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Isolation of Diabetes-Associated Kidney Genes Using Differential Display[1]

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Differential Display was used to isolate genes that show transcriptional changes in the kidney during the development of diabetes in the GK rat. Eight candidate diabetes-associated cDNA fragments, CDK1-8, were isolated and characterised. cDNA sequencing and subsequent database analysis revealed that CDK2, 4, 5 and 6 showed no significant sequence similarity to previously reported genes, suggesting that they represent novel genes, whereas CDK 1,3,7 and 8 showed significant similarity with rat lactate dehydrogenase, rat amiloride sensitive sodium channel, EST109013 and mouse ubiquitin-like protein respectively. The differential mRNA expression of CDK1-8 was confirmed using differential screening of slot blots. CDK1, 2, 4 and 8 mRNAs appeared to increase whereas CDK3, 5, 6 and 7 mRNAs decreased in the kidneys of GK rats with increasing hyperglycaemia. The altered renal mRNA expression of these genes in association with increased hyperglycemia in the GK rat suggest that they are candidates for a role in the development of diabetic nephropathy.

Diabetic nephropathy is the major cause of end-stage renal failure in the Western world and affects more than 30% of patients with both insulin dependent and non-insulin-dependent diabetes. We are interested in investigating the molecular changes that occur in the kidney during the onset and progression of diabetes mellitus in order to identify molecular markers for diagnostic and therapeutic use. It is well recognised that both in man and in animal models of diabetes mellitus, the rate of development of organ change is a relatively slow phenomenon but that early changes in gene function may significantly precede morphological changes of end organ damage. A strong correlation between duration of hyperglycaemia and rate of progression of diabetic microvascular disease has been demonstrated (1). We have investigated the molecular changes that occur in the kidney during the onset and early stages of diabetes mellitus using the Goto-Kakizaki (GK) rat, a well characterised genetic model of non-insulin dependent diabetes mellitus (2-5). The GK rat displays glomerular basement membrane thickening as well as proteinuria suggesting it to be a suitable experimental model for studying diabetic nephropathy (6,7).

To identify genes that display transcriptional changes in the kidney during the development of diabetes we have utilised the differential display technique (8,9). This technique is essentially a mRNA fingerprinting technique and involves the reverse transcription of a subset of the mRNA population with an anchored oligo(dT) primer (T_{12}MN where M is degenerate in A,C,G and N in all bases) followed by the polymerase chain reaction using a combination of arbitrary 10mers and T_{12}MN. Parallel comparison, by polyacrylamide gel electrophoresis, of the sets of cDNA fragments generated under different conditions being studied, provides a means of identifying differentially expressed cDNAs. This technique has successfully allowed the isolation and identification of a number of differentially expressed genes in eukaryotic cells of many types and a number of different diseased states (9-15). Moreover, glucose-regulated genes have been isolated from bovine retinal pericytes (10, 15), bovine aortic smooth muscle cells (11) and rat cardiac ventricles (12). To date there are no published reports of attempts to identify renal genes which change in levels of expression as a response to hyperglycaemia *in vitro* or *in vivo.* In this

[1] The DNA sequences described in this paper have been assigned the following accession numbers: CDK1-X89821; CDK2-X89823; CDK3-X89818; CDK4-X89820; CDK5-X89822; CDK6-X89817; CDK7-X89824; CDK8-X89819.

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study we have used the kidneys of the GK rat at progressive stages of diabetes in conjunction with the differential display technique to identify glucose-induced renal genes. Characterisation of such genes will aid in elucidating the underlying molecular mechanisms that are occurring in the diabetic kidney and may ultimately provide markers for diagnostic purposes or therapeutic intervention of diabetic renal complications.

