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Tracing the Origin of the RACTK1 K^+ Channel

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Potassium secretion by the kidney is vital for the maintenance of K^+ homeostasis. RACTK1, a putative inwardly rectifying potassium channel cloned from cultured rabbit collecting duct cells, has been proposed to play a role in this process. However, the lack of homology with any other cloned potassium channel and the inability to reproduce the results across different laboratories has brought into question the existence of RACTK1. Recently, it has been suggested that RACTK1 is a contamination from *Escherichia coli*. In this work we add conclusive evidence supporting the bacterial origin of RACTK1. Using both genomic PCR and RT-PCR we were unable to detect RACTK1 in a number of mammalian species. In addition sequencing of RACTK1 cDNA confirmed a complete homology between RACTK1 and a region of *E. coli* genomic DNA. Finally, a hypothesis on how RACTK1 could have been generated from a contamination by *E. coli* genomic DNA is presented.

Key Words: **potassium channel; kidney.**

The distal nephron of the kidney is an important site for the maintenance of K^+ homeostasis in mammals, since in the cortical collecting duct (CCD) system K^+ is secreted into the urine, thus controlling the urinary excretion of K^+. Although the identity of the channels mediating K^+ secretion in this region are not known for certain, there is considerable evidence to suggest that the ROMK family of ATP-regulated inwardly-rectifying K^+ channels are involved (1, 2, 15). Mutations in the *ROMK* gene that impair normal channel function have been found in patients with one type of Bartter's syndrome (3, 4), a genetic disorder resulting in hypokalaemia. Since impaired secretory channel function is not entirely consistent with increased urinary potassium loss, it may be that there are other channels in the distal nephron that are important in mediating renal K^+ secretion.

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In 1994 Suzuki and co-workers (5) reported the isolation of a novel cDNA (*RACTK1*) from cultured cells of rabbit CCD. The cDNA encoded a putative K^+-channel with a molecular weight of 31 kDa, two transmembrane domains and one H5 domain (5, 6). While some properties of RACTK1 matched quite well with those described for the CCD K-secretory channel, many others were dissimilar. Like the native secretory channel, RACTK1 is an inward rectifier, with high voltage-independent open probability, pH sensitivity and is inhibited by the nonspecific K^+ channel blocker Ba^{2+} but not tetraethylammonium. However, it differed from the native secretory channel by its intermediate conductance of around 80 pS, its activation by 500 nm Ca^{2+} and inhibition by PKA but not PKC (8). Although inhibited by mM concentrations of ATP, RACTK1 did not show channel run-down in the absence of ATP (5, 8). In 1994 a consideration of the amino acid sequence of RACTK1 by Sutcliffe and Stanfield (7) showed that RACTK1 possessed no homology (even at the pore region) with any known potassium channel. Subsequently, Shmukler and co-workers (9) reported their failure to find evidence for the expression of RACTK1 in a number of different tissues. They concluded that rabbit RACTK1 might originally have been the product of the accidental cloning of a bacterial contaminant. Comparison of the RACTK1 sequence with the *Escherichia coli* genome revealed a match with a stretch of sequence between 65 and 68 min. This sequence was 98% homologous with RACTK1, yet did not code for any similar open reading frame.

A number of studies concerning the function of RACTK1 have been published (8, 10, 11) which are difficult to reconcile with the idea that this gene may represent a bacterial contaminant lacking the open reading frame needed to encode a K^+ channel. Furthermore, the properties and possible functions of the RACTK1 K^+ channel continues to be discussed (1, 2, 12). We therefore thought it important to reconsider the claims of Suzuki *et al.* (5) and Shmukler *et al.* (9) and add further evidence to this issue. Here we report that by using RT-PCR and genomic PCR we have been unable to find any evidence for the existence of

