Am. J. Hum. Genet. 60:1399–1410, 1997

Identification of Mutations in the Duplicated Region of the Polycystic Kidney Disease 1 Gene (*PKD1*) by a Novel Approach

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Summary

Mutation screening of the major autosomal dominant polycystic kidney disease gene (*PKD1*) has been complicated by the large transcript size (>14 kb) and by reiteration of the genomic area encoding 75% of the protein on the same chromosome (the *HG* loci). The sequence similarity between the *PKD1* and *HG* regions has precluded specific analysis of the duplicated region of *PKD1,* and consequently all previously described mutations map to the unique 3′ region of *PKD1.* We have now developed a novel anchored reverse-transcription–PCR (RT-PCR) approach to specifically amplify duplicated regions of *PKD1,* employing one primer situated within the single-copy region and one within the reiterated area. This strategy has been incorporated in a mutation screen of 100 patients for more than half of the *PKD1* exons (exons 22–46; 37% of the coding region), including 11 (exons 22–32) within the duplicated gene region, by use of the protein-truncation test (PTT). Sixty of these patients also were screened for missense changes, by use of the nonisotopic RNase cleavage assay (NIRCA), in exons 23–36. Eleven mutations have been identified, six within the duplicated region, and these consist of three stop mutations, three frameshifting deletions of a single nucleotide, two splicing defects, and three possible missense changes. Each mutation was detected in just one family (although one has been described elsewhere); no mutation hot spot was identified. The nature and distribution of mutations, plus the lack of a clear phenotype/genotype correlation, suggest that they may inactivate the molecule. RT-PCR/PTT proved to be a rapid and efficient method to detect *PKD1* mutations (differentiating pathogenic changes from polymorphisms), and we recommend this procedure as a first-pass mutation screen in this disorder.

Received December 20, 1996; accepted for publication March 13, 1997.

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0002-9297/97/6006-0018$02.00

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder (incidence 1/1,000) and a frequent cause of end-stage renal disease (ESRD). ADPKD is characterized by the progressive development and enlargement of renal cysts, typically leading to ESRD by late middle age (Gabow et al. 1992). A variety of extrarenal manifestations also are associated with ADPKD, including hepatic cysts, cerebral aneurysms, and cardiac valve abnormalities, showing that this is a systemic disorder (Gabow 1990).

ADPKD is genetically heterogeneous, although the majority (~85%) of cases are due to mutation of the polycystic kidney disease 1 gene (*PKD1*) (Peters and Sandkuijl 1992). Most of the remainder are due to mutation of the *PKD2* gene, although a small number of families with disease unlinked to either of these loci have been described (Bogdanova et al. 1995; Daoust et al. 1995; de Almeida et al. 1995). Comparisons of PKD1 and PKD2 families have shown that PKD1 is typically a more severe disorder, with an average age at ESRD of ~56 years, compared with ~71.5 years for PKD2 (Ravine et al. 1992).

The *PKD1* gene has been identified (European Polycystic Kidney Disease Consortium 1994), and the genomic region (Burn et al. 1995) and cDNA have been sequenced fully (Hughes et al. 1995; International Polycystic Kidney Disease Consortium 1995). The gene covers ~52 kb of genomic DNA in 16p13.3 and is divided into 46 exons. The transcript is 14,136 bp, and the predicted protein, polycystin, consists of 4,302 amino acids. Polycystin is thought to be an integral membrane protein with a large extracellular region and multiple transmembrane domains (Hughes et al. 1995).

The *PKD2* gene, situated in 4q21-23, recently has been identified and characterized (Mochizuki et al. 1996). The transcript is ~5.4 kb, and the 968-amino-acid product is predicted to be an integral membrane protein with similarity to the α_1 subunit of voltage-activated Ca^{2+} and Na^{+} channels, suggesting a related role for the *PKD2* protein (Mochizuki et al. 1996). Similarity between parts of the membrane-associated regions of the PKD2 protein and polycystin suggests that they may have a related function or that they could interact.

Mutation detection in *PKD1* has been complicated by

the structure of the genomic region encoding the gene. Most of the *PKD1* gene lies in an area of DNA that is reiterated several times elsewhere on the same chromosome (in 16p13.1). This duplicate area also encodes three genes (the *HG* loci) that share substantial (~97%) homology with the first 32 exons of *PKD1* (European Polycystic Kidney Disease Consortium 1994). The degree of homology between the *PKD1* and *HG* regions means that both generally are visualized simultaneously when analyzed by hybridization, PCR, or reverse-transcription–PCR (RT-PCR) methods. Consequently, at present all of the 16 mutations characterized in *PKD1,* except the translocation that helped in identification of the gene (European Polycystic Kidney Disease Consortium 1994), are clustered in the 3′ 14 exons encoded by single-copy DNA. These mutations include three intragenic deletions, four splicing defects, six nonsense mutations, an insertion of a nucleotide, and a possible missense mutation (European Polycystic Kidney Disease Consortium 1994; Peral et al. 1995, 1996*a,* 1996*b;* Turco et al. 1995; Neophytou et al. 1996; Rossetti et al. 1996). A second type of *PKD1* mutation also has been described: large deletions that disrupt *PKD1* and the adjacent tuberous sclerosis gene, *TSC2* (Brook-Carter et al. 1994). In these contiguous gene–syndrome cases, a specific phenotype of tuberous sclerosis and severe, childhood-onset polycystic kidney disease was observed. These clearly are inactivating mutations of *PKD1* and indicate a central role for *PKD1* in cases of renal cystic disease in tuberous sclerosis.

Previous studies have shown that the proportion of mutations detected in the single-copy region of *PKD1* is consistent with mutations being spread evenly throughout the gene (Peral et al. 1996*b*) and, hence, that the majority are located within the duplicated area. Consequently, we now have extended our mutation screen to include the 25 most 3′ exons of *PKD1,* 11 of which lie in the duplicated area of the gene. We employed a novel approach to specifically amplify the duplicated area of *PKD1*. In this screen of more than half of the *PKD1* exons, 10 novel mutations were characterized, including the first changes detected within the duplicated area.

Subjects and Methods

Clinical Details

Pedigree 187 (P187).—OX40 had a positive family history but was the only living affected member of P187, with ESRD onset at age 53 years. Her affected father, grandfather, cousin, and great uncle had died at age 48, 63, 43, and 60 years, respectively.

Pedigree 193 (P193).—OX1055 (ESRD onset at age 57 years) had a negative family history and two daughters with negative renal ultrasounds at ages 37 and 34 years.

