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Long RT-PCR Amplification of the Entire Coding Sequence of the Polycystic Kidney Disease 1 (*PKD1*) Gene

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ABSTRACT

Characterization of mutations of the PKD1 gene has been limited by the fact that three-fourths of this gene at its 5' end is homologous to sequences of at least three other genes on the same chromosome. We have therefore developed a method of long reverse transcription PCR for selective amplification of the entire coding sequence of the PKD1 gene from its mRNA. A PCR primer specific to the sequence in the 3' unique region of the PKD1 gene was synthesized for use coupled with a primer binding to sequence in the homologous region at a distance of about 13.6 kb apart. The commercial availability of RNase H-free reverse transcriptase for long cDNA synthesis and of an enzyme mixture containing Taq and Pfu DNA polymerases for long-range PCR have made this development possible. The long PCR product was proven to be derived from PKD1-mRNA. The results clearly indicated that the long PCR product contained the coding sequence derived from PKD1-mRNA. To our knowledge, this is the first report of a procedure that can reproducibly isolate full-length PKD1 coding sequence from its mRNA transcript, which will prove useful for screening and characterization of mutations in the PKD1 gene.

INTRODUCTION

Characterization of mutations of a gene responsible for a human genetic disease requires the availability of its entire coding and genomic sequences. The polycystic kidney disease 1 (*PKD1*) gene, which has the size of approximately 52 kb containing 46 exons and encoding a 14-kb transcript (5,11,32) and whose defects account for about 85% of autosomal dominant polycystic kidney disease (ADPKD) cases (23), is one of the genes that are most difficult to isolate and identify (5,11,31,32). This is because of the presence of at least three highly homologous sequences of genes mapped more proximally on the same chromosome at the 16p13.1 region (31). Three-fourths of the *PKD1* gene, located in the 5' part extending to a length of approximately 40 kb of DNA, is duplicated. The three homologous genes (*HG*) encoding transcripts with sizes of 21 (*HG-A*), 17 (*HG-B*) and 8.5 kb (*HG-C*) share about 97% identity with the reiterated part of the *PKD1* gene. The only region of the *PKD1* gene that is different from its homologs is a 10-kb sequence at its 3' end, encoding a part of the transcript (3.5 kb) covering exons 33–46; whereas, all of the homologs have similar 3' regions (31). This has made *PKD1* gene isolation, identification and mutation analysis very difficult. Most of the mutations observed so far in the *PKD1* gene are clustered around the unique 3' region of the gene, which is the only easily accessible region (3,17,19,21,22,27,31,33–35). Therefore, the detection rate of muta-

tion is only 10%–15% of the cases analyzed, and all of them are different, indicating that there is no mutational “hot-spot” area. It is believed that most of *PKD1* gene mutations occur in the duplicated region (22).

The mRNA transcripts of several genes are present from their illegitimate or ectopic transcriptions in human peripheral blood lymphocytes (6), and these mRNAs are correctly processed; although, their levels are extremely low (28). The *PKD1*-mRNA has also been found to be present in human lymphocytes (17,25–27,33,34). Many research groups have attempted to isolate the coding sequence of the *PKD1* gene from its mRNA transcript by reverse transcription polymerase chain reaction (RT-PCR) (17,19–22,25–27,31,33,34), but only partial coding sequences were obtained with a maximum size of 2440 bp. Until now, to our knowledge, there is no report on a method for isolation of the entire coding sequence of the *PKD1* gene from its mRNA transcript. Limitations of the previous RT-PCR techniques have resulted from both the inability of reverse transcriptase to synthesize full-length cDNAs and from the failure of *Taq* DNA polymerase to efficiently amplify more than 4 kb of DNA fragment. Recently, the commercial availability of genetically engineered reverse transcriptases that lack RNase H activity allows the synthesis of full-length cDNA from a long mRNA (16). Furthermore, modified PCR conditions using the combination of *Taq* and *Pfu* DNA polymerases that contain proofreading activity have made it possible to amplify long DNA targets (1,7).

