Molecular analysis of the PDS gene in Pendred syndrome (sensorineural hearing loss and goitre)

Beth Coyle, William Reardon[1], Jo-Anne Herbrick[2], Lap-Chee Tsui[2], Eleanor Gausden, Jeffrey Lee[2], Rebecca Coffey[1], Annette Grueters[3], Ashley Grossman[4], Peter D. Phelps[5], Linda Luxon[6], Pat Kendall-Taylor[7], Stephen W. Scherer[2] and Richard C. Trembath*

Division of Medical Genetics, Departments of Genetics and Medicine and Therapeutics, University of Leicester, Leicester LE1 7RH, UK, [1]Department of Clinical Genetics and Fetal Medicine, Institute of Child Health, 30 Guildford Street, London WC1N 1EH, UK, [2]Department of Genetics, The Hospital for Sick Children, Toronto M5G 1X8, Canada, [3]University Children's Hospital, Berlin, Germany, [4]Departments of Endocrinology and Nuclear Medicine, St Bartholomew's Hospital, West Smithfield, London EC1A 7BE, UK, [5]Royal National Throat Nose and Ear Hospital, Grays Inn Road, London WC1X 8EE, UK, [6]Institute of Laryngology and Otology, Grays Inn Road, London WC1X 8EE, UK and [7]Department of Endocrinology, Royal Victoria Infirmary, Newcastle NE1 4LP, UK

Pendred syndrome is an autosomal recessive disorder characterized by the association between sensorineural hearing loss and thyroid swelling or goitre and is likely to be the most common form of syndromic deafness. Within the thyroid gland of affected individuals, iodide is incompletely organified with variable effects upon thyroid hormone biosynthesis, whilst the molecular basis of the hearing loss is unknown. The *PDS* gene has been identified by positional cloning of chromosome 7q31, within the Pendred syndrome critical linkage interval and encodes for a putative ion transporter called pendrin. We have investigated a cohort of 56 kindreds, all with features suggestive of a diagnosis of Pendred syndrome. Molecular analysis of the *PDS* gene identified 47 of the 60 (78%) mutant alleles in 31 families (includes three homozygous consanguineous kindreds and one extended family segregating three mutant alleles). Moreover, four recurrent mutations accounted for 35 (74%) of *PDS* disease chromosomes detected and haplotype analysis would favour common founders rather than mutational hotspots within the *PDS* gene. Whilst these findings demonstrate molecular heterogeneity for *PDS* mutations associated with Pendred syndrome, this study would support the use of molecular analysis of the *PDS* gene in the assessment of families with congenital hearing loss.

INTRODUCTION

Congenital hearing loss is common, with a fairly consistent prevalence estimate of one per thousand births in the developed world (1,2). Although aetiologically heterogeneous, it is thought that genetic factors contribute to at least 50% of such cases (3,4). Several reports over the last few years have contributed to a rapidly expanding understanding of the range and diversity of genes important in establishing and maintaining normal cochlear function (5). These reports, based on the identification of mutant genes in familial hearing loss, are also increasingly important in governing the approach to the investigation of the deaf child and to the provision of counselling to families (6). Many of these developments have arisen from a combined clinical and molecular approach focusing specifically on families in which the hearing loss is associated with other clinical features, representing a syndrome. Although many of the syndromes associated with hearing loss are individually rare, Pendred syndrome is probably the exception in that epidemiological data suggest that it may account for as much as 10% of hereditary hearing loss (7).

Pendred syndrome is inherited as an autosomal recessive condition. Classically, the disorder is characterized by congenital sensorineural hearing loss and goitre (7). However, exceptions to this presentation are not uncommon (8). While most patients will show alteration in the handling of iodide by the thyroid gland on formal testing by perchlorate challenge, the non-specificity of this investigation can lead to misdiagnosis (9). Equally, it has been established, in the study of familial cases, that perchlorate discharge testing is not 100% sensitive in Pendred syndrome and that genuine cases may be missed on this investigation (8,10). While subtle alterations in the radiological conformation of the cochlea are certainly useful diagnostic aids to recognizing the disorder in specialist centres (11), the non-specificity of these findings means that there is no single diagnostic investigation which can secure the diagnosis of Pendred syndrome. For this reason the availability of a DNA based diagnostic approach might bear a striking impact on clinical practice in the investigation of