Pedigree 157 (P157).—The proband (OX1190) had a positive family history, with elevated blood pressure and chronic renal disease at age 33 years. His MZ twin daughters were found to have a few cysts in each kidney at age 4 years, but the organs were not enlarged, and the twins remained normotensive.

Pedigree 161 (P161).—OX1097 (ESRD onset at age 47 years) had an affected sister and three children, ages 28, 23 and 22 years, with renal cysts but normal renal function.

Pedigree 130 (P130).—OX973 had a positive family history, was hypertensive, and received a renal transplant at age 52 years. His daughter was found to have bilateral small cysts, on screening at age 7 years.

Pedigree 179 (P179).—OX46 was adopted and died, at age 61 years, from a myocardial infarction, with ESRD onset at age 52 years. He had an affected daughter, age 28 years, who had normal renal function.

Pedigree 11 (P11).—P11 was a large family; ADPKD could be traced through three generations, and the proband (OX61; ESRD onset at age 51 years) had eight children, five of whom were affected. The five affected children (ages 25–45 years) had renal function within the normal range, but three were hypertensive.

Pedigree 229 (P229).—OX1056 (ESRD onset at age 63 years) had a negative family history and adopted children.

Pedigree 109 (P109).—ADPKD could be traced through four generations, with ESRD onset at age 53 years in OX980. Her affected brother and father had died at age 46 years and 42 years, respectively, and only a niece with renal cysts was available for study.

Pedigree 225 (P225).—ADPKD could be traced through three generations, but OX18 (ESRD onset at age 50 years), who is hypertensive, was the only surviving affected relative. His affected mother and grandmother had died at age 42 and 32 years, respectively.

Pedigree 3 (P3).—Three affected individuals were available for study: OX21 (ESRD onset at age 42 years), his sister (ESRD onset at age 42 years), and her daughter, who had normal renal function at age 25 years.

RT-PCR

Total RNA was extracted from lymphoblast cell lines by the method of Chomczynski and Sacchi (1987). Five micrograms of total RNA were employed in the first-strand cDNA synthesis in a total volume of 50 μl, according to the method described elsewhere (European Polycystic Kidney Disease Consortium 1994).

Nonisotopic RNase Cleavage Assay (NIRCA)

NIRCA was adapted from methods described by Myers et al. (1985) and Winter et al. (1985) (Mismatch Detect II™; Ambion). The initial pairs of primers used to amplify cDNA are listed in table 1. PCR amplification was performed either as described by Harris et al. (1991)

Table 1

Primers Used in Initial PCR

Name		Sequence (5′–3′)	Mg^{2+} Concentration (mM)	Annealing Temperature (°C)	Position of Product[a] (nt)	Product Size[a] (bp)	Exons
NIRCA:							
Mut 1	7.8F	TATGCCGCTCGTGCCTGAAGCAGAC	1.5	62	8247–10686	2,440	22–34
	AH3 B2	TCCATGTGGGTGTCTTGGGTAGGG					
Mut 2 and 3	AH3 F2	GCTTTGTGTTTCCTGAGCCGAC	1	60	9399–10686	1,288	26–34
	AH3 B2	TCCATGTGGGTGTCTTGGGTAGGG					
Mut 4	AH3 F9	TTTGACAAGCACATCTGGCTCTC	1	62	9983–11238	1,256	29–38
	AH3 B7	TACACCAGGAGGCTCCGCAG					
PTT:							
1 and 2	7.8F	TATGCCGCTCGTGCCTGAAGCAGAC	1.5	62	8247–10686	2,440	22–34
	AH3 B2	TCCATGTGGGTGTCTTGGGTAGGG					
3	Mut 4F	GGACTCGTCCGTGCTGGAC	1.5	60	10324–12381	2,058	31–45
	3A3 C2	ACGCTCCAGAGGGAGTCCAC					
4	1 Long F	CATGCTTTTTCTGCTGGTGACC	1	65	11239–13280	2,042	38–46
	1 Long R	TTAAAGTGCTGAAGCCCACAGAC					

[a] In cDNA.

or by use of a dimethyl sulfoxide (DMSO)–containing buffer (Dodé et al. 1990) and the conditions described in table 1. When the size of the expected product was >1 kb, the PCR additive *Taq* Extender™ (1 unit/kb amplified fragment; Stratagene) was added. To screen for mutations within the duplicated region, we used the reverse primer AH3B2 (table 1 and fig. 1), which is located in the single-copy area (exon 34), to specifically amplify *PKD1*. The specificity of products was tested with the somatic-cell hybrids P-MWH2A (HG only) and Hy145.19 (PKD1 only; European Polycystic Kidney Disease Consortium 1994).

An aliquot of the initial product was used as a template for a nested reaction employing the primers and conditions listed in table 2. These primers also contain, at their 5′ ends, the sequence of either the T7 phage promoter (forward), 5′-GATAATACGACTCACTATAGGG-3′, or the SP6 phage promoter (reverse), 5′-TCATTTAGGTGACACTATAGGA-3′. The PCR products were transcribed in vitro with the appropriate T7 or SP6 polymerase to produce sense (S) and antisense (AS) RNA probes, respectively. The S product from a PKD1 sample was hybridized to an AS product of the wild-type control, and vice versa, as was a control S with a control AS. All the hybridized samples were treated with as many as three different RNases (I–III), in separate reactions, and the cleaved products were analyzed on ethidium bromide–stained agarose gels (2.5%–3%).

Protein-Truncation Test (PTT)

The initial PCR reaction employed the primers and conditions shown in table 1. An aliquot of that product was used as a template in the second, nested PCR