Table 1. Nucleotide Sequences of PCR Primers for the Long RT-PCR and Nested PCR of *PKD1* Gene

Primer	Primer Sequences (5'→3') (nucleotide position ^a)	PCR Product Size (bp)	Annealing Temperature (°C)
TH1F	CTG GGG ACG GCG GGG CCA TGC G (nt 175–196)	13 634	68
TH1B	GGC CTG GGG CAA GGG AGG ATG ACA A (nt 13 808–13 784)		
SI2F	AGG AGC CTA GAC GTG TGG ATC G (nt 1595–1616)	1678	65
SI2B	CCT GCA TCC TGT TCA TCC GCT C (nt 3272–3251)		
SI4F	ATC TCT GCT GCC AAT GAC TCA G (nt 4562–4583)	1473	60
SI4B	GGG GAA GCT GTG GGA GAA AC (nt 6034–6015)		
SI9F	CTT CAG CAC CAG CGA TTA CGA CGT T (nt 11 533–11 557)	1650	65
SI9B	AGA AAG TAA TAC TGA GCG GTG TCC ACT C (nt 13 182–13 155)		

^aThe nucleotide positions are according to HUMPKD1A, GenBank Accession No. L33243 by Hughes et al. (11).

In this paper, we report a long RT-PCR method for amplification and isolation of the entire coding sequence of the *PKD1* gene from its mRNA transcript. Advantage was taken of the unique sequence at the 3' region of the *PKD1* gene (and hence in its mRNA transcript) to design a PCR primer for selective amplification by PCR. This long PCR product with the length of 13 634 bp was analyzed and shown to contain the full-length coding sequence of the *PKD1* gene, which could be used for screening, characterization of mutations and other types of studies.

MATERIALS AND METHODS

Peripheral Blood Samples and Lymphocyte Preparation

Blood samples were taken from 21 normal individuals and 15 ADPKD patients with prior informed consent. Approximately 15 mL of peripheral blood were collected into a tube containing

EDTA-disodium salt as anticoagulant. Lymphocytes were prepared from peripheral blood samples by Ficoll Hypaque[®] gradient centrifugation using Lymphoprep[™] (Nycomed Pharma AS, Oslo, Norway).

RNA Isolation

Total RNA was isolated from peripheral blood lymphocytes by using TRIzol[®] reagent (Life Technologies, Gaithersburg, MD, USA), following the manufacturer's instruction, with careful and gentle handling to preserve the integrity of long mRNA required. RNA pellet obtained in the final step was dissolved in 50 µL of sterile diethyl pyrocarbonate (DEPC)-treated water. RNA solution was diluted, and its concentration was determined from optical density (OD)₂₆₀ readings that were measured by a UV spectrophotometer. RNA was kept in DEPC-treated water at -70°C. For long-term storage, RNA was precipitated and kept in 75% ethanol at -70°C.

PCR Primers

Sequence of *PKD1*-mRNA (HUMPKD1A, Accession No. L33243) was retrieved from the Entrez database (GenBank[®]). PCR primers were designed by using the MacVector[™] 4.5.1 Program (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA) and OLIGO[®] Version 4.03 Primer Analysis Software (National Biosciences, Plymouth, MN, USA). The primers used for long RT-PCR (TH1F/TH1B), flanking the open reading frame (ORF) of *PKD1* (12 906 bp), were designed and selected with the additional criterion that their melting temperatures (T_m s) were high enough to be able to perform PCR with two-step cycling conditions that combine annealing and extension steps at 68°C. The backward (TH1B) primer is specific to the 3' unique sequence in the *PKD1* transcript; whereas, the forward (TH1F) primer is specific to the sequence in the reiterated region (Figure 1). Three pairs of primers for nested PCRs (SI2F/SI2B, SI4F/SI4B and SI9F/SI9B) were designed to amplify two regions in the homologous and one region in the unique sequences (Figure 1). These primers were custom-synthesized by BioService Unit, National Center for Biotechnology and Genetic Engineering (BIOTEC), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. Table 1 shows the sequences of PCR primers.