MATERIALS AND METHODS

Rats. Male and female GK rats at defined ages (6,16 and 26 weeks) were obtained from the University Hospital of Wales Biomedical Services Institute. They were housed in a temperature-regulated room with artificial light providing a 12h-light/12h-dark cycle. The rats were fed on a standard pellet diet, supplied *ad libitum*, or prior to blood glucose measurements, starved overnight. Glucose concentrations were measured (model 23AM glucose analyser) in blood samples from the tail vein of the rat. GK rats (6,16 and 26 weeks) displaying a steady increase of 6 (normoglycaemic) to 13 mM (hyperglycaemic) plasma glucose concentrations were used for further studies.

RNA isolation. Total cellular RNA was isolated from GK rat kidneys by acid-guanidinium isothiocyanate-phenol-chloroform extraction (16). At least 3 different GK rat kidneys at each age were used for isolation of total RNA. Removal of chromosomal DNA contamination from total cellular RNA was carried out (17) and the RNA was quantitated by absorbance at 260nm.

Identification of differentially expressed cDNA fragments using differential display. Differential display was performed essentially as described by Liang & Pardee (8). DNA-free RNA (1μg) was reverse transcribed in a 20 μl reaction volume using either T_{12}MA, T_{12}MC or T_{12}MG primers (synthesised by R&D Systems) where M represents a 3 fold degeneracy for A, C and G. Polymerase chain reaction was performed in reaction mixtures (20 μl) containing 1/10th volume of the reverse transcription mixture, 2 μl appropriate anchored oligo-dT primer (25 μM), 1 μL of 10μM random 10mer (OPB-01 to OPB-08, Operon Biotechnology Inc.; GC content 60 - 70%), 2 μl 10$\times$ PCR buffer, 2 μM each of dATP, dCTP, dGTP and dTTP, 0.5 μl [^{35}S]-dATP (>3000μCi/μM), 1.6 μl $MgCl_2$ (25mM), 0.2 μl Taq polymerase (5U/μl) and overlaid with 20 μl mineral oil. PCR was carried out in a Perkin Elmer 480 DNA Thermal Cycler using the following parameters: denaturation at 95 °C for 5 minutes followed by 40 cycles at 94 °C for 30s, 40 °C for 2 min, 72 °C for 30s and then a final extension at 72 °C for 10 min. The PCR products were separated on a 6% polyacrylamide gel, dried down and exposed to blue sensitive X-ray film (GRI Ltd, UK) to allow for identification of putative differentially expressed cDNA fragments. All appropriate controls were employed. To ensure that banding pattern and intensity of bands was reproducible, total RNA from kidneys of different rats at the same age was utilised and duplicate RT-PCR reactions were performed.

Band recovery, cloning, and DNA sequencing. Bands of interest were excised, rehydrated and the DNA was reamplified by PCR using the appropriate primer combination under the PCR conditions stated above except that the dNTP concentration was 20 μM and no radioisotope was included. Reamplified DNA was visualised on a 2% agarose ethidium bromide stained gel. The putative differentially expressed cDNA fragments were cloned using the pGEM-T Vector System (Promega Corporation, UK) and insert size of all subclones was confirmed by restriction enzyme digestion (Apa I and PstI; Promega Corporation, UK) of the recombinant plasmids. The pGEM-T clone inserts were manually sequenced, both strands, using Sequenase version 2.0 kit (Amersham International, UK). Sequence analysis was performed using the University of Wisconsin Genetic Computer Group software package.