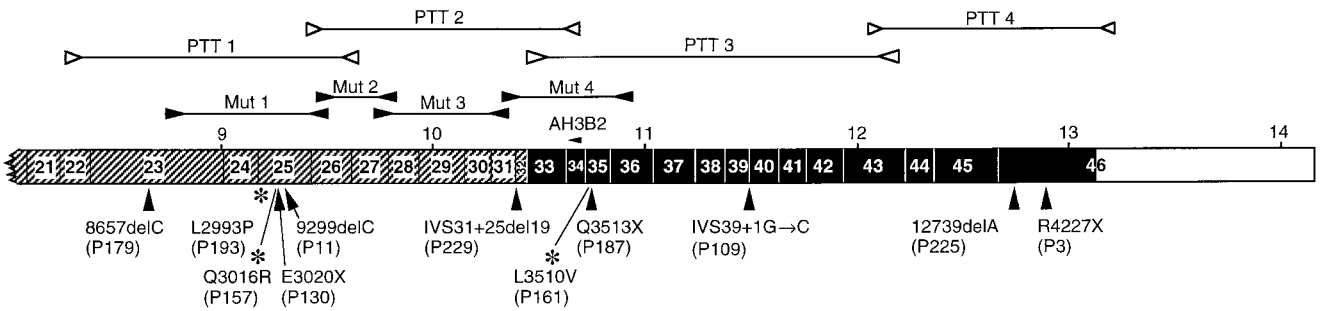

Figure 1 Map of the region of the *PKD1* transcript analyzed (exons 22–46), showing the locations of described mutations. The exons encoded by the duplicated area (*hatched section*), the single-copy region (*blackened section*), and the 3′ UTR (*unblackened/nonhatched section*), are illustrated. Each kilobase of the *PKD1* transcript is numbered above the exons. The regions analyzed with each of the eight sets of primers—four sets for NIRCA (Mut 1–Mut 4) and four sets for PTT (PTT 1–PTT 4)—are shown above the map. The primer AH3B2, used as an anchored primer to obtain *PKD1*-specific products for analysis of the duplicated area, is shown above exon 34. Below the map the designation of each mutation, the pedigree number (P), and the position of the change are shown. Arrows indicate stop or frameshifting mutations, and asterisks denote missense changes.

Table 2

Nested Primers

Name	Sequence (5′–3′)	Mg^{2+} Concentration (mM)	Annealing Temperature (°C)	Position of Product[a] (nt)	Product Size[a] (bp)	Exons	No. of Mutations/ No. of Samples Analyzed
Primers used in NIRCA:							
Mut 1 forward	CCTCGATGGCATTCCAGACAC	1.5	65	8757–9525	769 (813)[b]	23–26	2/60
Mut 1 reverse	CGGCTGGCATCCAACTGG						
Mut 2 forward	CATGTGCTGTGTGCCTGGTG	0.5	64	9450–9826	377 (421)	26–28	0/60
Mut 2 reverse	CAGGTCCCTGACGATGACGTG						
Mut 3 forward	ATCGCCACCCCGCACAG	1	65	9719–10358	640 (684)	27–31	0/60
Mut 3 reverse	ACGTGAGGAAGGAGCTGTCC						
Mut 4 forward	GGACTCGTCCGTGCTGGAC	0.5	65	10324–10941	618 (662)	31–36	2/60
Mut 4 reverse	GCACCCACCCACCCTGAG						
Primers used in PTT:							
PTT 1 forward	TCGTGCCTGAAGCAGACGC	0.9	62	8255–9644	1,390 (1,429)	22–27	3/100
PTT 1 reverse	CCCCATACAGCATGATGCCC						
PTT 2 forward	TTTGTGTTTCCTGAGCCGAC	1	60	9401–10686	1,286 (1,325)	26–34	1/100
PTT 2 reverse	TCCATGTGGGTGTCTTGGGTAGGG						
PTT 3 forward	GAGGGAACGCTCAGTTGG	1	64	10451–12211	1,761 (1,800)	33–43	0/100
PTT 3 reverse	CTTGACCAAAAGCAGGAAGAG						
PTT 4 forward	GCACTGGTACGCCTCGCC	1	65	12053–13222	1,170 (1,209)	43–46	3/100
PTT 4 reverse	CGTGCAGCCATTCTGCCTG						

[a] In cDNA.
[b] Plus extra primer sequence.

reaction, with the primers listed in table 2. The upstream primer also had the T7 promoter sequence and translation-initiation codon, with Kozac consensus, engineered 5′ to the gene-specific primer sequence: 5′-GGATCC-TAATACGACTCACTATAGGAACAGACCACC-ATG-3′ (Roest et al. 1993). A 250–500-ng portion of each PCR product was transcribed and translated in a TnT/T7-coupled reticulocyte lysate system (Promega), according to the protocol recommended by the manufacturers, with the exception that the reactions were carried out in a 12.5-μl final volume. Incorporation of ^{3}H-Leucine (TRK754; Amersham) in the in vitro translation reaction was used to detect the translated products after electrophoretic separation (16–20 h at 10 W) on a 15% or 17% SDS-polyacylamide gel. High-range ^{14}C Rainbow markers (Amersham) were used. The gel was fixed in a 25:65:10 mixture of isopropanol:water:acetic acid for 30 min, soaked in Amplify™ (Amersham) for 30 min, and finally was dried and autoradiographed for 16–48 h at −70°C.

DNA Sequencing

PCR products were sequenced directly by including a single 5′ biotinylated primer as described elsewhere (Peral et al. 1996*b*). For sequencing of mutations located within the duplicated area, either a 5′ biotinylated primer, 5′-GTGGGTGTCTTGGGTAGGG-3′ (10681–10662), from within the single-copy area was employed, for patients OX1056, OX973, and OX61, or an initial PCR was performed, with use of the primers 7.8F/AH3 B2 (table 1), to generate a *PKD1*-specific product, followed by a nested reaction with primers entirely within the duplicated region for OX46, OX1055, and OX1190. The nucleotide numbering and amino acid numbering described by (Hughes et al. 1995) were used throughout the study.

Primer-Template Mismatches to Detect Mutations

Primers to specifically amplify the mutant allele were designed to trace mutations that did not alter a restriction site in pedigrees P225 and P3. As well as a mutation-specific change to the 3′-terminal nucleotide, an additional mismatch was engineered within the last four bases, to ensure allele-specific amplification (Kwok et al. 1990). For the change 12739delA (P225), we used a downstream primer with two mismatches from the normal sequence—18-reverse, 5′-TCGGAGCCAGCG-CTGGCG-3′ (12756–12739)—and the normal upstream primer—18-forward, 5′-TTGCGTGGAGAG-CTGTACCG-3′ (12545–12564). A 212-bp product was obtained only from individuals with the mutation. For the change R4227X (12890C→T) in P3 (the same stop mutation as was seen in P89; Peral et al. 1996*b*), we used the modified upstream primer 21-forward—5′-TGCTCACCCAGTTTGAGT-3′ (12873–12890)—and the downstream primer 21-reverse—5′-CGTGCAGCC-ATTCTGCCTG-3′ (13222–13204)—giving a mutant-specific 350-bp product.