Synthesis of Full-Length cDNA

Full-length cDNA was synthesized by the reaction of RNase H-free reverse transcriptase and by using oligo(dT)_{12–18} primer. An aliquot of total RNA (1–5 µg) was used for cDNA synthesis. The reaction mixture in a total volume of 21 µL contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 500 µM dNTP mixture, 10 mM dithiothreitol (DTT), 0.5 µg of oligo(dT)_{12–18} and 200 U of RNase H-free SUPERSCRIPT[™] II RT (Life Technologies). RNasin[®] Ribonuclease Inhibitor (Promega, Madison, WI, USA) (30 U) was also added to protect mRNA template. The cDNA synthesis was performed at 42°C for 75 min and terminated by incubation at 70°C for 15 min.

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Then, 2 U of RNase H (Life Technologies) were added, and the reaction mixture was incubated at 37°C for 20 min.

Long PCR

The full-length *PKD1*-cDNA sequence was amplified by long PCR using a pair of primers flanking the *PKD1*-ORF (TH1F/TH1B). The cDNA was amplified by using *ELONGASE*TM Enzyme Mix (Life Technologies) following the manufacturer's instructions. The enzyme mixture included thermostable *Taq* and *Pfu* DNA polymerases; the latter has proofreading activity, which reduces error rate of DNA synthesis. The reaction mixture (50 μ L) contained 2 μ L of cDNA, 1 U of *ELONGASE* Enzyme Mix, 60 mM Tris-SO₄ (pH 9.1), 18 mM (NH₄)₂SO₄, 1.2 mM MgSO₄, 200 μ M dNTP mixture, 10% dimethyl sulfoxide (DMSO) and 200 nM of each primer.

A hot start at 95°C for 1 min and 30 s was performed before adding the enzyme mixture. PCR was carried out for 30 cycles in a GeneAmp[®] PCR System 2400 (PE Applied Biosystems, Foster City, CA, USA). Each cycle consisted of denaturation at 94°C for 15 s and a combined step of annealing and extension at 68°C for 15 min. A final extension step was carried out at 70°C for 10 min. The long RT-PCR product was analyzed by electrophoresis on 0.8% agarose gel in 0.5 $\times$ TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.3) and visualized after staining by ethidium bromide.

Restriction Endonuclease Analysis of Long PCR Product

Restriction endonuclease sites in the *PKD1*-mRNA sequence were first analyzed by the MacVectorTM 4.5.1 computer program. The restriction endonuclease digestions were performed by taking 20- μ L aliquots of the long RT-PCR product and adding the following: (i) 20 U of either *Bam*HI, *Kpn*I or *Xho*I enzymes (Promega), (ii) a suitable buffer as supplied by the manufacturer and (iii) 0.1 μ g/mL of bovine serum albumin (BSA) in a final volume of 30 μ L. The reaction mixtures were incubated at 37°C for 2 h. The restriction

fragments were then resolved by electrophoresis on 1% agarose gel followed by ethidium bromide staining.

Nested PCR

To reduce nonspecific amplifications caused by excessive templates, the long PCR product was diluted 100–1000-fold before amplification with each pair of primers in nested PCR. The nested PCR mixture (25 μ L) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs, 10% DMSO, 400 nM of each primer and 0.625 U of AmpliTaq GoldTM (PE Applied Biosystems). After an initial denaturation at 95°C for 10 min, 25 PCR cycles were performed as follows: (i) denaturation at 95°C for 20 s, (ii) annealing at 60°C for 20 s and (iii) extension at 72°C for 1 min and 30 s, followed by another 10 min of extension at 72°C in the final step. The sizes

of nested PCR products were analyzed by electrophoresis on a 1.2% agarose gel in TBE buffer with ethidium bromide staining.

Direct Sequencing of Nested PCR Products

The nested PCR products were purified from gel by using the QIAquickTM Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. Their sequences were determined by the manual direct-sequencing method using the AmpliCycleTM Sequencing Kit (PE Applied Biosystems).

