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Characterization of the Rabbit *HKα2* Gene Promoter

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Abstract

The *HKα2* gene directs synthesis of the *HKα2* subunit of the H^+ , K^+ -ATPase. In the kidney and colon, the gene is highly expressed and is thought to play a role in potassium (K^+) conservation. The rabbit has been an important experimental system for physiological studies of ion transport in the kidney, so the rabbit *HKα2* gene has been cloned and characterized. The genomic clones and the previously reported *HKα2a* and *HKα2c* subunit cDNAs provided a means to address several issues regarding the structure and expression of *HKα2* gene. First, the genomic organization established that the rabbit *HKα2* gene was unambiguously homologous to the mouse *HKα2* gene and the human *ATP1A1* gene. Second, the mapping of the transcription start site for the alternate transcript, *HKα2c*, confirmed that it was an authentic rabbit transcript. Finally, isolation of DNA from the 5' end of the *HKα2* gene enabled us to initiate studies on its regulation in the rabbit cortical collecting duct. The promoter and two putative negative regulatory regions were identified and the effect of cell confluency on gene expression was studied.

Keywords

HKα2; H^+ ; K^+ -ATPase; kidney; collecting duct; potassium; confluency

1. Introduction

Despite daily fluctuations in dietary K^+ intake, mammals maintain blood K^+ levels in the range of 3.5–5.5 mEq/L. H^+ , K^+ -ATPases are among the pumps expressed along the collecting duct of the kidney that play a role in K^+ conservation in hypokalemic individuals [1]. Indeed, a H^+ , K^+ -ATPase-deficient patient presented with severe hypokalemia [2]. H^+ , K^+ -ATPases have either a gastric (*HKα1*) subunit or a colonic (*HKα2*) subunit. Both types of α subunits are expressed in the collecting duct. Evidence from several sources suggested that *HKα2*, and not *HKα1*, was upregulated in the kidney when blood K^+ concentration decreases [3–6]. Furthermore, in wild type mice fed a K^+ deficient diet, *HKα2* mRNA was upregulated in the collecting duct [7]. When *HKα2* knockout mice were fed a K^+ deficient diet, the mice developed severe hypokalemia with much of the K^+ loss occurring in the colon. The *HKα2* H^+ , K^+ -ATPase has also been linked to bicarbonate absorption [8], ammonium secretion [9] and chronic

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adaptation to changes in sodium [10] and aldosterone [11]. In aggregate, the evidence favors an important role for the HK α 2 containing pump in K⁺ conservation.

Genomic clones for the human (*ATP1A1*) [12] and mouse [13] *HK α 2* genes, and the cDNAs encoding the human [14], rat [15], mouse [16], guinea pig [17], and rabbit [18,19] HK α 2 subunits have been previously reported. Analysis of the cDNA sequences left some doubt about whether these proteins should be considered homologous, and indeed, what subunits were expressed in tissues of various mammalian species. Although the deduced amino acid identity of the HK α 2 subunits was lower (87%) than the identity of the HK α 1 proteins (97%), distance analysis of the HK α 1, HK α 2, and NaK α subunits from several species showed that the HK α 2 proteins were more closely related to each other than to HK α 1, or any of the NaK α subunits [20]. The cDNAs cloned from rabbit and rat suggested that the *HK α 2* gene produced alternative transcripts that differed from the widely accepted HK α 2a sequence only at the 5' end. These mRNAs therefore encoded alternative HK α 2 subunit proteins with distinct amino termini (HK α 2b and HK α 2c) [13,18]. Alternative transcripts of this sort have not been reported in other mammals. A controversy over the rabbit alternative transcript (HK α 2c) arose when two laboratories cloned the HK α 2 cDNAs with differing results. Both laboratories used 5' rapid amplification of cDNA ends (5' RACE) to clone the 5' end of the HK α 2 transcript. Fejes-Toth et al. [19] obtained only a cDNA for the known colonic HK α 2a cDNA, but Campbell et al. [18] identified two cDNAs with distinct 5' ends yielding the HK α 2a and HK α 2c subunits. The latter appeared to be a product of differential splicing near the 5' end of the *HK α 2* gene transcript. The HK α 2c protein was therefore identical to the HK α 2a protein in all but the extreme amino terminus. The HK α 2c translation begins at an AUG codon encoded within intron 1 of the HK α 2a sequence producing a protein that lacks the first 2 amino acids of HK α 2a, but contains an additional 63 unique amino acids (Figure 1C). Inspection of intron 1 of the human genomic sequence did not reveal an HK α 2c-like open reading frame. In view of the absence of the alternative HK α 2c subunit mRNA in other mammalian species and one group identifying only the HK α 2a transcript in the rabbit, it remained controversial as to whether HK α 2c was in fact an authentic rabbit transcript.

