Splicing of a retained intron within ROMK $K^+$ channel RNA generates a novel set of isoforms in rat kidney

A. H. BEESLEY, B. ORTEGA, AND S. J. WHITE
Laboratory for Membrane Protein Function, Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN, United Kingdom

Beesley, A. H., B. Ortega, and S. J. White. Splicing of a retained intron within ROMK $K^+$ channel RNA generates a novel set of isoforms in rat kidney. Am. J. Physiol. 276 (Cell Physiol. 45): C585–C592, 1999.—The renal outer medulla $K^+$ channel family of $K^+$ channels may constitute a major pathway for $K^+$ secretion in the distal nephron. To date, four main isoforms of this gene have been identified in the rat that differ only in their NH$_2$-terminal amino acids and that share a common “core exon” that determines the remaining protein sequence. Using RT-PCR, we have identified a new set of ROMK isoforms in rat kidney that are generated by the deletion of a region within the ROMK core sequence that is identifiable as a typical mammalian intron. This splicing event was shown to be reproducible in vitro by detection of deleted ROMK mRNA in Madin-Darby canine kidney (MDCK) cells stably transfected with the gene for ROMK2. Translational deletion of the variant of ROMK2 was confirmed in vitro and visualized in MDCK cells following transient transfection with an enhanced green fluorescent protein tag. The deletion in this core region is predicted to generate hydrophilic proteins that are approximately one-third the size of native ROMK and lack membrane-spanning domains.

METHODS

Animals. Male Wistar rats (220–250 g) were anesthetized with 60 mg/kg ip sodium pentobarbitone (Sagatal, RMB Animal Health), and blood samples were taken by cardiac puncture. Both kidneys were removed, decapsulated, and snap frozen in liquid nitrogen for later use. Animals were subsequently killed with an overdose of anesthetic (Sagatal). RT-PCR. Total RNA was extracted from whole kidneys or cultured Madin-Darby canine kidney (MDCK) cells using TRIzol reagent according to the manufacturer’s instructions (GIBCO BRL) and then treated with DNase I (Promega) to remove genomic DNA. RT reactions (20 µl in volume) contained 2 µg of total RNA, 2.5 µM oligo(dT) primer, 200 µM mixed dNTPs, 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol (DTT), and 300 units Moloney murine leukemia virus RT (Promega). Samples were heated to 90°C for 2 min to denature RNA secondary structures, left on ice for 10 min to allow primer annealing, heated at 35°C for 1 h to reverse transcribe the RNA, and finally heated to 95°C to end the reaction. PCR was performed with primers selective for rat ROMK isoforms (ROMK1, ROMK2, ROMK3, and ROMK6) or the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR reactions (25 µl in volume) contained 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 0.1% Triton X-100, 200 µM mixed dNTPs, 3 mM MgCl$_2$, 2.5 units Taq DNA polymerase (Promega), 200 nM each primer (see below), and 1 µl of the above reverse transcription reaction product. Samples were heated to 94°C for 5 min and then subjected to 30–35 cycles of denaturation (94°C, 1 min), annealing (55–60°C, 1 min), and extension (72°C, 1.5 min). A final extension phase (72°C, 5–10 min) was included for all samples. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining under ultraviolet light (302 nm). Controls included the omission of RT enzyme in the RT step to test for residual contamination of the RNA with genomic DNA and omission of the cDNA sample from the PCR to test for contamination of the PCR reagents or primers.

PCR primers. Sense and antisense primers used for PCR amplification of rat ROMK isoforms were as follows: R1 (sense), CAATGCAAGTAAATGTCATT; R2 (sense), TTTAC...

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C586 NOVEL ROMK ISOFORMS EXPRESSED IN RAT KIDNEY

CCCAGCAATCCATGA; R3 (sense), GCCAGTACAGACATGGT; R4 (sense), GAGGTCGGCTGCGCACTGATGGGGCTAC; R5 (sense), ATCTGGGTAGCAGCTGACAGG; R5 (antisense), GGCAGCAGGCGCTGCTGCGCTCAGCACCCAGCAATCCATGA; R6 (antisense), CAGGTCGGCTGCGCACTGATGGGGCTAC; R7 (antisense), CTACATCTGGTGCTGCTGCTCC; R8 (antisense), GTCTCCACTCAGCTGACAGG; R9 (antisense), CAGGTCGGCTGCGCACTGATGGGGCTAC; R10 (antisense), TGATCACGCATTCTTCTGCTG. A schematic diagram showing the positions of these primers in relation to ROMK cDNA is shown in Fig. 1. Primers R1–R3 previously described by Boim et al. (4), as was the sequence of R6 and R4 target the 5' exons differentially used by specific ROMK isoforms [exon numbering as described by Kondo et al. (14)]. Primers R5–R10 bind to the ROMK core exon and therefore detect all ROMK isoforms.