Expression analysis. The method of Mou *et al.* (18), uses slot blot screening, to confirm differential expression, was performed with a few modifications. Plasmid DNA from putative differential cDNA fragments cloned in pGEM-T vector (15 μg) was blotted onto nylon membranes (Hybond nfp; Amersham International, UK) using the minifold II slot blot apparatus (Schleicher and Schvell, Inc., Keene). The slot blot membrane was then differentially screened using cDNA probes generated from 0.5 μg poly A^+ RNA from kidneys of 6 week old (GK6) and 26 week old (GK26) GK rats. 80% incorporation of [α-^{32}P]-dCTP for both probes was used to allow representation of less abundant mRNAs. The membranes were hybridised with the GK26 week probe, stripped (50% formamide in 50 mM sodium phosphate buffer (pH 6.5); 65 °C for 1.0 hour) and reprobed with the GK6 week probe. Membranes were hybridised in 6$\times$SSC, 1.25$\times$ denhardts, 0.5%SDS, 300ng denatured sonicated salmon sperm DNA and denatured probe (1.0×10^6 cpm/ml) at 65 °C for 16 hours then washed to a high stringency of 0.1$\times$SSC/0.1%SDS at 65 °C. The washed membranes were exposed to Kodak AR film with intensifying screens at -70 °C for 5 days. Quantification of the intensity of the bands was performed using the UVP system 5000 (Ultra Violet Products Ltd, Science Park, Cambridge, UK). The fold increase or decrease in expression was calculated by the ratio of GK26 to GK6 week measured intensities or GK6 to GK26 band intensities respectively. Measured intensities were corrected using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal standard and pGEM-T vector was used as the negative control since all candidate differentially expressed cDNA fragments were cloned into this vector.

RESULTS AND DISCUSSION

Differential display was carried out using total RNA from kidneys at progressive stages of hyperglycaemia. Comparison of the displays generated using GK kidneys at different stages showed that the majority of the bands were of equal intensity in all samples (data not shown). Using these bands as internal standards we were able to identify putative differentially expressed bands. The bands selected for further study included the following three types: bands present at specific stages of the disease and absent at others, bands which showed increased intensity with increasing glucose concentrations and bands which showed decreased intensity with increasing glucose concentrations. Approximately 5000 bands were observed and 1.5% of the mRNAs appeared to be differentially expressed in the kidneys of the GK rats during disease progression. A total of 70 putative differentially expressed cDNAs were detected and excised (data not shown). The majority of fragments excised showed changes in intensity but were present at all stages. These fragments were designated **c**andidate **d**iabetes-associated **k**idney genes (CDK).

From the 70 CDK fragments, 8 were randomly selected for detailed characterisation (Table 1). For these eight CDK fragments, re-amplification had resulted in the production of a single band of expected size (data not shown). CDK1,2,4,6 and 7 were selected on the basis of increasing intensity with increasing hyperglycaemia whereas CDK3 and 5 displayed a decrease in intensity with increasing hyperglycaemia. CDK8 was selected as a control as it appeared to be equally ex-

TABLE 1

Clones Selected for Detailed Characterization

Clone	Display detected in*	Size of fragment (bp)	Reason for excision†	Change in mRNA expression‡
CDK1	T_{12}MA/OPB-06	298	↑	4.20↑
CDK2	T_{12}MA/OPB-06	311	↑	1.08↑
CDK3	T_{12}MA/OPB-06	252	↓	2.20↓
CDK4	T_{12}MA/OPB-07	253	↑	5.00↑
CDK5	T_{12}MG/OPB-06	315	↓	4.00↓
CDK6	T_{12}MG/OPB-07	263	↑	5.00↓
CDK7	T_{12}MG/OPB-08	172	↑	4.00↓
CDK8	T_{12}MG/OPB-05	190	↔	4.40↑

* Primer combination (anchored oligo/random primer) used for differential display in which the CDK fragment was isolated.

† By differential display, the cDNA fragment showed either an increase (↑) or decrease (↓) in band intensity or remained at equal intensity (↔) with progressive hyperglycaemia.

‡ Increase (↑) or decrease (↓) in mRNA expression as determined using slot blot differential screening (see methods).

pressed at all stages investigated. CDK1 to CDK8 were cloned into pGEM-T vector and characterised further.