Although there was abundant in vivo data suggesting a role for the HK α 2 form of the H⁺,K⁺-ATPase in K⁺ conservation, little is known about the molecular mechanisms involved in regulating transcription from the *HK α 2* gene. Zhang et al. [16] cloned a 7.2Kbp fragment of DNA upstream of the mouse *HK α 2* transcription start site and performed a promoter deletion analysis. In two mouse medullary collecting duct cells lines (mIMCD3 and mOMCD3) high levels of reporter gene expression were observed. The activity was not significantly altered after deleting all but 177 bp upstream of the transcription start site, and no regulatory elements were identified outside of this minimal core promoter. Recently, these investigators identified a functional cyclic AMP response element (CRE) within the 177 bp promoter.

In this report, we address several lingering issues with respect to the structure and expression of the rabbit *HK α 2* gene. Rabbit *HK α 2* genomic clones have been obtained to show that the gene structure is comparable to the human and mouse genes that apparently produce only single transcripts. The transcription start sites for the *HK α 2* gene were mapped at single base resolution to demonstrate that it does indeed produce two transcripts encoding the HK α 2a and HK α 2c subunits. Additionally, studies on the regulation of the *HK α 2* gene were initiated. A series of promoter deletion and mutation experiments were performed to consider regulation of the gene beyond the minimal promoter. RT-PCR and QPCR experiments tested the effect of tissue culture cell confluency on *HK α 2* gene expression.

2. Experimental procedures

2.1 Cloning the rabbit HK α 2 gene

A rabbit genomic bacteriophage λ library (Clontech) was plated on *Escherichia coli* and plaques were lifted onto hybridization membranes. Three HK α 2a (accession# AF023128) cDNA probes (bp 16–93, 1264–1569, and 3265–4073) were radio-labeled by random priming and used to screen the membranes. Positive plaques were purified and λ DNA was isolated using the Qiagen large-scale λ DNA isolation kit. Sections of the gene not represented in the λ clones were obtained by PCR using rabbit genomic DNA as template. Nucleotide sequences were determined in the core facility at the University of Florida.

2.2 RNase protection assay

Transcription start sites for HK α 2a and HK α 2c were mapped by RNase protection using the RPAIII kit from Ambion. The probes for HK α 2a and HK α 2c mRNAs consisted of a 726bp EcoRI/SacII fragment and a 121bp XmnI/ApaI fragment from pDZ10, respectively. The RNase protection assay was carried out using total RNA from the rabbit colon. The protected fragments were run on a 6% polyacrylamide gel along side a reference DNA (Bacteriophage M13). The gel was dried for two hours and exposed to autoradiograph film at -80°C overnight.

2.3 Promoter deletion and mutation constructs

A 5.5 Kbp XhoI/SacII fragment of λ HK α 2.1 DNA was cloned into the pGL3-basic vector (Promega) to make the longest reporter gene construct extending to position -5394 upstream of the HK α 2a transcription start site. Existing restriction sites and *Mlu*I sites made by site directed mutagenesis (Quikchange kit, Stratagene) were used to create deletions of pDZ10. Quikchange mutagenesis was also used to create mutations at the TATA-like element upstream of the HK α 2a transcription start site. Primer DZ86 (5'GCGGGGCGCGCAGCGATCGAGGCGGACACCA CC) and its complement, DZ87, were used to destroy the element. Primer DZ57 (5'GCGG GCGCGCATATAAAAGGCGGACACCAC3') and its complement, DZ58, were used to create a consensus TATA box.