Genomic Analysis in P11

The mutation 9299delC in P11 abolished a *CvJi*I restriction site and was analyzed in genomic DNA from 11 family members, with an anchored PCR reaction. Genomic DNA (100 ng) was amplified initially with the primers 61-forward—5′-AGTCACTCCAGGGTGCTGACC-3′—and AH3B2 (table 1). An aliquot of the 5,696-bp product was used as template with the primers 25-forward—5′-GAGCAGAGACCCAGCGGG-3′—and 25-reverse—5′-AGGTGGCGGGTGAGGCAG-3′. The resulting 195-bp product was digested with *CvJi*I and was analyzed on a 4% agarose gel. Normal individuals had five fragments—61, 42, 36, 34, and 22 bp—but a restriction site was destroyed in mutation carriers, producing a novel fragment of 94 bp (61+34-1 bp).

Analysis of the Intronic Deletion in P229

Somatic-cell hybrids separating both chromosomes 16 from OX1056 were generated as described elsewhere (Deisseroth and Hendrick 1979). To trace the intronic deletion in P229, 50 ng of genomic DNA from three family members and the hybrids (BB4 and BB2-5) was amplified with the primers Mut 4F (table 2) and AH3B2 (table 1). An aliquot of the 753-bp product was amplified with the primers Mut4F and the reverse primer 5′-GGAACCCACCTCTTAGAATCATCC-3′, and the 203-bp product was analyzed in a 3% agarose gel. *PKD1* haplotypes were produced with the microsatellites KG8, SM6, 16AC2.5, CW2, W5.2, SM7, and VK5AC (Harris et al. 1991; Aksentijevich et al. 1993; Peral et al. 1994; Snarey et al. 1994).

Results

To screen for mutations within the duplicated area of *PKD1,* we developed an anchored RT-PCR approach. One unique primer (AH3 B2) situated within the single-copy area (exon 34; fig. 1) was employed to amplify *PKD1*-specific products extending ~2 kb into the duplicated region. The *PKD1* specificity of these products was tested by use of somatic-cell hybrids containing just *PKD1* or just the *HG* loci (for details, see Subjects and Methods). Mutations were screened in the single-copy region and in the specifically amplified duplicated area of *PKD1* by two different methods—NIRCA and PTT. NIRCA detects mismatches between in vitro–transcribed RNA molecules of ≤~1 kb, whereas PTT identifies mutations that alter the size of an in vitro–translated product. These studies identified 11 *PKD1* mutations, which are summarized in figure 1 (details are given in table 3).

Mutations Identified by NIRCA

cDNA from 64 patients (including 24 in whom no mutation had been detected in a previous, SSCP screen of exons 36–46; Peral et al. 1996*b*) was analyzed for mutations in exons 23–36, by NIRCA (for details of primers employed, see tables 1 and 2). Ten of these exons (23–32) lie within the duplicated area.

Nonsense mutation in P187.—Analysis with the Mut 4 primers showed abnormal fragments after digestion with RNase in individual OX40 (P187) (fig. 2*a*). Direct sequencing revealed a substitution (10748C→T) in exon 35, giving rise to the stop mutation Q3513X (fig. 2*c*). This mutation abolished restriction sites for both *Pst*I and *Alw*NI, and these were used to confirm the change in genomic DNA.

Missense mutations within the duplicated area.—Assay of exons 23–26 (primers Mut 1; table 2) revealed two samples with abnormal fragments: OX1055 (P193) (fig. 2*b*) and OX1190 (P157). Direct sequencing of OX1055 revealed the substitution—9189T→C, changing Leu (CTG) to Pro (CCG) (missense mutation L2993P) in exon 25. This mutation created a *Fok*I restriction site that was used to confirm the mutation in genomic DNA and to show that the two unaffected daughters in this pedigree did not inherited this change. Direct sequencing of OX1190 revealed the mutation 9258A→G, changing Gln to Arg (missense mutation Q3016R). This change creates a restriction site for *Msp*I, which was employed to confirm the mutation in the affected MZ twin daughters in this pedigree. Genomic DNA of additional unrelated patients was screened for the changes L2993P (103 individuals) and Q3016R (152 individuals), but no other examples of either of these transitions were observed on normal or affected chromosomes.

An abnormal digestion pattern was identified in the Mut 4 product from OX1097 (P161). Direct sequencing showed a 10739C→G transversion resulting in L3510V. This change abolished restriction sites for *Hga*I and *Cac*81, and segregation to the patient's three affected offspring was demonstrated. Analysis of 192 further affected and normal chromosomes showed no other examples of this change.

Polymorphisms.—By use of NIRCA, a number of other substitutions were detected, which were characterized by direct sequencing and which, in population studies, were shown to be polymorphisms; these include a substitution of two nucleotides, 9406GT/CC, resulting in F/L3066 (fig. 2*b*), which was found in 23.5% of the individuals analyzed, including one homozygote. A transition 9541T/C, conserving P3110, was detected in 31% of individuals assayed, whereas the substitution 9880G/A, conserving T3223, was found once. The polymorphism 10737C/T, changing threonine to methionine (T/M3509; fig. 2*a*) created a *Sfa*NI site and was found in 2/192 of the individuals screened (1 of these 2 was affected). A 10743C/T change resulting in A/V3511 was found in 14/150 individuals. Two further polymorphisms were identified in the cDNA of different patients during sequencing for mutations in exon 46. The first,