The mRNA expression of CDK1-8 in kidneys from 6 week and 26 week GK rats, representing progressive stages of hyperglycaemia, was investigated using a slot blot screening method (18). This method utilises mRNA from 2 stages of disease which is radioactively labelled and used as a probe against slot blots of candidate differentially expressed genes. Differential expression was evaluated using GAPDH as and internal standard. To confirm that GAPDH is not differentially expressed in the diabetic kidney northern blot analysis was performed and it was shown that GAPDH mRNA expression in the 6 and 26 week old kidneys of the GK rat not does not appear to change (Figure 1a). DNA prepared from the pGEM-T vector with no insert was used as a negative control and showed no hybridisation to either probes (Figure 1b). Positive signals for CDK1-8 and GAPDH were detected and confirmed their renal mRNA expression. After correction for GAPDH measured intensity, the results showed that CDK1, 2, 4 and 8 mRNA expression increase and CDK3, 5, 6 and 7 mRNA expression decrease with progressive hyperglycaemia (Table 1). The pattern of differential expression for CDK1-5 was the same as that for the original detection by differential display. However CDK6 and CDK7 display the opposite pattern of expression when compared to the original reason for their detection by differential display. Nevertheless CDK6 and 7 still appear to be transcriptionally modulated. Interestingly CDK8 which showed no alteration in expression with progressive hyperglycaemia by differential display does appear to be slightly upregulated (Table 1).

CDK1-8 were subjected to DNA sequencing and the deduced CDK insert sequences ranging from 173bp to 315bp are shown in Figure 2. Due to the nature of the differential display technique, these inserts should contain the random primer at the 5′ end and the anchored primer at the 3′ end and thereby should represent the 3′ regions of mRNAs expressed in the kidneys of GK rats. All the clones except CDK1 contained the anchored primer at the 3′ end and the random primer at the 5′ end. In the case of CDK4 the random primer contained 6 of the 10 bases, suggesting mismatches during PCR amplification. The presence of the 2 primers in the expected positions suggested that these clones represent the 3′ end of genes expressed in the kidneys of the GK rat. CDK1 contained the random primer at the 5′ end but not the expected anchored primer at the 3′ end of the clone. However, subsequent analysis revealed that CDK1 also represented the 3′ untranslated region of a specific gene (see below). CDK2, 3, 4, 5, 7 and 8 contain putative polyadenylation signals which are located at positions 10 to 20 bases upstream from the poly A^+ tail (Figure 2) further supporting the view that these cDNA fragments represent the 3′ untranslated end of the corresponding mRNAs.

In order to determine whether the CDKs represent previously reported DNA sequences, searches of GenEMBL database (Blast) were carried out for CDK1-8. CDK2, 4, 5 and 6 displayed no significant homologies

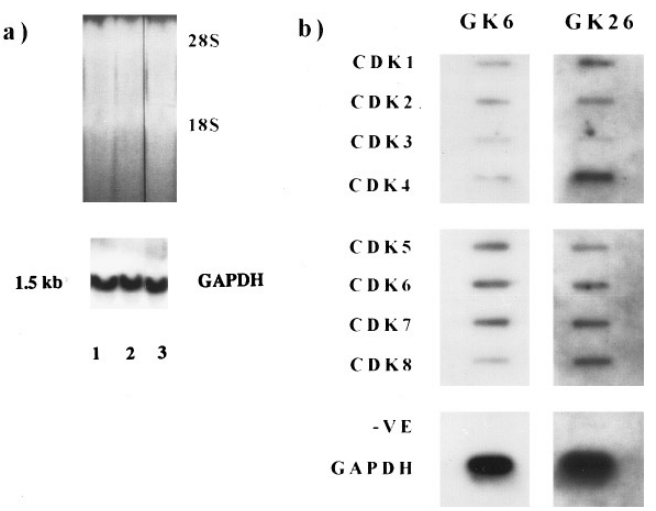

FIG. 1. (a) mRNA expression of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in kidneys of 6,16 and 26 week old GK rats. 20 μg of total RNA from GK 6 (lane 1), 16 (lane 2) and 26 (lane 3) week old kidneys were northern blotted and probed with GAPDH. The top panel represents the ethidium bromide stained agarose/formaldehyde gel containing the total RNA and the bottom panel represents the GAPDH mRNA present in the kidneys of the diabetic rats. **(b)** Slot blot screening of CDK clones. Plasmid DNA containing CDK1-8 cDNA fragments and GAPDH and pGEM-T vector were blotted onto nylon membrane and differentially screened using probes prepared from poly A^+ RNA isolated from GK rat kidneys at 6 weeks and 26 weeks which represented progressive stages of hyperglycaemia. GAPDH represents a positive control and pGEM-T vector represents a negative control for differential screening.