2.3 Transfection and luciferase assays

RCCT28A cells were grown in 24 well tissue culture dishes (Corning) in DMEM-F12 plus 10% FBS to 70% confluency. 250pMol of the promoter construct was mixed with non-specific DNA (to 1 μg) and with 0.2 μg of pRL control plasmid DNA. Transfections were carried out using 40 μl of the Superfect reagent (Qiagen). Cell lysis and the reporter gene assays were conducted according to the Dual Luciferase Reporter Gene Protocol (Promega). Luciferase data was normalized to the Renilla transfection control and the relative light units for the promoter construct with the highest reporter gene activity were set to one.

2.4 RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from 60mm dishes containing RCCT28A cells using the Trizol method (Invitrogen). Reverse transcriptase PCR (RT-PCR) was carried out with PCR Mastermix (Qiagen). The reactions were primed with oligonucleotides BC230 (5'CCGACACGAGTGA AGACAAT3) and BC231 (5'GCTTGTCATTGGGATCTTCC). This primer set amplified a 305 base pair band from the common region of the HK α 2 mRNAs (HK α 2a 1264–1569). The PCR products were analyzed on a 1% agarose gel with ethidium bromide staining.

2.5 RNA isolation and Quantitative Real-time Polymerase Chain Reaction (QPCR)

RNA was isolated from 30mm dishes that were either pre- or 30 days post-confluent using the RNAqueous RNA isolation protocol (Ambion, Inc.). Total RNA (1 μg) was converted to cDNA

using cDNA archive kit (Applied Biosystems). QPCR was performed using *Taq*-man universal PCR master mix (Applied Biosystems). The reactions were carried out in an ABI 7900HT sequence detection system with cycle parameters as follows: 50°C for 2min, 95°C for 10min, 45 cycles of 95°C for 15sec and 60°C for 1min. The Assay by Design reagents (Applied Biosystems) were created to amplify a portion of the common region of the HK α 2a and HK α 2c transcripts. The forward primer was CCCCTGGAAACAAAGAACATCACTT, the reverse primer was CCGTCACCCGTGTTGATGA, and the probe was FAM TTGCCGTGCCTTCCAG. Assay on Demand reagents for 18S were used as an internal standard. Samples were scored as either positive if a Ct value was obtained within 45 cycles or negative if the sample remained undetermined after 45 cycles.

3. Results

3.1 Rabbit HK α 2 gene structure

In order to obtain λ clones that contained the *HK α 2* gene, a rabbit genomic library was screened using probes corresponding to the 5' end, the middle, and the 3' end of the HK α 2a cDNA. Four λ clones hybridized to the 5' probe, three were detected with the mid-probe and two were identified using the 3' probe. Southern analyses were performed using all nine clones and all three probes. Three clones that spanned most of *HK α 2* gene were identified (Figure 1). Clone λ HK α 2.1 contained a DNA fragment that hybridized only to the 5' probe and extended at least 12 Kbp in the 5' direction. Clone λ HK α 2.5 was identified with the mid-probe, but also hybridized to the 5' probe indicating an overlap with λ HK α 2.1. None of the clones found using the 3' probe shared sequence with λ HK α 2.5. The downstream clone selected for sequence analysis was λ HK α 2.8. Together λ HK α 2.1, λ HK α 2.5 and λ HK α 2.8 contained genomic DNA covering >90% of the rabbit *HK α 2* gene. Genomic PCR was used to amplify fragments containing intron-exon boundaries within the gap between λ HK α 2.5 and λ HK α 2.8 (Figure 1). The sequence of the *HK α 2* gene was submitted to GenBank under the following accession numbers: AY552537 (exons 1–11), AY552538 (exon 12), AY552539 (exons 13 and 14), AY552540 (exons 15–17), AY552541 (exons 18–21), and AY552542 (exons 22 and 23).