Table 3

Mutations Identified in *PKD1* Gene

Name	Pedigree[a]	Nucleotide Change	Effect on Coding Sequence	Location	Reference(s)
Nonsense:					
E3020X	P130	9269G→T	Glu→Stop at 3020	EX25[b]	Present paper
Q3513X	P187	10748C→T	Gln→Stop at 3513	EX35	Present paper
Y3818X	P117[c]	11665C→A	Tyr→Stop at 3818	EX41	Peral et al. (1996*a*)
Q3837X	P198	11720C→T	Gln→Stop at 3837	EX41	Peral et al. (1996*b*)
R4020X	4137T	12269C→T	Arg→Stop at 4020	EX44	Rossetti et al. (1996)
Q4041X	VR4001	12332C→T	Gln→Stop at 4041	EX44	Turco et al. (1995); Torra et al. (in press)
C4086X	1608	12469T→A	Cys→Stop at 4086	EX45	Neophytou et al. (1996)
R4227X	P89	12890C→T	Arg→Stop at 4227	EX46	Peral et al. (1996*b*); present paper
Insertion/deletions:					
8657delC	P179	Deletion of C at 8657	Frameshifting after 2815	EX23[b]	Present paper
9299delC	P11	Deletion of C at 9299	Frameshifting after 3029	EX25[b]	Present paper
10262del 2 kb	P98 (OX114)[c]	Deletion of 2 kb, IVS30–IVS34	Frameshifting after 3350	IVS30[b]–IVS34	European Polycystic Kidney Disease Consortium (1994)
10708del 5.5 kb	P95 (OX875)	Deletion of 5.5 kb, IVS34–3′ UTR	Deletion of 3491–4302	IVS34–EX46	European Polycystic Kidney Disease Consortium (1994)
10947insT	P100	Insertion of T after 10947	Frameshifting after 3578	EX36	Peral et al. (1996*b*)
11457del15	P35	Deletion of 15 bp, 11457–11471	Deletion of 3749–3753	EX39	Peral et al. (1996*b*)
12739delA	P225	Deletion of A at 12739	Frameshifting after 4176	EX46	Present paper
Splicing:					
IVS31+25del19	P229[c]	19-bp deletion at 10378+25	Frameshifting after 3389	IVS31[b]	Present paper
IVS39+1G→C	P109	G→C at 11477+1	Frameshifting after 3717	IVS39	Present paper
IVS39+266del72	P167	Deletion of 72 bp, IVS39–EX40	Deletion of 3756–3772	IVS39–EX40	Peral et al. (1996*b*)
IVS43+17del18	S6046	18-bp deletion at 12211+17	Complex splicing	IVS43	Peral et al. (1995)
IVS43+14del20	P138	20-bp deletion at 12211+14	Complex splicing	IVS43	Peral et al. (1995)
IVS44+1G→C	P4 (OX32)	G→C at 12346+1	Deletion of 4001–4045	IVS44	European Polycystic Kidney Disease Consortium (1994)
Missense:					
L2993P	P193	9189T→C	Leu→Pro at 2993	EX25[b]	Present paper
Q3016R	P157	9258A→G	Gln→Arg at 3016	EX25[b]	Present paper
L3510V[d]	P161	10739C→G	Leu→Val at 3510	EX35	Present paper
E3631D[d]	P125	11104G→C	Glu→Asp at 3631	EX36	Peral et al. (1996*b*)

[a] In which mutation was first described.
[b] Mutation within the duplicated part of *PKD1*.
[c] De novo mutation occurs within the family.
[d] Conservative substitution that segregates with the disease and was not found in normal subjects.

12777C/T, changed serine to phenylalanine (S/F4189) and also was detected in the normal parent. The second was 12838T/C, conserving P4209.

Mutations Identified by PTT

A large number of polymorphisms were identified by NIRCA (see above), and significant effort was required to characterize these nonpathogenic changes. To overcome this problem, we employed PTT, which identifies aberrantly sized translation products and, hence, detects only disease-related mutations. We screened the entire region from exons 22–46 in four different, overlapping segments, by PTT (fig. 1 and table 2) in 101 unrelated ADPKD patients; these included 61 patients analyzed by NIRCA, plus 40 new families.

Frameshifting mutations in the duplicated area (P179 and P11).—PTT analysis with the PTT 1 primers revealed truncated polypeptides in two patients, OX46 (P179) and OX61 (P11) (fig. 3*a*). Direct sequencing showed that the mutation in OX46 was a deletion of a C nucleotide from a run of three C's (8657delC; fig. 3*b*). This generated a frameshift mutation that introduced 58 novel amino acids before premature termination. This mutation destroyed an *Apa*I restriction site, which was employed to confirm the mutation and to show segregation to the affected daughter. In the second patient, OX61 (P11), direct sequencing revealed deletion of a C nucleotide (9299delC). This frameshift mutation introduced 43 novel amino acids after position 3029. Destruction of a *CvJi*I restriction site was used to confirm the mutation and to trace the change in the family (for details, see fig. 3*c* and Subjects and Methods).

Nonsense mutation in the duplicated area (P130).—Analysis of exons 22–27 by use of primers PTT 1, showed a shorter peptide in patient OX973 (P130) (fig. 3*a*), and direct sequencing revealed a 9269G→T substitution changing Glu to a stop codon (E3020X) (fig. 3*d*). This change created an *Alu*I restriction site, which was

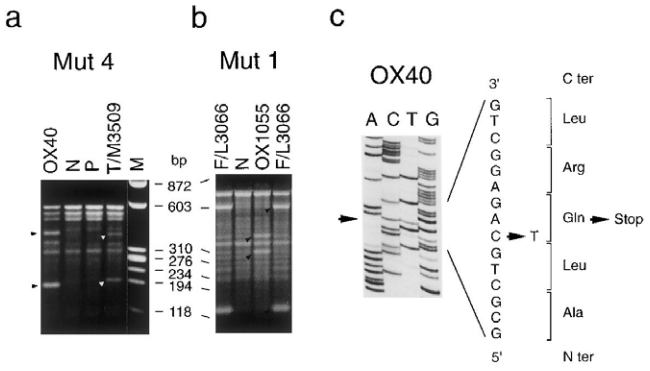

Figure 2 NIRCA analysis. *a,* 3% Agarose gel resolving NIRCA products generated with the Mut 4 primers. N = normal control; P = PKD1 patient (no mutation detected); and M = *Hae*III-digested ϕX174 marker. Two abnormal digestion patterns (*arrowheads*). These digestion patterns were revealed, after the sequence was determined, to be 10748C→T, giving rise to Q3513X in OX40 and to the polymorphism 10737C/T resulting in T/M3509 in an unaffected individual. *b,* NIRCA analysis with the primers Mut 1, showing two different abnormal digestion patterns (*arrowheads*), corresponding to 9189T→C (L2993P) in OX1055 and to the polymorphism 9406GT/CC (F/L3066) in two other individuals. *c,* Direct sequencing of the nonsense mutation in OX40 (P187), showing the C→T transition at position 10748.

used to confirm the mutation in genomic DNA and to show that it also was inherited by his affected daughter.

Deletion of an A nucleotide in OX18 (P225).—A shorter polypeptide in OX18 (fig. 3*e*) was shown to be due to a deletion of an A nucleotide at position 12739 (12739delA). This frameshifting mutation resulted in the addition of 19 novel amino acids before premature termination, leaving a protein 107 amino acids shorter than normal. This mutation was confirmed by use of a mismatch primer (see Subjects and Methods).

Nonsense mutation in P3.—A shorter PTT product in OX21 (fig. 3*e*) was revealed to be due to 12890C→T, converting Arg4227 to a stop codon (R4227X). This mutation previously had been detected in another *PKD1* family (P89), by SSCP (Peral et al. 1996*b*). By use of a mutation-specific primer pair (see Subjects and Methods), the nonsense mutation was confirmed in three affected members of P3 and also in the three *PKD1* patients from P89.