*To whom correspondence should be addressed. Tel: +44 116 258 6042; Fax: +44 116 258 6057; Email: rtrembat@hgmp.mrc.ac.uk
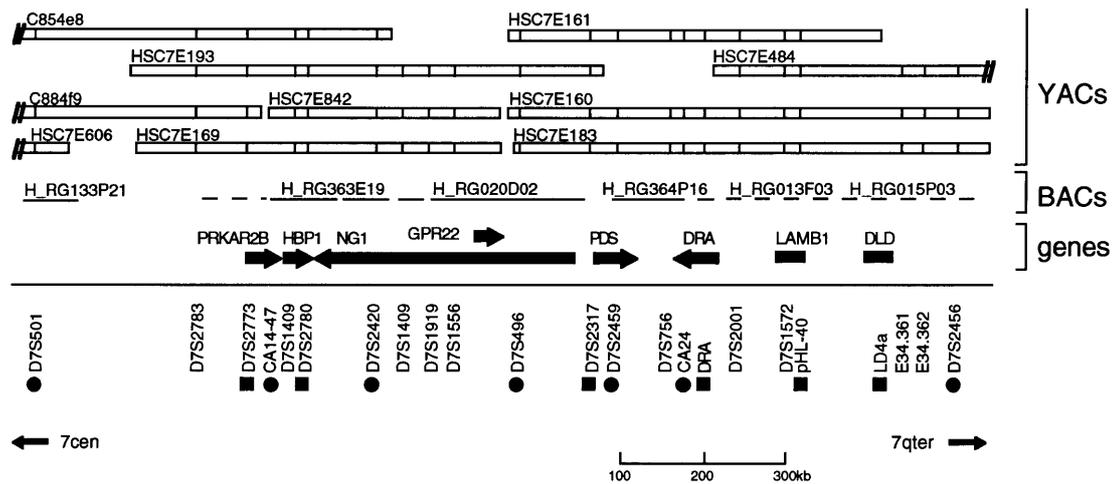
Figure 1. The Pendred syndrome region. The position of seven genetic markers (●) and eight genes (the arrow depicts the orientation of the gene, if known, from 5′ to 3′) within the Pendred syndrome region is shown. PRKAR2B (21), the human orthologue of HBP1 (18,19,20), GPR22 (22), DRA (15,23), LAMB1 (24) and DLD (25) represent previously described genes. The precise position of the PDS gene at 7q22.3–q31.1 with respect to the genetic markers used in our study, is shown. NG1 is a novel and previously uncharacterized gene that encompasses >300 kb. The dashed lines under each BAC clone name represent the genomic segments for which contiguous stretches of DNA sequence has been determined.

hearing-impaired children with particular reference to this, the most common syndromic form of hearing loss.

The locus for Pendred syndrome was mapped to chromosome 7q in 1996 (12,13) and mutations in a putative sulfate transporter gene, *PDS*, have recently been identified (14). This gene, organized in 21 exons, encodes a predicted 780 amino acid protein, hereafter referred to as pendrin, which bears striking homology to two other human proteins, DRA and DTD, which are mutant in the autosomal recessive conditions of hereditary chloride diarrhoea and diastrophic dysplasia, respectively (15,16). Neither of these disorders is associated with hearing loss or with goitre and it has been speculated that the common aetiological factor between these disorders and Pendred syndrome is a likely dysfunction in ion transport (14).

To date, three distinct homozygous mutations have been identified in five consanguineous Pendred syndrome families. Two of these mutations result in frameshift with premature truncation and the third, a missense mutation, causes substitution of a highly conserved Phe by Cys. Since this gene has diagnostic potential for identifying Pendred syndrome, we report our experience in mutation analysis in patients with a confirmed or suspected diagnosis of this condition.

RESULTS

The *PDS* gene resides within a relatively gene-dense region at the boundary of the 7q22.3–q31.1 and within 40 kb of the *DRA* gene with which it shares >70% homology for the predicted protein (14,15,17). Using various gene identification techniques, it has been possible to position at least seven additional transcripts (including the known genes *PRKAR2B*, *GPR22*, *DRA*, *LAMB1* and *DLD*, and two new genes tentatively named *HBP1* and *NG1*) within the critical region (Fig. 1). The *HBP1* gene contains an HMG-Box DNA binding domain motif and has been shown to rescue K$^+$ transport-deficient yeast mutants (18) as well as serve

as a transcriptional repressor of N-MYC (19,20). *NG1* is a novel gene that spans ~300 kb of genomic sequence. Although the two latter genes were shown to be expressed in thyroid and/or cochlear RNA (data not shown), our analysis failed to detect any structural abnormalities in them, so detailed analysis of the *PDS* gene was performed.