Nucleotide sequence data from the three λ clones and the four PCR products were used to determine the genomic organization for the rabbit *HK α 2* gene (Table 1). The rabbit *HK α 2* gene contained 23 exons organized much like the human *ATP1A1* [12], the mouse *HK α 2* [16] and the rat *HK α 2* genes (NCBI database). The exon sizes were identical in all except for three 5' exons (1, 2 and 14) and the last exon (23). This strongly supported the conclusion that the genes were homologous, and focused our attention on the differences at the extreme 5' end of the gene.

3.2 Transcription start sites for HK α 2a and HK α 2c

Our report of the HK α 2c cDNA and the subunit [18] had been attributed to an artifact of an in vitro system [19]. In order to determine if the HK α 2c transcript existed in the animal, the HK α 2a and HK α 2c mRNAs were mapped at single-base resolution using the RNase protection assay. HK α 2a and HK α 2c specific probes were designed and annealed to rabbit colon total RNA. Colon RNA was chosen because it was the most abundant in vivo source of HK α 2 mRNAs [18]. The experiments yielded protected fragments for both HK α 2a and HK α 2c (Figure 2). The protected fragments for HK α 2a were 94 and 95bp (Figure 2A). This placed the transcription start site for HK α 2a mRNA 10–11bp upstream of the cDNA end reported by Fejes-Toth et al. [19]. More importantly, the HK α 2c protected fragments were 117 and 118bp (Figure 2B), corresponding to 6–7 bp upstream of the cDNA end obtained by Campbell et al. [18]. The result represented an independent approach to showing the existence of the HK α 2c subunit mRNA in vivo.

3.3 HK α 2 gene promoter

Sequence 1500bp upstream and 900bp downstream of the HK α 2a transcription start site was analyzed using CPGplot (www.ebi.ac.uk/cpg/) and TFSearch [21]. A CpG island characteristic of eukaryotic promoters extended from -80 to +483 and covered the transcription start sites for both HK α 2a and HK α 2c. The TFSearch program identified many putative transcription factor binding sites, and these were sorted for transcription factors known to be expressed in kidney tissue (Figure 3). An element with weak homology to a TATA box was located just upstream of the HK α 2a transcription start site and an apparent CAAT box was located upstream of the HK α 2c transcription start site.

In order to identify elements important for expression and regulation of the *HK α 2* gene, a luciferase reporter gene strategy was adopted. A 5.5 Kbp genomic fragment containing 5400bp of sequence upstream of the HK α 2a transcription start site and 93bp downstream of the transcription start site was cloned from λ HK α 2.1 into pGL3-basic to direct expression of luciferase. This fragment did not contain the transcription start site for HK α 2c or the translation start sites for HK α 2a and HK α 2c. A series of deletion constructs were made by progressively removing segments of DNA from the 5' end of the fragment (Figure 4). *HK α 2* deletion constructs were transiently transfected into rabbit cortical collecting tubule RCCT28A cells along with a *Renilla* luciferase transfection control DNA. Surprisingly, activity increased as the *HK α 2* DNA was reduced to position -345. Control experiments involving replacement of the deleted DNA with bacterial DNA demonstrated that this was not a product of the size of the construct (Figure 4, dotted line). Two statistically significant increases in reporter gene activity were observed. These increases correspond to deletions of DNA between positions -2471 to -1919, and -881 to -639. A transcription factor database search using the deleted sequences revealed several possible binding sites for potential transcriptional repressors known to be expressed in kidney. Between positions -2471 and -1919 there were four potential GATA-1 binding sites (-2385, -2111, -2052, -1980), one NF κ B binding site (-2471), one AP-1 binding site (-2428) and two C/EBP binding sites (-2031, -1983). Similarly, between positions -881 and -639, there were two GATA-1 binding sites, one CREB binding site and one AP-1 binding site (Figure 3). The functionality of these binding sites was not tested. Additionally, there were short sequences conserved between the two regions, mutation of those bases did not result in an increased luciferase activity (data not shown).