The rat housekeeping gene GAPDH was amplified using the primers G1 and G2 (CGGCAAGTTCACAGGCGCAGTC and GTTTTCTCAGGGCCAGTC, respectively).

DNA sequencing. Single-banded PCR products were purified from agarose gels using the Geneclean III kit (Anachem) and completely sequenced in both directions using the ABI PRISM dye-terminator cycle sequencing system (Perkin-Elmer).

In vitro transcription and translation of ROMK isoforms. Full-length cDNA for ROMK2 was amplified from rat kidney by RT-PCR and cloned into the vector pEGFP-C2 (Clontech). On translation, this construct (pC2-ROMK2) generates a ROMK2 fusion protein tagged at its NH2 terminus by enhanced green fluorescent protein (EGFP). The same procedure was then used to clone the cDNA for the deletion-containing isoform of rat ROMK2 (pC2-RDEL2). Both clones were fully sequenced in both directions using the ABI PRISM dye-terminator cycle sequencing system (Perkin-Elmer).

Further RT-PCR was performed on the vectors pEGFP-C2, pC2-ROMK2, and pC2-RDEL2 to amplify the full coding sequences for EGFP, EGFP-ROMK2, and EGFP-RDEL2, respectively. Composite primers were used to add a 5' T7 RNA polymerase sequence and a 3' poly(A) tail to the amplified cDNAs for purposes of synthesizing RNA. In vitro transcription and translation were performed with each of these PCR-amplified cDNAs using the TNT coupled reticulocyte lysate system (Promega) using [35S]methionine (in vivo cell-labeling grade, Amersham) according to the manufacturer’s instructions. Subsequent protein samples were separated by electrophoresis on a 10% SDS-polyacrylamide gel and then visualized by autoradiography on Hyperfilm-MP (Amerham).

Expression of ROMK-EGFP fusion proteins in MDCK cells. MDCK cells were grown in 5% CO2 and maintained in a 1:1 mixture of Ham's F-12 and DMEM (Sigma) containing 12.5 mM glucose, 5 mM HEPES, 25 mM NaHCO3, and 10% FCS (Sigma). Cells were transiently transfected with pC2-ROMK2, pC2-RDEL2, or the empty pEGFP-C2 vector (see preceding section) using SuperFect (Qiagen) according to the manufacturer's instructions. These cells or control (untransfected) MDCK were subsequently examined for the expression of green fluorescence following exposure to blue light (480 nm).

In vitro transcription of ROMK RNA. The vector pSPORT1-ROMK1 was digested with proteinase K to remove residual RNase activity, linearized with the restriction enzyme Not I, and subsequently purified by phenol-chloroform extraction and ethanol precipitation. In vitro transcription reactions (50 µl in volume) contained 1 µg of linearized pSPORT1-ROMK1, 500 µM each rNTP (Promega), 10 mM DTT, 30 units of RNasin RNase inhibitor (Promega), 40 mM Tris-HCl (pH 7.9), 6 mM MgCl2, 2 mM spermidine, and 15 units of T7 RNA polymerase (Promega). Reactions were incubated at 37°C for 2 h before the addition of 1 unit of RQ1 RNase-free DNase enzyme and a further 30 units of RNasin inhibitor. Reactions were then incubated for a final 15 min at 37°C to destroy the plasmid DNA. The RNA product was purified by phenol-chloroform extraction and ethanol precipitation and analyzed on a 0.8% denaturing agarose gel. In vitro-transcribed ROMK RNA (35 ng/reaction) was then subjected to RT-PCR as described above for total rat kidney RNA.

PCR of genomic DNA. Genomic DNA was extracted from rat blood using the Wizard genomic DNA purification kit (Promega) and quantified by spectrophotometry. PCR was performed as described previously for rat kidney cDNA, using 125 ng genomic DNA/reaction.