Inclusion of intron 31 (P229).—PTT analysis of OX1056 by use of the PTT 2 primers showed a shorter polypeptide, and analysis with primers flanking the predicted position of the mutation revealed a larger fragment in the cDNA (fig. 4*a*). Sequencing showed that this was due to the insertion of intron 31 into the cDNA. Amplification of OX1056 genomic DNA by use of primers flanking the intron showed a smaller fragment (fig. 4*b*), and sequencing revealed an intronic deletion of 19 bp (IVS31+25del19), which did not affect either the 5′ or 3′ splice sites or the branch-point sequence (fig. 4*c*). The ends of the deletion lay within corresponding positions in a 12-bp direct repeat within the intron (fig. 4*c*), and the deleted intron was 71 bp. The aberrant splicing resulted in inclusion of the deleted intron, causing a 71-bp frameshifting insertion in the cDNA, which introduced 29 novel amino acids after position 3389, before premature termination (fig. 4*c*). This intronic deletion and resulting aberrant splicing was similar to that elsewhere described in two *PKD1* patients with mutations in intron 43 (Peral et al. 1995). In all these cases, defective splicing probably resulted because the deleted intron was too small to be processed efficiently (Wieringa et al. 1984). Genomic DNA of a brother and sister of OX1056 was available for study, and analysis showed that neither sibling had the genomic deletion. Somatic cell–hybrid analysis indicated that the haplotype associated with the deleted chromosome also was present on the chromosome of the unaffected brother, demonstrating that this was a de novo mutation (fig. 4*d*).

Mutation Detected by Direct Analysis of an RT-PCR Product: Skipping of Exon 39 (P109)

An RT-PCR product, ~120 bp smaller than normal, was generated with the 1 Long primers (table 1) in patient OX980 (P109). Direct sequencing showed a frameshifting deletion of 113 bp that was due to skipping of exon 39. Electrophoretic analysis of genomic DNA showed no abnormal fragment, but sequencing demonstrated a G→C substitution at +1 of the splice-donor site following exon 39. This mutation, IVS39+1G→C, created a *Dde*I site, which was used to confirm the mutation and to show that it also was present in the only other available affected individual. This frameshifting change introduced 58 novel amino acids, from position 3717 of polycystin.

Discussion

We have described the first examples of base-pair mutations within the duplicated area of the *PKD1* gene, the likely location of most mutations at this locus. These have been identified and characterized by use of a novel anchored RT-PCR strategy to generate *PKD1*-specific products extending ~2 kb into the reiterated region. The development of such strategies is central to screening the entire *PKD1* gene for mutations, and the anchored RT-PCR approach will allow the analysis of at least ~4 kb of the duplicated part of the gene. Other methods, such as exploiting the rare differences between the *HG* and *PKD1* sequences, will, however, be required in order to analyze the 5′ region of *PKD1*.

PKD1-specific products have been assayed, for mutation, by two different methods, NIRCA and PTT, which previously had not been employed in analysis of this gene. An RT-PCR approach has been used so that larger fragments can be screened without inclusion of intronic DNA; PTT requires that the reading frame be preserved.

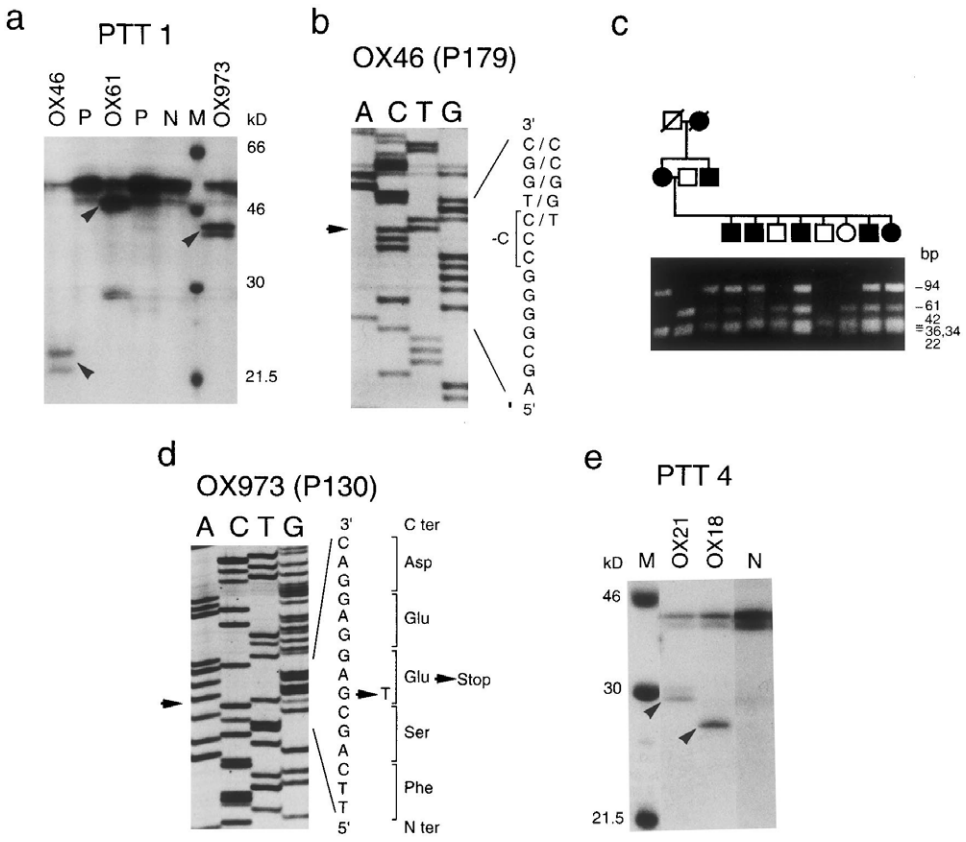

Figure 3 PTT analysis. *a*, SDS-PAGE (17%) analysis of PTT products, with use of the PTT 1 primers. Arrowheads indicate truncated polypeptides generated from mutant alleles in OX46 (P179), OX61 (P11), and OX973 (P130). P = PKD1 patient (no mutation detected); N = normal control; and M = C^{14}-labeled Rainbow molecular-weight marker (Amersham). *b* and *d*, Direct sequencing of two *PKD1* mutations found by PTT (as shown in *a*): deletion of a C nucleotide (8657delC), visualized as doublets in the sequencing gel 3′ to the deletion in OX46 (*b*) and the nonsense mutation 9269G→T (E3020X) in OX973 (*d*). *c*, Agarose gel showing amplified genomic DNA from 11 members of P11 whose DNA was digested with *CvJi*I. Affected individuals with the mutation 9299delC have lost a *CvJi*I site and therefore show the larger, 94-bp fragment (for details, see Subjects and Methods). *e*, Analysis of PTT products in a 15% SDS-PAGE with the primers PTT 4. Arrowheads indicate truncated polypeptides generated from mutant alleles in OX21 (P3; R4227X) and OX18 (P225; 12739delA).