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TABLE 1

5′ and 3′ Primer Sequences for Standard PCR

Gene	Primer name	Sequence (5′ to 3′)
ROMK1, 2	ROMK 5′	GCGGCCGGATCCAAGATCTCTAGACCCAAAAAACG
ROMK1, 2	ROMK 3′	ACGAATTCGATGGTCTCGCCTTCAGGAGT
ROMK1	ROMK-1 5′	CAATGCAAGTAAATGTCATT
ROMK1, 2	ROMK-2 5′	TTTACCCGAGCAATCCATGA
ROMK3	ROMK-3 5′	GGCAGTACAGACAATGGTGT
ROMK1, 2	ROMK-1 3′	GGCGCACTGTTCTGTCACAA
ROMK3	ROMK-2 3′	GTACCTCCATTTCAGGTCCA
RACTK1	RTK1 5′	CGATGTTCAGAGCGATGAC
RACTK1	RTK1 3′	TCATTGAAGCATCGGCAC
RACTK1	RTK2 5′	TACGAAGAATTCGATGCCGTTCATCAGATCCGAAC
RACTK1	RTK2 3′	TTAGATCTCGAGTCAATTACGGTTTCTTCGGCGACATC
RACTK1	RTK3 5′	GTAATCCATATTGTCGCAAATCTTG
RACTK1	RTK 3′	GCCGGTAATATGCTGGATAACC
GAPDH	GAPDH 5′	CGGCAAGTTCAACGGCACAGTCA
GAPDH	GAPDH 3′	GGTTTCTCCAGGCGGCATGTCA
β-actin	β-actin 5′	TCCTAGCACCATGAAGATC
β-actin	β-actin 3′	AAACGCAGCTCAGTAACAG

Note. Rat ROMK primers were based upon those published previously (14, 15). Primers for GAPDH and cytoplasmic β-actin were designed against the published rat sequences (GenBank X02231 and V01217, respectively). RACTK1 primers (RTK1-3) were designed against the published rabbit sequence (GenBank D16216).

RACTK1 in the mammalian genome. Furthermore, we present direct evidence that the original RACTK1 clone may have been incorrectly sequenced to generate the ORF encoding rabbit RACTK1. Finally, on the basis of a detailed consideration of the sequence we present a hypothesis on how RACTK1 could be the result of PCR amplification from an *E. coli* contamination.

METHODS

RT-PCR and genomic PCR. Whole kidneys were obtained from adult male Wistar rats and Dwarf-Lop rabbits anaesthetised with sodium pentobarbitone (Sagatal 60 mg kg^{-1} i.p. & 120 mg kg^{-1} i.v., respectively). Total RNA extraction and reverse transcription were carried out as previously described (15). Primer pairs are listed in Table 1.

Human genomic DNA was obtained from the Centre for Human Genetics (Sheffield University), and mouse and hamster genomic DNA from the UK HGMP Resource Centre (Cambridge). Rabbit genomic DNA and bacterial DNA (*E. coli* strains DH5α, BL21 and MC1061) were prepared as described by Ivinson and Taylor (13). PCR of RT product or genomic DNA was performed with primers selective for RACTK1 and isoforms of the ROMK K^+ channel family (ROMK 1–3), or the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. PCR reactions (25 μl volume) contained 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 200 μM mixed dNTPs, 1–5 mM $MgCl_2$, 2.5 units *Taq* DNA polymer-

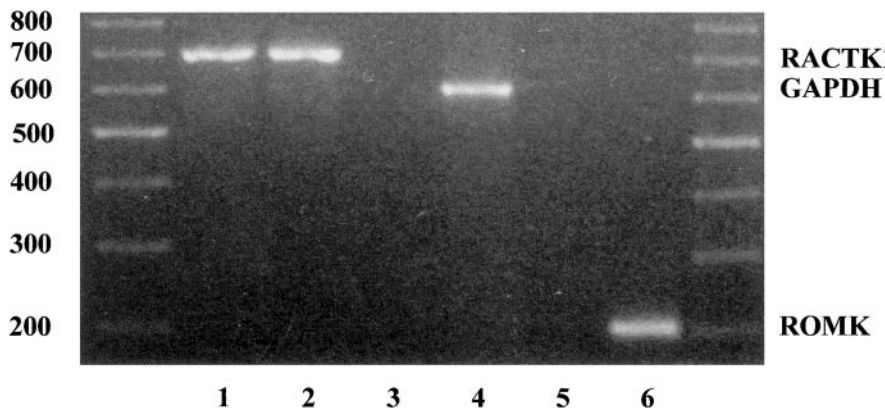

FIG. 1. PCR amplification of RACTK1 with primers RTK1 5′ & 3′. Using pCR2-RACTK1 (lane 2) and *E. coli* genomic DNA (lane 3) as templates. In both cases the size of the resulting products corresponded to the *E. coli* sequence (687 bp) rather than to the predicted size for rabbit RACTK1 (747 bp). RT-PCR from rabbit kidney failed to generate any RACTK1 product (lane 4), despite correct amplification of GAPDH (lane 5) from the same RNA sample. Similarly, RACTK1 primers failed to generate a PCR product from rabbit genomic DNA (lane 6), despite correct amplification of ROMK with primers ROMK 5′ and ROMK 3′ (lane 7). Lanes 1 and 8 are markers (enhanced band at 500 bp).