The two shortest deletion constructs -345 and -26 had a dramatic decreases in luciferase activity (Figure 4). A transcription factor database search of the region between -639 and -345 revealed potential binding sites for NF κ B, C/EBP and GATA-1 (Figure 3). Furthermore, an alignment of the DNA sequence from -345 to -26 with the same region of the human *ATP1A1*, the mouse *HK α 2*, and the rat *HK α 2* genes revealed a great deal of conservation that included a completely conserved CATTTAA element located at the appropriate distance from the transcription start site to serve as a TATA box (-31). In order to test the functionality of the element, two mutations were made to the -639 reporter gene construct (Figure 5). The first mutation converted the CATTTAA to a randomized sequence (CGATCGA) and the second mutation converted it to a consensus TATA box sequence (TATAAAA). Approximately half of the promoter activity was lost upon mutation of the CATTTAA element. Other putative core promoter elements found in the *HK α 2* promoter likely contribute to the remaining reporter gene activity. These include SP1 binding sites, an initiator sequence and a downstream promoter element. Promoter activity was restored by conversion to a consensus TATA box sequence indicating that the element probably serves this role in the *HK α 2* promoter.

3.4 RCCT28A cell confluency and HK α 2 gene expression

Surprisingly, the *HK α 2* gene promoter was repressed under the conditions of the reporter gene assay. Our laboratory had previously reported that the *HK α 2* gene was in fact expressed in

RCCT28A cells [18]. The one major difference between the earlier experiments and the reporter gene experiment was the confluency of the cells. The luciferase reporter experiments were necessarily conducted under conditions where the cells were not confluent because of the need to obtain efficient transient transfection. Therefore, it seemed plausible that the repression of the *HKa2* gene observed was due to the level of cell confluency with the gene only maximally expressed in polarized cells. RT-PCR was performed on total RNA isolated from RCCT28A cells grown to 70% and to 100% confluency to monitor expression of the endogenous chromosomal *HKa2* gene, rather than relying on a reporter. A RT-PCR product was only observed when RNA from the 100% confluent cells was used as template (Figure 6). Additionally, mRNA was isolated from 30 culture dishes containing RCCT28A cells grown to either pre- or 30 days post- confluency. QPCR analysis showed that 10 of 12 pre-confluent dishes had an undetermined C_t values after 45 cycles, whereas 15 of 18 post-confluent produced determined C_t values after 45 cycles. Culture confluency clearly favored *HKa2* gene expression, however, there must be additional factors that affect *HKa2* gene expression.

4. Discussion

Here we report the cloning and characterization of the rabbit *HKa2* gene. The genomic organization of the rabbit *HKa2* gene provided additional evidence that the rabbit gene is indeed homologous to the mouse *HKa2* gene, the rat *HKa2* gene, and the human *ATP1AL1* gene. In rabbit colon, the *HKa2* gene had transcription start sites for both *HKa2a* and *HKa2c*. This unambiguously demonstrated that the *HKa2c* transcript was an authentic rabbit transcript present in RNA derived from rabbit tissue. Moreover, the result established that use of alternative transcription initiation sites was the mechanism for generation of two mRNAs. Reporter gene analysis of the *HKa2* gene promoter identified the core promoter and two regions upstream with respect to the promoter that account for negative regulation of the promoter.

The computer analysis of the sequence immediately upstream of the *HKa2a* and *HKa2c* transcription start sites revealed a large number of putative transcription factor binding sites. Several transcription factor binding sites may be of particular interest. One apparent site was the CATTAA element that appeared to serve as the TATA box and was completely conserved between human, mouse, rat, and rabbit. However, none of the other putative transcription factor response elements appeared to be conserved across mammalian species at exactly the same positions. Nevertheless, many of the factor binding sites were found at different locations on all three genes. For example, in rats cyclic AMP increases when blood K^+ levels decrease [22]. Putative CRE were found at -217, -869, and -1245 in the rabbit gene providing a plausible mechanism for stimulation of transcription from the *HKa2* gene in response to hypokalemia. In fact, it was recently reported that the CRE at -177 in the mouse *HKa2* promoter played a functional role in regulating gene expression [23]. Similarly, NF- κ B inhibits transcription of *HKa2* in a mouse medullary collecting duct cell line [24]; a putative NF- κ B site is located at -486 in the rabbit *HKa2* promoter.