Detection of ROMK RNA splicing in MDCK cells. MDCK cells were grown as described above and transfected with the vector pcDNA3 containing the gene for ROMK2 using SuperFect reagent (Qiagen) according to the manufacturer’s instructions. Stable transfectants were selected on the basis of geneticin resistance. Confluent flasks of control (untransfected) or positive (ROMK2-transfected) MDCK were subsequently used for RNA extraction and RT-PCR as described above.

RESULTS

A common feature of RT-PCR experiments is the amplification of spurious products resulting from the chance binding of the PCR primers to DNA sequences other than the desired target. For this reason, some primer combinations are more successful than others in amplifying a single-banded RT-PCR product from a particular source. During the course of routine experiments, we observed that RT-PCR of rat kidney RNA with a range of different primers for ROMK frequently

![Diagram of rat renal outer medulla K⁺ channel (ROMK) isoform cDNAs indicating the positions of primers (R1–R10) used in PCR. Differential splicing of 5' exons (shown as boxes) generates sequence differences within each isoform (exons numbered as described by Kondo et al. (14)).](http://apc.cell.physiology.org/)

Fig. 1. Schematic diagram of rat renal outer medulla K⁺ channel (ROMK) isoform cDNAs indicating position of primers (R1–R10) used in PCR. Differential splicing of 5' exons (shown as boxes) generates sequence differences within each isoform (exons numbered as described by Kondo et al. (14)).
resulted in the amplification of two separate products: a larger band consistent with the size predicted for ROMK and an unknown smaller product. A more systematic study using ROMK primers R1–R10 (Fig. 1) revealed only one primer (R9 in combination with any other) capable of generating a single-banded product from rat kidney RNA.

Although the generation of spurious products by RT-PCR is quite common, the secondary products observed in these experiments were consistently estimated by agarose gel electrophoresis to be 150–200 bp smaller than the predicted product in each case, regardless of the primer combination used. Such consistency is not something one would predict for the random binding of primers to nonspecific sequences, and we therefore decided to purify these secondary products for DNA sequencing. Interestingly, these products were found to be an exact match for the rat ROMK sequence apart from the deletion of a 179-bp region normally present within the core exon (Fig. 2A). This deletion accounted for the smaller size of the secondary products generated by RT-PCR and also for the ability of the R9 primer to amplify only a single product, since its target sequence lies within the deleted region (Fig. 2A).

These data suggested the existence of novel ROMK mRNA isoforms in rat kidney that would be predicted to generate peptides significantly different from those translated by native ROMK. An alignment of the protein sequences for rat ROMK2 and that predicted for the deletion variant of ROMK2 is shown in Fig. 2B. Although the first 59 amino acids of the core peptide sequence (equivalent to the NH₂-terminal end of rat ROMK Core Sequence