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RACTK1      (1)  TTGCCTGAATCACTGCTGCCGCCG ATG CCGTTCATCAGATCCGAACCCCG
N-RACTK1    (1)  TTGCCTGAATCACTGCTGCCGCCGATGCCGTTCATCAGATC--gACCCCG
E.coli      (1)  cgttCaaAATCACcGCTGCCGCCGATGCCGTTCATCAGATC--gACCCCG

RACTK1     (51)  GCTACGTGTGTTGAGTTGGCATGCCCGTAAATATCAAACTCCAGGCCCGA
N-RACTK1   (49)  GCTACGTGTGTTGAGTTGGCATGCCCGTAAATATCAAACTCCAGG-CCGA
E.coli     (49)  GCTACGTGTGTTGAGTTGGCATGCCCGTAAATATCAAACTCCAGG-CCGA

RACTK1    (101)  TGTTCAGAGCGATGACGCCCAGACGACGGATGATTTCCGGGTTATTGGAA
N-RACTK1   (98)  TGTTCAGAGCGATGACGCCCAGACGACGGATGATTTCCGGGTTATTGGAA
E.coli     (98)  cGTTCAGAGCGATGACGCCCAGACGACGGATGATTTCCGGGTTATTGGAA

RACTK1    (151)  ATCTCCTGCGGACGCAACACAATGCGGCTGGCAA-GTAATCCATATTGTC
N-RACTK1  (148)  ATCTCCTGCGGACGCAACACAATGCGGCTGGCAAaGTAATCCATATTGTC
E.coli    (148)  ATCTCCTGCGGACGCAACACAATGCGGCTGGCAAaGTAATCCATATTGTC

RACTK1    (200)  GTAAATCTAGCGCAGGGAATCGGCCGAGATTGTCAGGCTGGAGGCGCTGG
N-RACTK1  (198)  GTAAATCTtGCGCAGGGAATCGGCCGAGATTGTCAGGCTGGAGGCGCTGG
E.coli    (198)  GTAAATCTtGCGCAGGGAATCGGCCGAGATTGTCAGGCTGGAGGCGCTGG

RACTK1    (250)  CCC-GCTGATTTGGCCGGTTTC-AGTAAATGCACCACCGATTCCTGTAGC
N-RACTK1  (248)  CCCcGCTGATTTtGCCGGTTTCcAGTAAATGCACCACCGATTCCTGTAGC
E.coli    (248)  CCCcGCTGATTTtGCCGGTTTCcAGTAAATGCACCACCGATTCCTGTAGC

RACTK1    (298)  ACTTCCGAATGCATCATAAACGGAGGAATTACCGGGTTTTCCCCCAGACG
N-RACTK1  (298)  ACTTCCGAATGCATCATAAACGGAGGAATTACCGGGTTTTCCCCCAGACG
E.coli    (298)  ACTTCCGAATaCATCATAAACGGAGGAATTACCGGGTTTTCCCCCAGACG

RACTK1    (348)  CGCCATTACCGCATTATTGATATTGCCCACGCCACTTTGCAGCGGCAGCA
N-RACTK1  (348)  CGCCATTACCGCATTATTGATATTGCCCACGCCACTTTGCAGCGGCAGCA
E.coli    (348)  CGCCATTACCGCATTATTGATATTGCCCACGCCACTTTGCAGCGGCAGaA

RACTK1    (398)  GTGATTCAGGCGGAATACGCCCATGCGCCATTTCCCCACGCCACTTGGCA
N-RACTK1  (393)  GTG--------------------------------------------------
E.coli    (398)  --------------------------------------------------