cDNA has been generated from lymphoblast cell lines, but expression in blood leukocytes is sufficient for cDNA synthesis directly from a peripheral blood sample. One potential disadvantage of the RT-PCR approach is that mutations eliminating expression from the aberrant allele would not be detected. However, previous studies have shown expression from all mutated *PKD1* alleles tested (European Polycystic Kidney Disease Consortium 1994; Peral et al. 1996*b*). Both NIRCA and PTT allow the analysis of larger fragments than does SSCP (which is limited to ~200 bp), with NIRCA accurately detecting fragments ≤~1 kb and with PTT accurately detecting fragments >2 kb. The advantage of PTT is that only changes that alter the size of the translated product are identified—and hence most will be pathogenic mutations. This is particularly important in the case of *PKD1,* in which a high polymorphism:mutation ratio has been found by methods designed to detect all mismatches (see discussion of results from NIRCA). RT-PCR/PTT could prove to be an efficient first-pass method for mutation screening in PKD1; >80% of the mutations described so far would be detected by this method. Clearly, other approaches would be required for the detection of missense mutations, and a second screen of those samples in which no PTT aberration had been detected could employ NIRCA.

The advantage of generating *PKD1*-specific products was that confusion between bona fide mutations in the duplicated part of *PKD1* and polymorphic changes at the *HG* loci was avoided. The mutations detected in this study were mainly frameshifting or stop mutations, eliminating significant portions of the gene and, hence, clearly were pathogenic changes. In all cases (9 of 11) in which samples were available, segregation of the mutation with the disease has been demonstrated. It was more difficult, however, to determine whether the three detected substitutions were pathogenic mutations, especially since it presently is impossible to screen the entire *PKD1* gene to exclude other changes. Clearly, the changes must segregate with the disease and must not

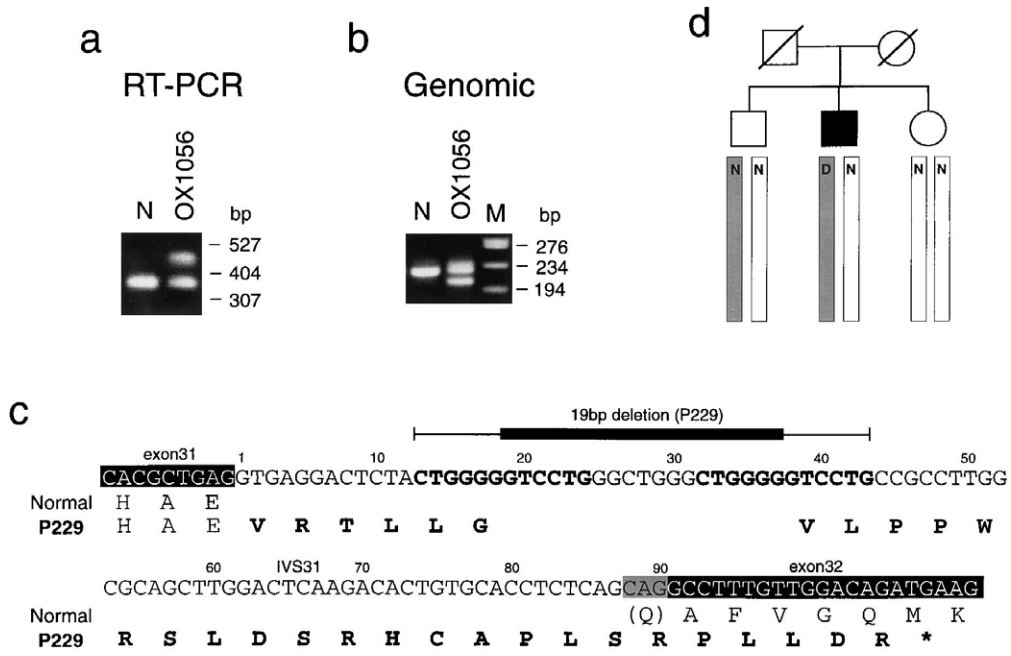

Figure 4 Splicing mutation in IVS31. *a,* RT-PCR products amplified with use of primers flanking the position of the mutation (Mut 4F and AH3B2), from a normal individual (N) and from patient OX1056 (P229). In addition to the normal fragment of 362 bp, a novel, larger fragment of 433 bp is detected in OX1056 *b,* Amplified genomic DNA from OX1056 and a normal control, which reveals a smaller fragment of 184 bp (normal size 203 bp) in the patient. *c,* Diagram of sequence of IVS31 (numbered 1–90), flanked by exons 31 and 32 (*blackened region*) and showing the amino acid sequence. The position of the 19-bp genomic deletion in OX1056 (P229) is shown, with the breakpoints in corresponding positions of a 12-bp direct repeat in the intron (*boldface*). In the P229 cDNA the deleted intron is included, and 29 novel amino acids (in boldface below the sequence) are introduced before premature termination, which is denoted by an asterisk. The gray-shaded codon is an alternative splice variant in the *PKD1* transcript (Harris et al. 1995). *d, PKD1* haplotype analysis of P229 (for details, see Subjects and Methods), showing that the haplotype found on the deleted chromosome (D; *gray shaded*) also is found in the normal brother without the deletion (N), implying that this is a de novo change.

be found in a sizable screen of normal individuals. Even these criteria, however, will not exclude all rare polymorphisms. The L3510V change, for instance, passed the segregation and population tests, but the conservative nature of the change still makes it likely that it is a polymorphism. In contrast, the L2993P and Q3016R changes are much less conservative. The introduction of proline can affect the secondary structure of the molecule, which may disrupt α helixes or β stands. Likewise, the substitution of the neutral residue glutamine for a positively charged arginine may be significant. Comparison of these residues to *PKD1* sequence from other species (once they become available) will show whether these are highly conserved residues. Both changes are in a part of polycystin in which no homology with other proteins originally had been identified (Hughes et al. 1995; International Polycystic Kidney Disease Consortium 1995). However, a region of homology with a sea urchin receptor protein for egg jelly (REJ), which is present on sea urchin sperm and is important in the acrosome reaction, recently has been found in polycystin (Moy et al. 1996). Although the region of described homology does not cover the two missense mutations, extention of the homology over this region can be discerned (authors' unpublished observation). If we look at the residues in the REJ protein that correspond to those which are mutated, we can see that Leu2993 is a very similar aliphatic residue, isoleucine (REJ 1329), suggesting that mutation to proline may be significant. Similar analysis shows that the amino acid corresponding to polycystin Gln3016 is asparagine (REJ 1357), a very conservative substitution, indicating that the introduction of a charged residue may be important. Ultimately, a functional test for polycystin, in which missense changes can be assayed, will be required to determine whether they are mutations.