RESULTS

We have developed a long RT-PCR method to isolate the entire coding region of the *PKD1* gene from its mRNA

Figure 1. The schematic illustration of *PKD1* mRNA, PCR and nested PCR products, and restriction fragments. The entire mRNA containing sequences of exons 1–46 is represented by horizontal bar. The boundaries of exons in the mRNA are indicated by vertical lines, and exon numbers are given for some exons. The ORF consisting of 12 906 bp is located between the nt positions 212 and 13 117 (HUMPKD1A; GenBank Accession No. L33243; Reference 11). The light shade area (between exons 1 and 32) represents the reiterated region, which is highly homologous to sequences in at least three other genes on the same chromosome. The darker shade area (between exons 33 and 46) represents the 3' unique region. Sites of restriction endonucleases *Bam*HI, *Kpn*I and *Xho*I, used to digest *PKD1* cDNA, are indicated by vertical arrows above the bar of mRNA, and the lower part of the figure shows sizes of fragments after digestions with the restriction enzymes. The extents of long PCR products are represented by solid lines under the bar of mRNA and of nested PCR products by solid lines under the long PCR product. Locations and directions of oligonucleotide primers for long PCR and nested PCR are indicated as solid horizontal arrows.

transcript that was prepared from peripheral blood lymphocytes. The first-strand cDNA synthesis was primed by oligo(dT)₁₂₋₁₈. The cDNA was subjected to amplification by using the forward (TH1F) primer specific to the reiterated region and the backward (TH1B) primer specific to the unique sequence in the coding region of *PKDI* transcript. The expected PCR product amplified by this pair of primers would be 13 634 bp in length (Figure 1). The

conditions for long RT-PCR were optimized by varying amounts of RNA, concentrations of MgSO₄ and numbers of PCR cycles. The technique described herein can efficiently amplify the long PCR product by using 1–5 µg of good-quality total RNA. Amounts of RNA greater than 9 µg were found to be less efficient in generating the long PCR product. The optimal conditions for long cDNA synthesis and PCR are described in Materials and Methods.

Figure 2. (A) Agarose gel-electrophoresis of PCR products from amplification of the entire coding sequence of the *PKDI* gene from its mRNA by the long RT-PCR method. Lanes M1 and M2 are 1-kb DNA Extension Ladder and λDNA/*Hind*III markers (Life Technologies), respectively. Lanes 1 and 2 are PCR products from samples of normal individuals. Lanes 3–10 are PCR products from samples of patients with ADPKD. The PCR product with the size of about 13.6 kb was detected in all samples. A smaller product with the size of <3 kb, present in lane 7, was also irregularly observed in other samples from both normal individuals and patients; it is possibly a nonspecific PCR product. (B) Gel electrophoresis of restriction fragments of the long PCR product. Lane M is 1-kb DNA Extension Ladder. Lane 1 is undigested PCR product. Lanes 2–4 are products after digestions with *Bam*HI, *Kpn*I and *Xho*I, respectively. Digestions of the long PCR product with *Bam*HI (lane 2) and *Kpn*I (lane 3) resulted in two restriction fragments with the lengths of 8922 and 4712 bp, and of 9438 and 4196 bp, respectively, and with *Xho*I (lane 4), five restriction fragments with the lengths of 6546, 3583, 1989, 976 and 540 bp. (C) Agarose gel-electrophoresis of nested PCR products from amplifications of the long PCR product by using three sets of *PKDI*-specific primers. Lanes M1 and M2 are 100-bp DNA Ladder and 1-kb DNA Extension Ladder, respectively. Lanes 1–3 are the nested PCR products from amplifications of the long PCR product with three sets of primers, SI2F/SI2B, SI4F/SI4B and SI9F/SI9B, resulting in DNA fragments sizes of 1678, 1473 and 1650 bp, respectively.

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We have successfully tested this procedure with RNA samples that were prepared from peripheral blood lymphocytes of 21 normal individuals and 15 patients with ADPKD. Figure 2A shows part of the data.