CTTGACACCTGTGGCCTGAAATGTCCGACGTTATGCATACAGAATGGAGGAG
CTTGACACCTGTGGCCTGAAATGGAGGAG

405
ACAGCTCTTGGGATGTTTCCTCTTGGTCTCTGTATGTCGATG

457
CGATGTCATAAGGACACTCCAGTGTCCTACCGCTGACAACGGACCTCC

509
TTGTGGGAGAAGCATATGCCCAGTTGCTCCTTGTCTAG

561
ACTCAAGTGACCATAAGCTTTTAGCAGTTGTGTGGACAGAAAGCTGGAAGCAGCTGACAGAAC

613
CTGGATTTTCAGGCTTACCACTCCGACCTGCTCTGTACGATCAATCC

665
CTTCCATCGTGGTGGTCATTTAGAC

CTTCCATCGTGGTGGTCATTTAGAC

Fig. 2. A: partial alignment of ROMK core exon with novel cDNA sequence obtained from rat kidney by RT-PCR (numbering as for ROMK1; GenBank no. X72341). Deleted region in novel sequence is shown by dashes and contains target sequence for primer R9 (bold lowercase letters). GT and AG base pairs for donor and acceptor sites are indicated in bold uppercase letters. Boxes and asterisks represent regions involved with RNA splicing (DS, donor site; AS, acceptor site and pyrimidine-rich sequence; BPS, branch point sequence; ** last occurrence of AG base pair before acceptor site). Arrows, splice sites. B: alignment of protein sequences of ROMK2 (equivalent to ROMK core peptide) and that predicted for deletion variant of ROMK2. Lowercase letters indicate differences created by a shift in reading frame for ROMK2 caused by deletion, and dashed lines represent missing residues. Boxed regions with bold letters are consensus sites for phosphorylation within new sequence.
that full-length fusion proteins could be generated for both normal ROMK2 and its deletion variant (bands at 67 and 41 kDa, respectively). Translation of EGFP alone generated a predicted protein band at 27 kDa (Fig. 4). Expression of these proteins in a living system was visualized following transient transfections in MDCK cells with constructs that were designed to express either EGFP on its own (pEGFP-C2), EGFP linked to rat ROMK2 (pC2-ROMK2), or EGFP linked to the deletion variant of rat ROMK2 (pC2-RDEL2). In each case, fluorescence could be observed following excitation by exposure to blue light (488 nm), indicating that all three constructs are successfully translated in this system (Fig 5, B–D). No fluorescence was observed in control (untransfected) MDCK cells (data not shown).

To ensure that the amplification of the ROMK RNA homologue could not be attributed to an artifact of the RT-PCR process, we generated full-length ROMK RNA from the vector pSport1-ROMK1 by in vitro transcription and subjected it to RT-PCR under the same conditions that were used to amplify total RNA from rat kidney. As can be seen from Fig. 6A, RT-PCR of in vitro-transcribed ROMK RNA resulted in the production of a single-banded product of the size predicted for full-length ROMK (lane 2). This is in contrast to that of rat kidney RNA, which generated both the full-length and smaller secondary products (437 and 258 bp, respectively), as well as a third, larger band (−720 bp) that is a heteroduplex formed from the other two products (Fig. 6A, lane 1). This product runs anomalously, since the difference in size between its two cDNA strands results in the formation of an irregular secondary structure. DNA sequencing (data not shown) confirmed the identity of the heteroduplex.

These data indicate that the amplification of the deleted ROMK cDNA from rat kidney represents the expression of novel ROMK mRNA isoforms rather than an artifact of RT-PCR. To test whether this resulted from the expression of an alternative “ROMK-like” gene, we extracted genomic DNA from rat blood and

![Fig. 3. A: schematic diagram of structure of native ROMK protein as described by Schwalbe et al. (23), showing point at which deletion occurs and MO, H5 (see text), and membrane-spanning domains 1–4 (M1–M4). B: protein hydropathy plot for predicted deletion variant of ROMK2 calculated as described by Kyte and Doolittle (16). Positive values indicate hydrophobic regions; negative values indicate hydrophilic regions.](http://ajpcell.physiology.org/)

**Fig. 4.** Autoradiography of enhanced green fluorescent protein (EGFP; lane 1), EGFP-ROMK2 (lane 2), and EGFP linked to deletion variant of ROMK2 (lane 3) generated by in vitro transcription and translation.
performed PCR with core exon primers that were previously successful in amplifying the ROMK homologue from rat kidney by RT-PCR (Fig. 6B). Although a product of the correct size for full-length ROMK was successfully amplified in each case (from both rat kidney RNA and the vector pSport1-ROMK1), no secondary product could be detected under any conditions. This serves, first, to confirm the previous conclusion that the generation of smaller ROMK products is not an artifact of the PCR process itself and, second, to indicate that the deleted ROMK isoform does not exist within the rat genome. This means that the deleted ROMK isoform detected in rat kidney by RT-PCR must be generated by the splicing of normal full-length ROMK mRNA.

To test whether this splicing event could be reproduced in vitro, we performed RT-PCR on MDCK cells that had been stably transfected with the gene for ROMK2 and that have previously been shown to exhibit $\text{Ba}^{2+}$-sensitive $K^+$ channel activity (20). Unlike control MDCK (which did not exhibit $\text{Ba}^{2+}$-sensitive channel activity), transfected cells expressed normal full-length ROMK mRNA (Fig. 6C, lane 2). The failure to amplify ROMK message from control MDCK cells could not be attributed to RNA degradation, since a product for the housekeeping gene GAPDH could be successfully amplified from both cell types (Fig. 6C, lanes 3 and 4). In addition, however, RT-PCR of transfected MDCK revealed the expression of a smaller secondary product (Fig. 6C, lane 2) that was subsequently shown by DNA sequencing to be the same as the ROMK deletion isoform previously detected in rat kidney. This indicates that the sequence of the published ROMK core exon (of which ROMK2 is entirely composed) must contain generic signaling sites for the activation of RNA splicing that are recognized by different mammalian species.