All of the mutations so far described in typical *PKD1* patients are summarized in table 3. In this limited data set, no clear hot spot for mutation is apparent, although some clustering is observed. Four changes have been described in the 253 bp of exon 25, whereas no mutations have been detected in the 965 bp of exons 26–31. In a region of 12 bp (nt 10737–10748) in exon 35, one mutation, a missense change, and two polymorphisms were identified. Several mutations also have been described in exons 43 and 44, but this may be because primers to amplify this region were described at an early stage (European Polycystic Kidney Disease Consortium

1994). The most striking feature is the variety of different mutations, with only two mutations—R4227 (Peral et al. 1996*b*) and Q4041X (Turco et al. 1995; Torra et al., in press)—described more than once. Although Q4041X was found in three families, their geographical and *PKD1*-haplotype differences indicated that the mutation had recurred, rather than that the families were ancestrally related (Torra et al., in press). The present study describes the third case of a de novo *PKD1* mutation (P229) (also see European Polycystic Kidney Disease Consortium 1994; Peral et al. 1996*a*), and one other pedigree (P193) had a clearly negative family history. All of these findings illustrate both the problem that PKD1 poses for genetic diagnosis and that new mutation at *PKD1* is occurring at a significant rate.

The sequence similarity of *PKD1* and the *HG* loci raises the possibility that mutation in the duplicated part of *PKD1* sometimes may be due to gene-conversion events inserting deleterious mutations from the *HG* loci. Examples of gene conversion being a major cause of mutation have been described. Most mutations of 21-hydroxylase deficiency are due to gene-conversion or deletion events involving recombination between the steroid 21-hydroxylase gene (CYP21) and a pseudogene with 98% sequence similarity, which are separated by 30 kb (White et al. 1994). Clearly, in the case of *PKD1* and the *HG* loci, the separation is much greater (~15 Mb), since they lie in different chromosome bands (i.e., 16p13.3 in the case of *PKD1* and 16p13.1 in the case of *HG*). In a limited analysis of available *HG* cDNA sequence, most of the described changes within the duplicated area of *PKD1* were not found at the *HG* locus. Interestingly, however, the rarer nucleotide (T) found at one frequent *PKD1* polymorphism (9541C/T) was found in the two different *HG* cDNAs analyzed. It is not clear whether this represents a gene-conversion event, a polymorphism present before the original duplication, or the same change occurring independently.

An important question that mutation detection will answer in the case of *PKD1* is whether there is a genotype/phenotype correlation. Both the limited number of affected members in many of the families studied and the localization of mutations to only part of the gene mean, however, that this study provides only further anecdotal evidence. Many of the probands experienced ESRD onset close to the age described as average for *PKD1* (i.e., seven had ESRD onset at age 50–57 years), although their mutations ranged from stop mutations, either in the middle of the gene or close to the 3′ end of the gene, to missense changes. One family (P3) with two individuals with ESRD onset at age 42 years had the same mutation (R4227X) as elsewhere had been described in another family, P89 (Peral et al. 1996*b*). In the P89 pedigree, three individuals experienced ESRD onset at ages 57, 54, and 53 years, showing an apparent difference between these two families with the same mutation. Two families with individuals with renal cysts detected in infancy also were analyzed in this study. However, these children had no clinical symptoms of *PKD1,* and their ultrasound results probably were no more severe than is typical for PKD1 in childhood. Overall, as previous studies have indicated (Peral et al. 1996*a,* 1996*b*), no clear correlation between the severity of renal disease and the type or position of mutation was obvious.

Previous studies have not resolved whether *PKD1* mutations are inactivating or whether they generate a protein with a dominant negative or gain-of-function effect. As frameshifting or stop mutations are identified further 5′ in the gene (as in this study), the possibility that the mutations are inactivating seems more likely. Furthermore, recent evidence of loss of heterozygosity (although at a low level) in cystic epithelia in ADPKD has suggested a two-hit mechanism of disease, with inactivating germ-line and somatic mutations (Qian et al. 1996; Brasier and Henske 1997). The limited available analysis of *PKD2* mutations (Mochizuki et al. 1996; Peters et al. 1996) also shows frameshifting and stop mutations throughout the gene, indicating that *PKD2* mutations probably are inactivating.

An area of similarity between part of the transmembrane regions of polycystin and the PKD2 protein suggests that they may have related roles (Mochizuki et al. 1996). Since polycystin is much larger (>4×) than the PKD2 protein, however, the unique extracellular portion of polycystin may have additional functions. It is possible that some of these may be mediated by alternatively spliced or cleaved products. Recent evidence of possible alternative splicing in the mouse, resulting in the production of proteins terminating after exon 12, has been described (Löhning et al., in press). The severe polycystic kidney disease associated with the *TSC2/PKD1* contiguous-gene syndrome may be due to simultaneous mutation of both the *TSC2* gene and the *PKD1* gene. Alternatively, if some products (such as truncated N terminal proteins) are generated from the *PKD1* gene in typical PKD1 patients—but not in *PKD1/TSC2* patients, who have null mutations at this locus—these may explain the differences, in disease presentation, between the two disorders. Ultimately, for determination of the mutational mechanism in *PKD1,* it will be essential to screen the whole gene for changes. This study has made important progress toward that aim, with both specific amplification of the duplicated part of *PKD1* and the first description of mutations from that region.

Acknowledgments

We thank Sue Butler for assistance with tissue culture, Milly Graver for preparing the manuscript, Prof. Sir D. J. Weatherall for his support, and the many nephrologists who have supplied clinical information on their patients. This work was financially supported by the Wellcome Trust, the Medical Research Council, the Oxford Kidney Unit Trust Fund, the National

Kidney Research Fund, and the Polycystic Kidney Research Foundation.

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