The size of mRNA identified in these tissues was approx. 2.1 kb. The molecular mass of the HGRG-14 polypeptide is predicted to be 13 kDa. However, in Western blot analyses, HGRG-14p-specific antibodies cross reacted with a native protein in mesangial cell lysate of approx. 26 kDa. This might be due to either protein dimerization or post-translational modification. HGRG-14 has a putative myristylation site at glycine-28 but we have no evidence that it is myristylated; in any case the addition of a myristate chain would not account for an additional 13 kDa. Similarly, HGRG-14 has an N-linked glycosylation motif close to the C-terminus. Whether it is actually glycosylated is unknown. However, the observation that recombinant HGRG-14 is a substrate for CKII kinase suggests that the native protein is intracellular, in which case N-glycosylation is unlikely. Thus dimerization is the most likely explanation for the observed 26 kDa band.

Although HGRG-14 has putative phosphorylation sites for both PKC and protein kinase CKII, the recombinant protein was a substrate only for the latter. CKII kinase is expressed in all eukaryotic cells and phosphorylates numerous cytoplasmic and nuclear proteins. Many have regulatory roles in signal trans-

duction or cell cycling that are modified by phosphorylation [32]. However, the phosphorylation of HGRG-14 *in vivo* has yet to be demonstrated. The role of *HGRG-14* is currently unknown. However, it has 65% identity at the nucleotide level with the murine *tctex-1* gene [14], one of four genes of the mouse t-complex, which is concerned with male germ-cell differentiation [29]. There is no human equivalent of the t-complex but the similarity of *HGRG-14* with *tctex-1*, the rapid turnover of HGRG-14 mRNA in hyperglycaemic mesangial cells, its mode of regulation, and the phosphorylation of the recombinant protein by CKII kinase, all point to a regulatory role for *HGRG-14*. Thus decreased HGRG-14 polypeptide expression in hyperglycaemic mesangial cells may have significant effects on cell function and be a contributory factor in the mechanism leading to diabetic nephropathy *in vivo*. Whether or not this proves to be so, we believe our results are a significant advance in understanding the mechanisms by which hyperglycaemia modulates cell function. This is the first paper showing that high-glucose conditions confer instability on a specific mRNA and it underlines the importance of RNA processing and its regulation as a factor affecting gene expression in hyperglycaemic cells.

This work was supported by project grants from The British Diabetic Association and the Medical Research Council.

REFERENCES

1 Mauer, S. M., Steffes, M. W., Ellis, E. N., Sutherland, D. E. R., Brown, D. M. and Goetz, F. C. (1984) J. Clin. Invest. **74**, 1143–1155
2 The Diabetic Control and Complications Trial Research Group (1993) New Engl. J. Med. **329**, 977–986
3 Abdel Wahab, N., Harper, K. and Mason, R. M. (1996) Biochem. J. **316**, 985–992
4 Abdel Wahab, N. and Mason, R. M. (1996) Biochem. J. **320**, 777–783
5 Liang, P. and Pardee, A. B. (1992) Science **257**, 967–971
6 Liang, P., Averboukh, L and Pardee, A. B. (1993) Nucleic Acids Res. **21**, 2369–3275
7 Bauer, D., Muller, H., Reich, J., Riedel, H., Ahrenkiel, V., Warthoe, P. and Strauss, M. (1993) Nucleic Acids Res. **21**, 4272–4280
8 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) J. Mol. Biol. **215**, 403–410
9 Recline, I. L., Florence, B., Denaro, M. and Alexander, M. (1988) J. Biol. Chem. **263**, 15335–15341
10 Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6133–6137
11 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
12 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
13 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14 Roux, A. F., Rommens, J., McDowell, C., Anson-Cartwright, L., Bell, S., Schappert, K., Fishman, G. A. and Musarella, M. (1994) Hum. Mol. Genet. **3**, 257–263
15 Shaw, G. and Kamen, R. (1986) Cell **46**, 659–667
16 Edwalds-Gilbert, G., Veraldi, K. L. and Milcarek, C. (1987) Nucleic Acids Res. **25**, 2547–2561
17 Lee, J. S., Lalli, E., Masquilier, D., Schlotter, F., Molina, C. A., Foulkes, N. S. and Sassone-Corsi, P. (1995) in Inducible Gene Expression, 2nd edn. (Baeuerle, P. A., ed.), pp. 9–12, Birkhauser, Boston
18 Foulkes, N. S., Schlotter, F., Pevet, P. and Sassone-Corsi, P. (1993) Nature (London) **362**, 264–267
19 Chen, C.-Y. and Shyu, A.-B. (1995) Trends Biochem. Sci. **20**, 465–470
20 Wennborg, A., Sohlberg, B., Angerer, D., Klein, G. and Von Gabain, A. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7322–7326
21 Mather, E. L., Nelson, K. J., Haimovich, J. and Perry, R. P. (1984) Cell **36**, 329–338
22 Amara, S. G., Evans, R. M. and Rosenfeld, M. G. (1984) Mol. Cell Biol. **4**, 2151–2160
23 Wang, Y. C. and Rubenstein, P. A. (1992) J. Biol. Chem. **267**, 2728–2736
24 Peattie, D. A., Hsiao, K., Benasutti, M. and Lippke, J. A. (1994) Gene **150**, 251–257
25 Batista, F. D., Efremov, D. G., Tkach, T. and Burrone, O. R. (1995) Nucleic Acids Res. **23**, 4805–4811
26 Edwards-Gilbert, G. and Milcarek, C. (1995) Nucleic Acids Symp. Ser. **33**, 229–233
27 Green, M. R. (1991) Annu. Rev. Cell Biol. **7**, 559–599
28 Keller, W. (1995) in Pre-RNA Processing (Lamond, A. I., ed.), pp. 113–134, Springer-Verlag/R. G. Landes Company, Heidelberg, Germany/Austin, Texas, U.S.A.
29 Wahle, E. and Keller, W. (1996) Trends Biochem. Sci. **21**, 247–250
30 Keller, W. (1995) Cell **81**, 829–832
31 Manley, J. L. (1995) Curr. Opin. Genet. Dev. **5**, 222–228
32 Allende, J. E. and Allende, C. E. (1995) FASEB J. **9**, 313–323

Received 14 July 1998/19 August 1998; accepted 28 September 1998