**DISCUSSION**

The data presented in this paper indicate that rat kidney expresses previously unknown isoforms of
ROMK that are generated by the splicing of normal full-length ROMK mRNA. The most common type of RNA splicing event is the removal of introns from pre-mRNA molecules, and the signaling sites that direct this process have been well documented (10). More than 99% of all mammalian introns begin at the 5' end with a GT base pair combination (the "donor" site) and terminate at the 3' end with an AG base pair (the "acceptor" site) (25). These bases contribute to consensus sequences [AGGT(A/G)AGT and (T/C)(T/C)-TT(T/C)6NCAGG, respectively, where N is a nonspecific base], the composition of which affects the degree of efficiency of the RNA splicing event (7, 19). The upstream component of the acceptor site is a pyrimidine-rich region that extends well into the intron and is important for the correct localization of the acceptor site for intron splicing. The absence of any alternative upstream AG base pair within this vicinity (usually a minimum of 10 bases from the acceptor site) assists with this process (25). Finally, a consensus branch point sequence (YNYYRAY, where Y is a pyrimidine and R is a purine) 18–37 bases upstream of the acceptor site (19) binds to the cleaved 5' end of the intron during formation of the splicing lariat (10). Analysis of the region of the ROMK core exon that is deleted in the novel mRNA isoforms expressed in rat kidney demonstrates that it matches every one of these criteria (Fig. 2A) and is therefore most likely to represent an intron that for some reason is retained in the majority of rat ROMK transcripts. Although the GT/AG base pair rule for the donor and acceptor sites is observed in this intron (bold, uppercase letters in Fig. 2A), the surrounding sequences do not exactly match the normal consensus (see Fig. 7), and this may explain why the frequency of splicing in this transcript is relatively low. Final proof that these sequences represent signaling sites for RNA splicing comes from the reproduction of this event in MDCK cells that were stably transfected with normal, full-length rat ROMK. It should not be surprising that cells derived from dog kidney are capable of splicing RNA derived from rat, since the signaling sites that define intron-exon boundaries are highly conserved across mammalian species (25).

Because of the exponential nature of the PCR reaction, it is difficult from these experiments to precisely calculate the relative levels of expression of the different isoforms in rat kidney, but initial estimates suggest that the deleted isoforms may be expressed at levels between 1 and 10% of native (intron-retaining) ROMK mRNA. However, because the intron lies within the "core" sequence of ROMK, the splicing of this message should in theory affect all known rat ROMK isoforms and therefore effectively double the number of transcripts generated from the rat ROMK gene. We have called the novel isoforms identified in the present study ROMK1.1, ROMK2.1, ROMK3.1, and ROMK6.1. Retention of introns within mammalian transcripts is a relatively uncommon event but has been previously documented for a number of genes, including bovine (11) and human (18) growth hormone, the β1 subunit of insulin-like growth factor (12), and the α1C subunit of the α2-adrenergic receptor (13).

Fig. 6. A: RT-PCR of rat kidney RNA (lane 1) and in vitro-transcribed ROMK RNA (lane 2) using primers R5 and R10. B: PCR of vector pSport1-ROMK1 using R1 and R6 primers (lane 1) and of rat genomic DNA using R5 and R8 primers (lane 2) and R5 and R10 primers (lane 3). C: RT-PCR of control MDCK cells (lanes 1 and 3) or cells stably transfected with gene for rat ROMK2 (lanes 2 and 4), using primers for either ROMK (R5 and R10; lanes 1 and 2) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; G1 and G2; lanes 3 and 4). M, 100-bp marker ladder (Promega).