RACTK1    (448)  GCGGCCAGCAGTGATTCAGGCGGAATACGCCCATGCGCCATTTCCTGCGA
N-RACTK1  (393)  -------------ATTCAGGCGGAATACGCCCATGCGCCATTTCCTGCGA
E.coli    (398)  -------------ATTCcGGCGGAATACGCCCATGCGCCATTTCCTGCaA

RACTK1    (498)  TAAGAACGTGACCACGTTATCGGCAATCTGCTGGCACATGGGATTTTTCT
N-RACTK1  (438)  TAAGAACGTGACCACGTTATCGGCAATCTGCTGGCACATGGGATTTTgCT
E.coli    (435)  TAAGAACGTGACCACGTTATCGGCAATCTGCTGGCACATGGGATTTTgCT

RACTK1    (548)  TATCCAGCATATTACCGGCGTCGGGTAAGTTGGTTTCCACGACGGCGACA
N-RACTK1  (488)  TATCCAGCATATTACCGGCGTCGGGTAAGTTGGTTTCCACGACGGCGACA
E.coli    (485)  TATCCAGCATATTACCGGCGTCGGGcAAGTTGGTTTCCACGACGGCGACA

RACTK1    (598)  ATCTTTTTCGGATCCGATTTGAACATAGCGGGTACCGACGCGATCCATTG
N-RACTK1  (538)  ATCTTTTTCGGAT-CGATTTGcACATAGCGGGTACCGACGCGATCCATTG
E.coli    (535)  ATCTTTTTCGGAT-CGATTTGcACATAGCGGGTACCGACGCGATCCATTG

RACTK1    (648)  CATGGAAGATCGACACGCTATTGCGCCGCGGTGGCGCGCCAGGAATCACA
N-RACTK1  (587)  CATGGAAGATCGACACGCTATTGCGCCGCGGTGGCGCGCCAGGAATCACA
E.Coli    (584)  CATGGAAGATCGACACGCTATTGCGCCGCGGTGGCGCGCCAGGAATCACA

RACTK1    (698)  ATATCCGCCAGTTCTGCAACGCGCGGATCGTGATAGTGATTGAGT-CAAT
N-RACTK1  (637)  ATATCCGCCAGTTCTGCAACGCGCGGATCGTGATAGTGATTGAGTtCAAT
E.COLI    (634)  ATATCCGCCAGTTCTGCAACGCGCGGATCGTGATAGTGATTGAGTtCAAT

RACTK1    (747)  GATCACTTTCTTCGCCCGCAGTAGCCAGGTCGGCGCATTACCGATCCCGC
N-RACTK1  (687)  GATCACTTTCTTCGCCCGCAGTAGCCAGGTCGGCGCATTACCGATCCCGC
E.COLI    (684)  GATCACTTTCTTCGCCCGCAGcAGCCAGGTCGGCGCATTACCGATCCCGC

RACTK1    (797)  TGGTTAACCAGACTCGACCATCCGGTGCCAGTGCCGATGCTTCAATGACG
N-RACTK1  (737)  TGGTTAACCAGACTCGACCATCCGGTGCCAGTGCCGATGCTTCAATGACG
E.COLI    (734)  TGGTTAACCAGACTCGACCATCCGGTGCCAGTGCCGATGCTTCAATGACG

RACTK1    (847)  A-AACATCGATGTCGCCGAAGAAACCGTAATTGACCATTTGCGCCACTTC
N-RACTK1  (787)  gcAACATCGATGTCGCCGAAGAAACCGTAATTGACCATTTGCGCCACTTC
E.COLI    (784)  gcAACATCaATGTCGCCGAAGAAACCGTAATTGACCATTTGCGCCACTTC

RACTK1    (896)  GCTCAAATGCAGGTCAACGAAACTCACCGCGCCCTGATTGATCTTTTTAC
N-RACTK1  (837)  GCTCAAATGCAGGTCAACGAAACTCACCGCGCCCTGATTGATCTTTTTAC
E.COLI    (834)  GCTCAAATGCAGGTCAACGAAACTCACCGCGCCCTGATTGATCTTTTTAC

RACTK1    (946)  GTAAACCGGACGATGTTTGATATGGCGCACGCCAGGAAACAGCATCGGCG
N-RACTK1  (887)  GTAAACCGGACGATGTTTGATATGGCGCACGCCAGGAAACAGCATCGGCG
E.COLI    (884)  GTAAACCGGACGATGTTTGATATGGCGCACGCCAGGAAACAGCATCGGCG