To prove that the long PCR product contained *PKDI* coding sequence, we initially analyzed restriction endonuclease sites known to be present in the sequence of *PKDI* mRNA in the long PCR product. The three restriction enzymes selected for this analysis were *Bam*HI, *Kpn*I and *Xho*I, which have 1, 1 and 4 sites, respectively, in the *PKDI* coding sequence (Figure 1). The predicted sizes of fragments after digestions of the *PKDI* coding sequence with the restriction enzymes were 8922 and 4712 bp for *Bam*HI, 9438 and 4196 bp for *Kpn*I and 3583, 976, 540, 6546 and 1989 bp for *Xho*I (see Figure 1). When the long PCR product that was prepared from a normal RNA sample was digested with the three selected enzymes, it was found that the digested DNA fragments had predicted lengths (Figure 2B).

To further prove the authenticity of the long-PCR product, DNA fragments were amplified by nested PCR using 3 pairs of primers (SI2F/SI2B, SI4F/SI4B and SI9F/SI9B) that were specific to sequences in the reiterated and unique regions of the *PKDI*-mRNA transcript (Figure 1) to observe whether the PCR products with expected sizes would be produced or not. The expected lengths of the nested PCR products amplified by these three pairs of primers were 1678, 1473 and 1650 bp, respectively (Figure 1). In this experiment, when the long PCR product was amplified without dilution, both larger PCR products on a smear of background and also product with the expected length were obtained. The larger PCR products and smear seemed to result from excessive DNA templates. Amplifications of the homologous sequences would produce single-stranded products and their amounts should be minute since the backward primer in the primary PCR (TH1B) was specific only to the 3' unique sequence of the *PKDI* gene. Serial dilution of the long PCR product from 10–10000-fold was performed to reduce the template before using it in the nested PCRs. We found that 100–1000-fold dilutions of

the primary PCR product generated the best results. Less dilution still produced nonspecific products, and more dilution resulted in less or no specific products. The results showed that the three nested PCR products had the lengths as expected (Figure 2C).

Finally, to verify that the long PCR product was derived from *PKDI* mRNA, we performed direct sequencing of the three nested PCR products amplified by the three pairs of primers (SI2F/SI2B, SI4F/SI4B and SI9F/SI9B). The nucleotide sequences thus determined were identical to the corresponding regions in the *PKDI* transcript. The nucleotide sequences obtained were located between the positions 1633–1841 (209 nucleotides [nt]), 4610–4709 (100 nt) and 11575–11699 (125 nt) in the *PKDI* mRNA (with reference to the sequence of HUMPKD1A; GenBank Accession No. L33243; Reference 11) (data not shown).

DISCUSSION

The main problem that has delayed progress in studies of the *PKDI* gene is the presence of homologous sequences in at least three potentially active genes on the same chromosome, which interfere with isolation of both the gene and its transcript. We first attempted to isolate *PKDI*-mRNA, which was ectopically transcribed in peripheral blood lymphocytes, by capture with a biotinylated specific-oligonucleotide probe and streptavidin-coated magnetic beads. However, this was unsuccessful, probably because of a minute amount of the *PKDI* transcript present in the peripheral blood lymphocytes. We have therefore developed a long RT-PCR method to selectively amplify the entire coding sequence of the *PKDI* gene from its transcript, by using one PCR primer specific to the sequence in the 3' unique region of the *PKDI* gene together with the primer specific to the sequence located within the reiterated area at a distance of 13634 bp. The long RT-PCR method could successfully amplify the entire coding sequence of *PKDI* gene from its mRNA. The long RT-PCR product was proven to be derived from *PKDI*-mRNA by demonstrating the presence of (i) identical re-

striction endonuclease maps, (ii) correct sizes of the nested PCR products and (iii) identical nucleotide sequences in three separated regions.

Although nucleotide sequences of the three homologous genes (*HG-A*, *HG-B* and *HG-C*) have not been available for comparison with the sequences obtained from sequencing experiments, the presence of 100% identity between sequences analyzed in the three regions (altogether 434 nt) with those of the corresponding regions in the reference *PKDI* mRNA confirms that the sequence of amplified product is the bona fide *PKDI* sequence. The most compelling evidence is the absolute identity of the 125-nt sequence of the nested PCR product from the unique region, amplified by SI9F/SI9B primers, to the corresponding region in the reference *PKDI* mRNA.