Fig. 7. Alignment of consensus (C) mammalian intron signaling sites, with equivalent regions of rat (R) and human (H) ROMK. Bold lowercase letters represent deviations from consensus sequence. Boxes highlight GT and AG bases involved in intron splicing that are absent in human sequence.
the rat, mouse, and human voltage-dependent Na⁺ channel (6), and the murine vitamin D receptor (8). In the latter case, the alternatively spliced transcript was found to be expressed at ~5% of normal levels (a figure similar to that estimated for the expression of intron-deleted ROMK isoforms in rat kidney) and was found to generate an alternative receptor peptide that negatively modulates the normal vitamin D signaling pathway.

The present study indicates that the novel ROMK transcripts described in this paper can be successfully translated in vivo, since full-length proteins were generated following in vitro transcription and translation and since fluorescence was observed following transient transfection of MDCK cells with the cDNA for the ROMK2 deletion variant (ROMK2.1) when linked to EGFP. However, the removal of the intron generates a frame shift that significantly alters the sequence of the protein that is predicted from the normal ROMK translation start site (Fig. 2B). These peptides would be approximately one-third of the size of native rat ROMK and would possess a unique COOH terminus that is predicted to contain three new consensus sites (21) for phosphorylation (two protein kinase C or cAMP-dependent sites and one casein kinase II site; Fig. 2B) but has no significant homology to other known proteins in the SwissProt database. An alternative open reading frame (ATG at position 669 in Fig. 2A), which could theoretically code for a larger polypeptide, is unlikely to be translated, since its start codon lies several hundred bases after the normal initiation site and does not lie within a consensus Kozak sequence (15). The function of these proteins is as yet unknown, but, since they do not possess transmembrane-spanning domains, it is unlikely that they would be capable of forming any kind of independent channel activity. However, the hydrophilic nature of these peptides would not necessarily preclude them from affecting the activity of native K⁺ channel function. The β-subunits of voltage-gated K⁺ channels, for example, are also small, hydrophilic proteins that lack membrane-spanning domains, and yet they are known to significantly affect channel kinetics (13, 24). Alternatively, the novel peptides described in the present paper may have intracellular functions unrelated to the regulation of membrane-bound ROMK K⁺ channel activity. The resolution of these possibilities awaits further experimentation with in vitro expression systems.

The importance of ROMK channels in K⁺ homeostasis in humans has been recently highlighted by the discovery that mutations in this gene are associated with Bartter’s syndrome, a genetic disorder resulting in hypokalemia (5, 22, 27). The human ROMK gene has a different exon structure from that of rat, which results in the generation of five human ROMK transcripts (hROMK1–hROMK5) that differ from those previously identified in the rat (3, 26). However, there is a great deal of homology between these two species (e.g., >90% amino acid identity for ROMK1), with the majority of the hROMK peptide sequence being determined, as in the rat, by a common core exon at the 3’ end of the gene. Despite this, analysis of the human ROMK sequence reveals that it is unlikely to possess an intron within its core sequence equivalent to that described in this paper for rat (Fig. 7). Although the consensus branch point sequence is conserved, as is the pyrimidine-rich tract, there are specific base changes within the donor and acceptor sites that make it unlikely that this region would be identified as an intron by the splicing machinery.

In the human sequence, the GT base pair of the donor site is changed to AT and the terminal AG base pair of the acceptor site to GG. However, not all intron boundaries are defined by the classical GT/AG consensus sites (25), and whether hROMK transcripts undergo alternative splicing processes similar to that described here for rat remains to be determined empirically. Interestingly, a DNA sequence recently deposited in the GenBank database for a mouse isoform of ROMK2 (no. AF012834) does share the same signal sequences that define the retained intron in rat ROMK, suggesting that this type of RNA splicing may also occur in mouse ROMK transcripts.

In summary, we have identified a previously unknown intron within the rat ROMK core sequence that is normally retained in the majority of ROMK transcripts but that is subject to low-frequency alternative splicing to generate a new set of ROMK isoforms in rat kidney. The role of these novel isoforms (named ROMK1.1, ROMK2.1, ROMK3.1, and ROMK6.1) and their pattern of expression in both renal and extrarenal tissues remain to be determined.

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The cDNA sequences for rat ROMK1.1, ROMK2.1, ROMK3.1, and ROMK6.1 have been deposited in the GenBank database (nos. AF081365, AF081366, AF081367, and AF081368, respectively).

Address for reprint requests: S. J. White, Laboratory for Membrane Protein Function, Dept. of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, UK.

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