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CDK1
TGCTCTGCCC TGCTAAGTGG TACTTGTGTA GTGGTAACCT GGTTAGTGTG ACGATCCCAC
TGTCTCCAAG ACACACTGCC AACTGCATGC AGAGACTTTG ATTACCCTGT GAGCCTGACT
GCATTGACTG TGCTACGCAC CCTCACCAAA CATGCCTAGG CCATGAGTTC CCAGTTAGTT
ATAAGCTGGC TCCAGTGTGT AAGTCCATCG TGCATATCTT GTGCATAAAT GTTCTACAGG
ATATTTTCTG TATTATATGT GTCTGTAGTG TACATTGCAA TATTATGTGA AATGTAAG
CDK2
TGCTCTGCCC AACTCTATGA ACTCTATGAA CTCCAAAGCT TTACAAGACA ACAGGAAAAA
AGCAAATGAT CAAGAAATTG CCAGAAAAAA GTGATCTAAC AAAAAGAGTC TGCCCTATGC
CTACTCAGAG TTTGAAGATC AACTTCCAAT TGACAAGGTT CTAGAGTTTG GATCATCTGG
GGGAAACAAA ACAAAAGCTG GTCTGCTGAA AGCCCAGCTC CCACTGGAGC CCTGTGTCCC
AGTGCTGTTT CCTTGAGCTC TTCCAACTTG AAGAAATAAA GCCGGCTCTG TAGCCCCTCA
AAAAAAAAAA A
CDK3
TGCTCTGCCC TGACTCAGCG TCTTGCTCTG CCCTCACCCA CGCCAGTCCT CCCTTGCTCT
GCCCTCACCC CCACAGTGTA CTGTATTATA TGTTCACATT CTTTTGTGTC TGCCTCCCTG
GGTAAACTGA GCTCCTTGTG TGGTCAGGGA TGAGATTTGC TCTGTTTTGT ATCCTTCCGG
TCTAGCCCAG TCTCCCACTT GGACGGGTAG GCAGGTACTC AATAAAGGCC TTGTTCCATC
AAAAAAAAAA AA
CDK4
GGcGACGaga ACAGATCAAT ACCGATCCGT CCAAAATGGA GGATTCTGTC TCCGCTCCAG
CTGCCCATCT CATACCAAAC TACAAGGAAC ATGTAAACCA GATAAGCCCA ACTGTTGCAG
GAGTTGACAG TGGTTTGAAG AATGGACATA AAGGACAAGC AAGGGATTGT AAAATTAGTG
TTTTAATAAA GTAAATGTTT TTGAGTTTAT TTACATCATC TCAAATAAA TTTTATGCCT
CAAAAAAAAA AAA
CDK5
TGCTCTGCCC AACTCTATGA ACTCCAAAGC TTTACAAGAC AACAGGAAAA AAGCAAATGA
TCAAGAAATT GCCAGAAAAA AGTGATCTAA CAAAAAGAGT CTGCCCTATG CCTACTCAGA
GTTTGAAGAA TCAACTTCCA TTGACAAGGT TCTGGAACCT TCTAGCGTTT GGAATCATCT
GGGGGAAAAC AAAACAAAGC TGGTCTGCTG AAAGCCCAGC TCCCACTGGA AGCCCTGTGT
CCCAGTGCTG TTTCCTTGAG CTCTTCCAAC TTGAAGAAAT AAAGCCGGCT CTGTAGCCCC
CCAAAAAAAA AAAAA
CDK6
GGTGACGCAG GGTCTACTTG AAGCTGCTGT CCTGGCCCAT CTGGACGTCT TGCATCAGCC
CACTCAGATA CTCAGGATGC TGACTTGCTG AGACCTTGAA TGGCCTTCCC TCTGTTGTCT
GCCTCTGTCC TCCCCACCTG GGACATATAA ATAGATTCTA GCTGTACTTA ATGGCCTTCC
CTCTGTTGTC TGCCTCTGTC CTCCCCACCT GGACATATAA ATAGATTCTA GCTGTACTTA
TTTTTCCTCC CAAAAAAAAA AAA
CDK7
CAGGCGTGCT TAATTCCTGG GAGTGGTCTC TGTTCTTCCT CTGGGACGTG CACAGCCCCG
AGTTCATTCA AGAAAGCTAC CAGAGGTGGT TTGGGAATGT GACGGTGTAT AAATTTTAGA
TAATGAGGTT TTAAAAAATT AAACGGAATT GTTACTCCCA AAAAAAAAAA AA
CDK8
TGCGCCCTTC CTTGGATGCC CAATCTTGTG TGTCTACTGG AGGGAGAATG TGAGGACCCC
AGGATGCAGT GTTCCTGCCC AAATGCCCTT GCTGGCCATT GGGTTTTAGT TTGCAGTCCT
GTGTGCTTCC CTCTCTTATG GCTATATCCT TGGTTGTCAA TAAAATATTT CCTGGCCAAA
AAAAAAAAAA
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FIG. 2. Nucleotide sequences of CDK1-8. Flanking primer sequences are underlined and putative polyadenylation signals are in bold. Mismatches in 5′ 10 mer of CDK4 are shown in lower case letters.