One important observation was that in RCCT28A cells, the *HKa2* gene was not expressed until the cells reach confluency. The promoter deletion analysis suggested that the *HKa2* gene was largely repressed under the conditions of the assay. Removal of two negative regulatory regions of DNA stimulated the reporter gene, but no consensus sequences for known repressor binding sites were found in those regions. Differential *HKa2* gene regulation was also observed in mouse kidney and colon using an *HKa2* promoter-reporter transgene [25]. Therefore, specific cell types may express unknown factors that act on the *HKa2* gene promoter. Indeed, comparison of this study with the results from Zhang et al. [16] indicated that the regulation in the cortical collecting duct cells may differ from medullary collecting duct cells. Zhang et al. observed significant reporter gene activity in their assays using a mouse medullary collecting duct cell line [16] whereas we observed repression with a similar promoter construct in

RCCT28A cells. Furthermore, our observation that cell confluency plays a role in regulation of *HKa2* gene expression is in line with work from other laboratories showing increased gene expression with increasing confluency [26,27].

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Figure 1.

Organization of the rabbit *HKα2* gene. Panel A. Phage lambda clones and PCR products used to determine the gene structure are indicated by the bars, and locations of exons are drawn to scale. Panel B. Intron-exon structure of the rabbit *HKα2* gene. The arrows show the transcription start sites as mapped in Figure 2 (see below). Panel C. Splicing of exon 1 to exon 2 yields the HK α 2a mRNA, and transcription initiation within intron 1 results in the HK α 2c mRNA. Stars indicate positions of the HK α 2a and HK α 2c probes for the RNase protection assay. Panel D. Computer analysis of transcription factor binding sites in the 5' region of the *HKα2* gene. The putative transcription factor binding sites included seven GATA binding sites [28], two Octamer motifs (Oct-1) [29], three cyclic AMP response elements (CRE) [22], three CCAAT enhancer binding protein (C/EBP) [30], seven stimulatory protein 1 elements (SP1) [31], two activating protein-1 elements (AP-1) [32], one serum response element (SRE) [24], one early growth response element (Egr-1) [33] and one NF- κ B binding site [34].

2a start site:

CAG**CATTTAA** GGC GGACACC ACCTCCCCTG GGCAGCGGCT GGCGATCGGC TCGGAGGTG

2c start site:

GGT**CAATCCA** GACACGCGGG GAAGGAGTTC CAGGGGTCAG CTCCGCCCTC GCACCTGCGG

Figure 2.

The *HK α 2* gene transcription start sites for HK α 2a (panel A) and HK α 2c (panel B). GATC represents those nucleotides for the M13 control sequence. R represents the protected fragment from the RNase protection assay performed with rabbit colon RNA. A portion of the genomic sequence 5' of the *HK α 2* gene is shown below each figure. Arrows indicate the position of the transcription start sites. Bolded nucleotides represent putative core promoter elements upstream of each transcription start site.