RACTK1    (996)  TCAGAAAGTACATCGTCAGCGGCAGCAGTGATTCAGGCA
N-RACTK1  (937)  TCAGAAAGTACATCGTCAGCGGCAGCAGTGATTCAGGCA
E.COLI    (934)  TCAGAAAGTACATCGTCAGCGGCgGCgcTGATTgAcGCA
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FIG. 2. Alignment of RACTK1, the sequence obtained from pCR2-RACTK1 (N-RACTK1) and the *E. coli* homologous genomic sequence. The **ATG** at position 25–27 of RACTK1 is the starting site for translation (Met). Bases in italic, at the 3′ and 5′ ends of RACTK1 and N-RACTK1 are likely to be derived from pCR2 sequence. Underlined, at the 5′ and 3′ ends, there are 20 bases that match exactly the inverted sequence of each other, and to a lesser extent also match the *E. coli* sequence (see also Fig. 3).

ase (Promega), 200 nM of each primer (see Table 1), and either 5 μl of the RT product or 100–250 ng of genomic DNA. Samples were heated to 94°C for 4 min then subjected to 30–40 cycles of denaturation (94°C, 1 min), annealing (51–72°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 2% agarose gels and visualised by ethidium bromide staining under ultraviolet light (302 nm).

DNA sequencing. Primers used for sequencing are listed in Table 1. DNA was sequenced using the ABI PRISM automated sequencer (Perkin–Elmer) according to the manufacturer's instructions.

RESULTS

Detection of RACTK1 by PCR. Using the RTK1 primer pair, no RT-PCR product for RACTK1 could be generated from either rat or rabbit kidney RNA under conditions that successfully amplified product for the K^+ channel isoforms ROMK1-3, or the housekeeping genes β-actin and GAPD. Figure 1 shows representative examples for rabbit (rat data not shown). Failure to detect RACTK1 in kidney was not affected by changing the Mg^{2+} concentration (1–5 mM), annealing temperature (51–59°C) or cycle number (30–40) of the PCR reaction, or by the use of a gene-specific primer (RTK1 3′) to reverse transcribe kidney RNA prior to PCR (data not shown).

Further PCR was performed on genomic DNA from a number of sources using RTK1, RTK2 and RTK3 primer sets. No products of the predicted size for full length RACTK1 were observed using rabbit (Fig. 1), mouse, hamster, or human DNA (not shown), despite using a range of Mg^{2+} concentrations (1–5 mM) and temperature profiles (52–72°C, and both standard and touch-down PCR). Under these conditions, Products were consistently obtained for K^+ channel gene ROMK (Fig. 1).

To confirm the efficacy of the RTK primers, PCR was performed using the rabbit clone pCR2-RACTK1 (provided by Dr. M. Suzuki). Surprisingly, the product amplified from this vector was smaller than that predicted from the published rabbit sequence (747 bp) but matched exactly that generated from genomic DNA isolated from three different *E. coli* strains: DH5α, BL21 and MC1061 (687 bp: Fig. 1).

Sequencing of pCR2-RACTK1. Following the observation that pCR2-RACTK1 contained an insert smaller than that published for rabbit RACTK1, we sequenced this vector. The resulting sequence is displayed in Fig. 2 along with the published sequence for rabbit RACTK1 (5) and the *E. coli* sequence described by Shmukler *et al.* (9). It is apparent that pCR2-RACTK1 does not contain the 60 bp direct-repeat element contained within the rabbit RACTK1 sequence, thus explaining the smaller product amplified following PCR. In addition, there are three base deletions, five insertions and six substitutions that result in the generation of several stop codons along the sequence length,