Many research groups have previously attempted to isolate the coding sequence of the *PKDI* gene from its mRNA for characterization of mutations. However, this was mainly successful in isolation of its unique sequence in the 3' region (17,19,21,22, 27,31,33–35). Three groups could carry out amplifications of the unique sequence in the 3' region (between exons 33–46) and part of the reiterated region (located before exons 32) of the *PKDI* gene. Roelfsema et al. (26) isolated the *PKDI* coding sequence from its mRNA prepared from peripheral blood lymphocytes by cDNA synthesis and amplifications of eleven overlapping fragments (each fragment of less than 1 kb) covering 6336 bp (between exons 16 and 46), and seven out of eight novel mutations were identified in the repeated part of the *PKDI* gene. Peral et al. (20) developed an anchored RT-PCR to amplify the *PKDI* coding sequence from its mRNA isolated from lymphoblastoid cell lines, using one primer situated within the single-copy region and one within the reiterated area to identify mutations in the duplicated region. The size of longest PCR product that could be isolated was 2440 bp, extending from exons 22–34 in the *PKDI* transcript, and six out of eleven mutations were identified within the duplicated region. A long-range PCR using a primer specific to the unique region was developed by Watnick et al. (36) to

amplify the *PKDI* gene spanning 10 kb (between exons 23–34) from genomic DNA. Although the methods of these workers could amplify parts of the sequence in the duplicated region, the entire coding sequence of the *PKDI* gene was not obtained.

To our knowledge, the long RT-PCR method developed in this report is the only one that could successfully and reproducibly generate the entire coding sequence of the *PKDI* gene as a single PCR product from the mRNA that was prepared from peripheral blood lymphocytes. This method is rapid—the whole process can be finished within 15 h.

The full-length *PKDI* coding sequence generated by long RT-PCR can be used for cloning or mutation analysis. Some mis-incorporations or errors might be introduced during generation of the full-length *PKDI* cDNA by the long RT-PCR method. However, the use of a mixture of *Taq* and *Pfu* DNA polymerases would alleviate this problem since *Pfu* polymerase contains proofreading activity (1). The clone containing full-length *PKDI* cDNA generated by the long RT-PCR method should be thoroughly evaluated by complete sequence analysis before using it in other work or further study. In cloning complete cDNAs generated from genomes of three RNA viruses including tick-borne encephalitis (10), hepatitis A (30) and hepatitis C (24) by the long RT-PCR method similar to the one described in this work, it was found that the cDNA clones had characteristics of the original genomes, confirming the high fidelity of cDNA generation by the long RT-PCR method.

The long RT-PCR method for analyzing *PKDI* mutations can be of limited usefulness in some cases in which mutations diminish the quantity of *PKDI* mRNA (14); although, the normal and stable expression of mutant alleles at the mRNA level has been observed in peripheral blood lymphocytes (17,25,27,33), cell lines and tissues (22,31). Analysis of mRNA for *PKDI* mutations can have an advantage over analysis from the *PKDI* gene, because mutations that cause abnormal RNA processing (such as exon skipping, RNA deletion or insertion) could also be detected (20,31). However, one should be aware of aberrantly spliced

transcripts that can be unrelated to hereditary defects and can complicate detection and analysis of truly abnormal transcripts (2,13). Therefore, the mutations causing abnormal RNA processing must be demonstrated.

The entire coding sequence of the *PKDI* gene that was amplified and isolated by the long RT-PCR method can be used for screening mutations by the following methods: (i) single-strand conformation polymorphisms (SSCP) (18), (ii) heteroduplex analysis (HA) (12), (iii) denaturing gradient gel electrophoresis (DGGE) (9), (iv) ribonuclease (RNase) cleavage (15), (v) chemical cleavage of mismatch (CCM) (8), (vi) dideoxy fingerprinting (ddF) (29) and (vii) cleavage fragment-length polymorphism (CFLP) (4). The study of mutations of the *PKDI* gene will provide insight into the functions of polycystin and its peptide domains, the molecular pathogenesis of ADPKD and the effect(s) of the mutations on clinical phenotypes of the patients.

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