-1500 gccagctctctgctgtggccagggtgcagtggaggatggcccagcacttgggagaccaggataaagtacctggctcctgccatcccatcagcacggtg
GATA-1 GATA-1, 2, 3

-1401 cgctggccgcagcacgcgcagccatggcgccattggagggtgaaccaacggcaaaggaagacctttctctctgtctctctctctcactgtctctctgccc

-1301 tgtcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaagagttaaatgaagaagtaactcactaaacttcacccctgtcccatagccagagaatccccagacttaaat
Oct-1 CREB

-1201 tctgctcttttgatttaggggtgttctaatggattttcttttgttaacttcaagtatttatctatttaattgtatttgaaagacaagacacagagatgtc
CRE-BP

-1101 ttcccccttactgggttactctccaatgtccccaaagcaggggctgggccaagaggagccaggggatccaagggtcgggagctcaacctaggtgtccca
C/EBP

-1001 tgtgggaagccaggaccacaataacttgagcagtcctctgctgctcccagggtatgtattagcaggaagtcagaattgggaggagggggcggtgcttgaatc
GC box/SP1

-901 caggtactctgataagaccaggcagcgtctccagcgtgcaccaaattggtcaccccagatcctggccttgaggagccaagggaagggcgaaaacttgtg
GATA-X SREBF GATA-1

-801 tgtgcagtaactgtgggtcggggtctccatcgatccggagctctgggggtgtcactctaattctgctaacaactgggcgcagcaccacgcagcccggaacc

-701 ccagagccctccacgctcactgaccagacccctccatccccaacccctctctcctccaaaatggcttcagaattcccaaatctgatgtagatgtgtcac
GATA-1 AP-1 C/EBP Oct-1

-601 aggggtataccagcgtctccttagagtctctcgcggtggggccacagtggcggttaaccagatccgctcccaagcgacttgaccttactctgagagt

-501 gcagctgctactggaactcgcaatttctctctctgcttacatatctgtataaacccctttatgggttagcaaatgaaattttataaagacaagtgtgta
NF-kb C/EBP

-401 ggggttccccacgaaagcttgaacaggaggtgggagcaccggagcgcggagcctcagcagccccggggcgcttggggacttgggggtccggatcctggg
GATA-1

-301 gccccggggtgggggtgctgagcacagagggtactgcgagctgaaggcgttgttccaagcgccaaggatttgggaccggcccgagacgccccacgc
CREB

-201 cgctgtgttcggctcctggaaggaattgggggtcccagccccggactctccctgcctcttgccatagccagcccgggtcccggactgcgcactcctcggtt
SP1 SP1 SP1

-101 cccagccccctgggggtgtctgcaggccgggctacttgcacagcagcaggtgcgtaggcggggcgcgcagcattttaagcggacaccacctcccctgggca
GATA-1 Erg-1 TATA box

+1 gcggtggtggcgatcggtgctgcggaggtgcgcgcagggcccgctggctgtgggtacctccttcgcagcaccgtcgccactaccaacgcgcgccaccgcggga
GATA-1, 2

+101 cctaccgccatcggtcgccgcgcaccgcaggtcccacgacctcctgcccccgcgccccctgcccgcgacccgcggcgctccagcgcgacAT
SP1 SP1

+201 GCGCCAGTgtgtgtgaggaagtgcgcgggtgcggactggagagaagtgcgggaagggtgaagggtccgtccgggggtcttactctgcaacctgttcc
AP1

+301 agccgcgagcaccccgctgtgtcactcggaactggctgggttaaagaggtTaaTccagacacgcggggaaggagttccagggtcagctccgccctcgcac
+401 ctgcggggtcggattcggagaaaagtgtctagactggagctacacgtatgcgtagcgggtctggaaaatgcccagggtcgggtctgaggggcccaggtcta
+501 tgacccgctggtgtgaccccgagggaaccccgcggttaacttctctcctgccacccttagaggtgtcttctgggaagacgATGGCAGGCGGTGCC

+601 ACCGAGCCGACCGTGCAACAGGGGAAGAGAGGAAGGAGGGAGGTGGAGGTGGCGCGCTCCCCACAGCCCTTCCCCTCCTGGCCCGGAGGGTGTCCGGT

+701 CCCACTCAAGGCAGCTGCGCAGAGCCTGTGCAAAATACCACCTGGGCGCGGTATTGCACTCTGCTTCTTTTCAGAGAAAAGCTGGAATTTACTCCGTG

+801 GAGCACCATGCAGCTACAGATATCAAGAAGAAGGAGGGGCGAGATGGCAAGAAAGACAATGACTTGGAACTCAAAGGAATCAGCAGAAAGAGGAGCTTA

Figure 3.