to previously reported sequences, suggesting that they may represent novel diabetes-associated renal genes. However, since the 3′ untranslated regions of genes usually display little conservation between species it is possible that these genes have been cloned in other species. CDK7 displayed 93% identity to an EST fragment (data not shown) isolated from rat PC-12 cells stimulated with nerve growth factor (19). EST109013 shows no significant homologies to previously reported sequences suggesting that CDK7 may represent a novel diabetes-associated renal gene. CDK1, CDK3 and CDK8 displayed significant homology to rat lactate dehydrogenase [LDH; 98% identity] (20), alpha subunit of rat amiloride sensitive sodium channel [αENaCh; 89% identity] (21) and mouse ubiquitin-like protein [UbLP; 78% identity] (22) respectively (Figure 3). The strong identity indicates that we have isolated the 3′ untranslated region of the LDH, αENaCh and UbLP genes from the kidneys of GK rats. Differences between CDK3 and αENaCh isolated from rat colon (21) and CDK8 and UbLP detected in mouse brain (22) were observed at the 5′ end of the two sequences. The mismatches could be the result of tissue specific splicing, errors which arise during the amplification procedure in differential display or species differences between rat and mouse as in the case of CDK8.

Although CDK1-8 show changes in levels of renal mRNA expression during progression of diabetes (Figure 1), this does not necessarily mean that the corresponding protein changes. Therefore CDK1-8 need to be treated as potential candidate diabetes-associated renal genes and further detailed studies are required to implicate these genes with a role in diabetes. However, recent reports concerning LDH, αENaCh and UbLP leads to exciting possibilities of their import in diabetes and diabetic renal injury. Firstly, it has been known for a long time that the LDH isoform pattern changes in diabetic patients with vascular complications (23) and recently a major disturbance in intracellular lactate/pyruvate metabolism in NIDDM has been estab-