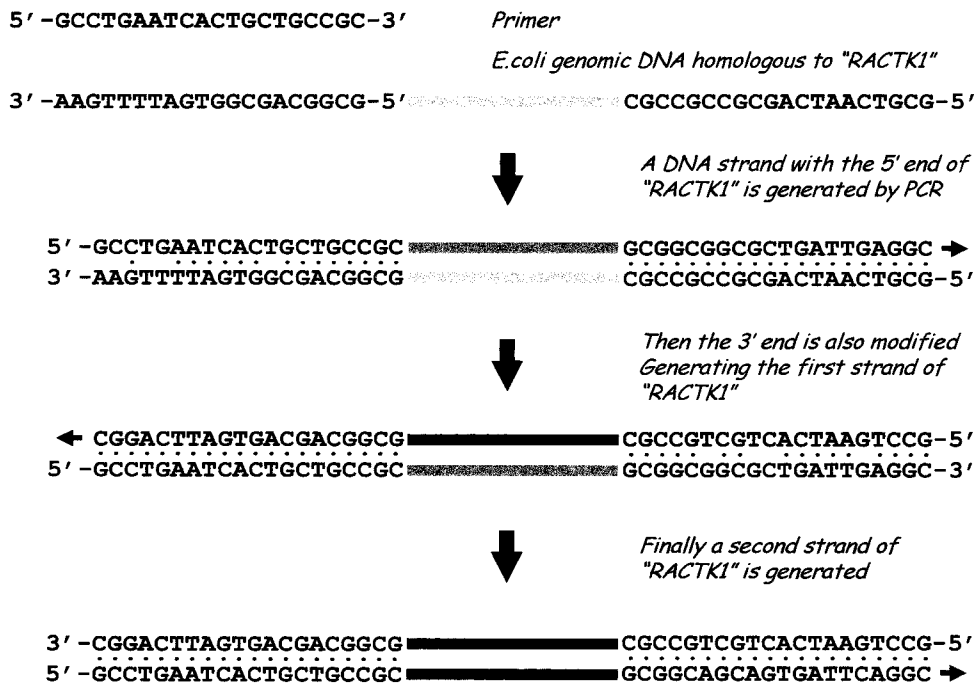

FIG. 3. A hypothesis on the generation of RACTK1 from *E. coli* genomic DNA by PCR using a single primer.

which are absent in rabbit RACTK1. In short, the pCR-2RACTK1 sequence is very similar to that of the *E. coli* sequence described by Shmukler *et al.*, suggesting that the published rabbit RACTK1 sequence (5) may have been the result of incorrect sequence analysis.

DISCUSSION

The main finding of our study is that it is not possible to detect RACTK1 in either rat or rabbit kidney or in genomic DNA of several mammalian species. The failure to detect either kidney mRNA encoding RACTK1 by RT-PCR or genomic DNA by PCR of several species is supported by the observations of Shmukler *et al.* (9) and suggests that RACTK1 is not present within the mammalian genome. Our sequence data from the clone pCR2-RACTK1 suggests that the published sequence for RACTK1 (5) arose as the result of errors in sequence analysis following the cloning of a bacterial contaminant. Although the sequences for pCR2-RACTK1 and the *E. coli* sequence described by Shmukler *et al.* (5) are not 100% identical (there are 8 mismatches and an additional codon at position 398: Fig. 2), they are similar enough for the differences to reflect the normal sequence variation that would be expected to occur between different strains of *E. coli.*

A closer examination of the data that describes the expression cloning of rabbit RACTK1 (5) reveals inconsistencies that are difficult to interpret. Firstly, the arginine repeats (RXXRXX) motif targeted with the probe used to clone RACTK1 is extremely generic since arginine is encoded by 6 different codons. Even more striking is the fact that neither the arginine repeats, or any sequence with homology to the probe described is present in the RACTK1 sequence, either in the sense or antisense strands (5). Interestingly, we have identified a 20 bp region at the 5′ end of the published rabbit RACTK1 (and the pCR2-RACTK1) sequence sense strand that is 100% identical to a 20 bp region at the 5′ end of the complementary strand (underlined regions in Fig. 2 and shown in Fig. 3). This exact match between 5′ ends is not observed in the *E. coli* sequence, suggesting that they have become identical in the "rabbit" sequence following PCR amplification of bacterial DNA with a single primer that has sufficient homology to the 5′ ends of both strands (Fig. 3). Again, this suggests that bacterial DNA is the source of the original "rabbit" RACTK1 clone.

In conclusion, our present study points to the isolation of *RACTK* being an experimental artefact caused by bacterial contamination. However, the origin of both the reported RACTK1 K^+ channel activity under a variety of circumstances (5, 6, 8, 11) as well as the reported expression of RACTK1 polypeptide in a variety of mammalian tissues by immunocytochemistry (10) has yet to be explained.

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