Nucleotide sequence of the 5'-flanking region of the *HKα2* gene. This sequence of 2400 bp represents 1500 bp upstream and 900 bp downstream of the *HKα2a* transcription start site. Bold letters indicate the 5'-most transcription start sites for *HKα2a* (+1) and *HKα2c* (+382). Boxed sequences represent a TATA-like element and a CCAAT box element that may serve as core promoter element for *HKα2a* and *HKα2c* respectively. Capital letters indicate the nucleotide bases that code for exon 1 and exon 2 amino acids of *HKα2a*. Italics indicate nucleotides that code for amino acids in exon 1 of *HKα2c* that are not included in exon 2 of *HKα2a*. Underlined sequences represent binding sites that were identified by TFSearch for transcription factors that have been shown to be expressed in kidney tissue.

Figure 4.

Deletion analysis of the *HKα2* promoter. Relative light units for reporter gene constructs that contain the *HKα2a* transcription start site. Error bars indicate standard error for $N \geq 6$. Stars represent constructs that had a statistically significant difference in luciferase activity when compared to the previous construct using a one way ANOVA analysis. Dotted line represents the insertion of bacterial DNA.

Figure 5.

Mutation of the apparent TATA box. Relative light units for reporter gene constructs with mutations in the CATTTAA element. X represents the mutation that converts the CATTTAA element to a random sequence (ATCGAGG). Triangle represents a mutation that converts the CATTTAA element into a consensus TATA element (ATATAAAA). Error bars indicate standard error for $N \geq 6$.

Figure 6.

RT-PCR products indicating the presence or absence of HK α 2 transcripts. Lane designations are as follows: Lane 1: 1Kbp ladder, lane 2: blank, lane 3: 70% confluent RCCT28A cells without reverse transcriptase (-RT), lane 4: 70% confluent RCCT28A cells + RT, lane 5: 100% confluent RCCT28A cells -RT, lane 6: 100% confluent RCCT28A cells + RT, lane 7: plasmid control showing the expected size of the PCR product using primers BC230 and BC231.

Table 1
Exon and intron sizes for the rabbit, rat, mouse and human (ATPAL1) HK α 2 genes

Exon	Rabbit ^a	Rat ^b	Mouse ^c	Human ^d	Intron	Rabbit ^a	Rat ^b	Mouse ^c	Human ^d
1	208	287	262	195	1	569	677	658	(700)
2	141	153	150	159	2	2702	2133	2286	(2300)
3	60	60	60	60	3	3061	2511	2321	(2900)
4	201	201	201	204	4	1049	746	762	738
5	114	114	114	114	5	1032	742	726	937
6	135	135	135	135	6	115	124	129	123
7	118	118	118	118	7	173	233	227	258
8	269	269	269	269	8	854	939	970	1187
9	199	199	199	200	9	142	144	134	157
10	110	110	110	110	10	2282	1324	1269	(1700)
11	135	135	135	135	11	(4200)	4231	2010	(4200)
12	193	193	193	193	12	(1600)	1484	1471	(1600)
13	176	176	176	176	13	105	834	639	(900)
14	138	137	137	137		(4200)	1599	1597	(4200)
15	151	151	151	151	15	365	*	1419	557
16	169	*	169	169	16	88	*	90	87
17	155	*	155	155	17	(700)	1425	1311	(1900)
18	124	124	124	124	18	171	184	195	193
19	146	145	146	146	19	445	594	590	(600)
20	134	134	134	134	20	167	174	168	195
21	102	102	102	102	21	(11550)	434	388	431
22	92	92	92	92	22	87	137	161	83
23	658	658	658	905	23	-	-	-	-

Sources:

^a this work,

^b NCBI database,

^c [16],

^d [18] Notes: () indicates introns with sizes determined by estimating the size of restriction fragments.

* indicates sizes that could not be determined due to incomplete database sequence.