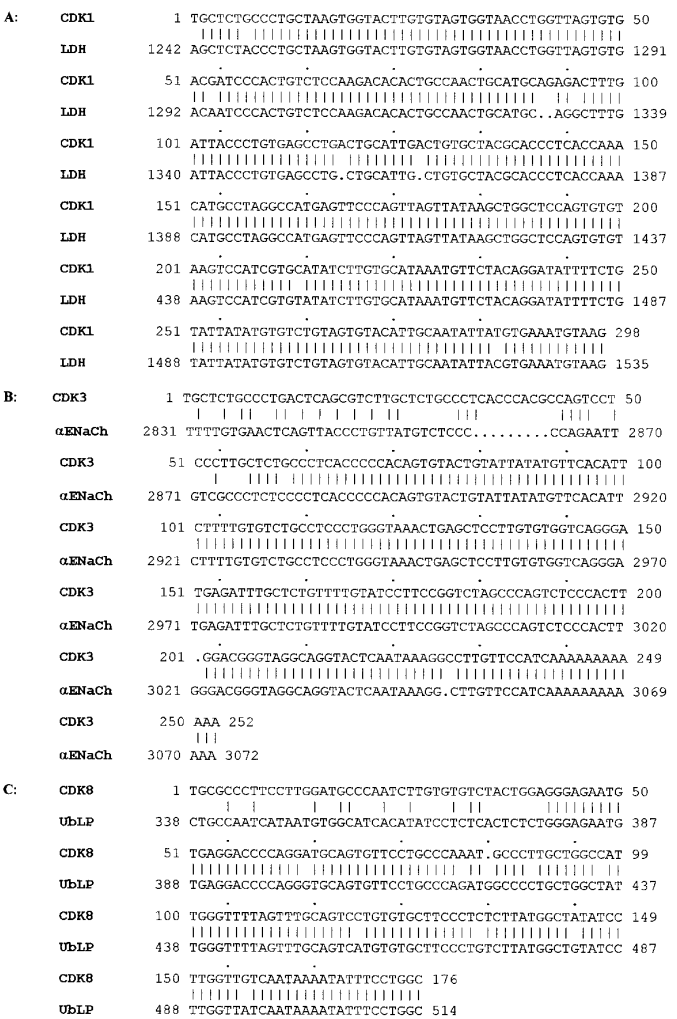

FIG. 3. Nucleotide sequence homology of (A) CDK1 and rat lactate dehydrogenase (LDH; Accession No. X01964) (B) CDK3 and alpha subunit of rat amiloride sensitive epithelial sodium channel (αENaCh; Accession No. X70521) and (C) CDK8 and mouse ubiquitin like protein (UbLP; Accession No. D10918). Identical residues are shown as | and gaps inserted are represented by dots.

lished (24). These factors plus our detection of altered LDH mRNA expression in the diabetic kidney pinpoints this enzyme for further investigation. Secondly, ENaCh, is involved in mediating Na^+ reabsorption in the epithelial cell (25) and has been implicated to have a pathophysiological role in diseases where dysfunction of Na^+ reabsorption such as hypertension occurs (26,27). We have detected changes in mRNA expression of the alpha subunit of ENaChl. Patients with diabetes mellitus, especially those with renal complications, display abnormalities in Na^+ balance, blood volume and blood pressure (28), metabolic activities regulated by ENaCh, which infers possible dysfunction of this channel in the diabetic kidney. Thirdly, several UbLP have been identified recently (22,29). Their functional significance is not known but it is thought that they may be involved in specialised ubiquitin related functions such as protein-protein interactions (29). Our detection of a UbLP in the kidney warrants more detailed studies of this protein into its metabolic function and potential role it has in diabetes. Interestingly, a recent report concerning the identification of Nedd4 (30) a possible regulator of ENaCh shows that this protein contains a ubiquitin ligase domain. It is suggested that Nedd4 binds to ENaCh, leading to ubiquination of the channel and subsequent degradation. This connection between ENaCh and Ubiquitin mediated functions emphasises the need for detailed studies on these metabolic systems in the diabetic kidney.

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