Single strand conformation polymorphism (SSCP) analysis included the 2342 bp coding region from exons 1 to 21 of the *PDS* gene and, with the exception of exon 19, encompassed the flanking intronic sequences (see primers and conditions in Table 1). One affected member of the 20 familial cases and 36 patients in whom either no additional family member was affected or details of the extended family history was unavailable, was analysed. Abnormal SSCP bands were detected in 17 families and 14 of the apparent sporadic cases with a putative Pendred phenotype. The nature of the band migration differences was determined by direct DNA sequence analysis. In addition, direct sequencing of all 21 exons of the *PDS* gene was performed on probands from five families, each fulfilling recognized clinical criteria for the diagnosis of Pendred syndrome, but in whom SSCP analysis had failed to identify all disease alleles. In combination, these studies revealed 10 mutations from eight families linked to the *PDS* locus (five consanguineous and three extended kinships), 16 mutations in 12 families consistent with *PDS* linkage and 21 mutations in 36 isolated patients with a possible diagnosis of Pendred syndrome. A total of 16 point mutations were identified, of which 10 were single base changes in addition to four small deletions and two insertions. These alterations predict a total of nine independent missense mutations, one donor splice site and six frameshift mutations distributed across 12 of the 21 *PDS* exons (Table 2). Segregation of mutations was confirmed in familial cases either by restriction digest analysis of novel cut sites or gel electrophoresis (Table 2). No sequence variant was detected in at least 50 non-Pendred disease chromosomes.

Table 1. Characterization of mutations

Exon	Ta	Size	Sequence
PDS1	60	445 *Bam*HI 162+283	for TTCCTCTTCTCCTCCCCATGTG* rev GTGTGGGCATCTTCAGGG
PDS2	57 (1.25 mM)	507 *Acc*I 221+286	for CAGGACGCGGACCAGACT rev CGAGACTGATGGAGCCACCCTC*
PDS3	56	218	for GCAAATTGGTTGTGACTGAG rev GTAAACTGCTGCCAACATC
PDS4	56	190	for TAATCACTTTGCATGTGCTTT rev GCCAAAACACTTTAAACATGA
PDS5	53	242	for CCTATGCAGACACATTGAACATTTG* rev ACCTGTATAATTCCAACCAGCA
PDS6	53	248	for GGTTTCTATCTCAGGCAAACAT rev ATTGTTTCTGGAATGAACAGTGACC*
PDS7	50	388 *Sau*3AI 172+216	for CATGGTTTTTCATGTGGGAAGATTC* rev AATGGCAGTAGCAATTATCG*
PDS8	50	408 *Rsa*I 245+163	for ACACAAAATCCCAGTCCC* rev CAAATGGCTTGACGTTTATCTACACAC*
PDS9	64	283	for GTGGTCAAATCTTCACAGCA rev CCCTTCTTTAGCTGACACCA
PDS10	64	250	for AAATACTCAGCGAAGGTCTTGC rev CGAGCCTTCCTCTGTTGC
PDS11/12	64	403 *Hin*dIII 124+279	for ACACATCCAGTGAGCTGGAA rev AGGTGGTAGGTGACTTACAGCA
PDS13	64	226	for TTGTTTGTGGATCATTGATCTT rev TCTGGGTTTTACGTTACTCACA
PDS14	64	186	for CAAAATACGGCTGTTCCAAA rev AATGGAGCTGCTGAAACTTC
PDS15	64	199	for CCCAGACAATTTCTTTTATGC rev TTGGACCCCAGTAAATACTTGT
PDS16	58	250	for TTGAGAAATAGCCTTTCCAGAT rev GACCCTCTAACTGCTCTCATCA
PDS17	64	287	for TCTTCGTTTAGAATGGCATCA rev ATTGCCAAAGCTCCAAATGT
PDS18	58	183	for TGAATGCTACTGAATTATGGGC rev AGATAGGAGAAAGGGCTTACGG
PDS19	60	342 *Eco*RII 106+236	for GGTAGGGTGTGCCCTGTAGT rev CGTTTCTAAAATGGAACCTTGA
PDS20	58	222	for ACCCTGTGTTCTCTTTTTCAAG rev GCATTTGGGGGAATTATGTT
PDS21	54	109	for ACACTTTGTTTTCCCCTTGCrev GCATTGAGGAGTTTTGTCTTG

The primers used to amplify each exon and flanking splice sites are given here, only the exon 19 reverse primer does not encompass the donor splice site due to the presence of an Alu repeat in the intron. Some of the primers used for exons 1–8 (*) were those previously described by Everett *et al.* (14), although the published exon 7 antisense primer and exon 8 sense had been reversed. Primers for exons 9–21 were designed using partial genomic sequence from the BAC RG364P16 available on the Washington University Genome Sequencing Centre Web site (http://genome.wustl.edu/gsc). In some cases it was necessary to digest the products hence the restriction enzymes and resulting fragment sizes are given. All products were amplified in buffer containing 1.5 mM magnesium unless otherwise stated and at the indicated annealing temperature.

Several of the mutations were observed in unrelated patients with Pendred syndrome. A 1001+1G→A change predicted to lead to a donor splice site alteration in the consensus sequence of exon 8, was observed on 10 disease alleles. Two of these were in family demonstrated to share identical disease haplotypes during the linkage study (Fig. 2). In a further family (*PDS* 21; Table 2C), this mutation was present as a compound heterozygote with a further recurrent missense mutation E384G, a substitution at an evolutionary cons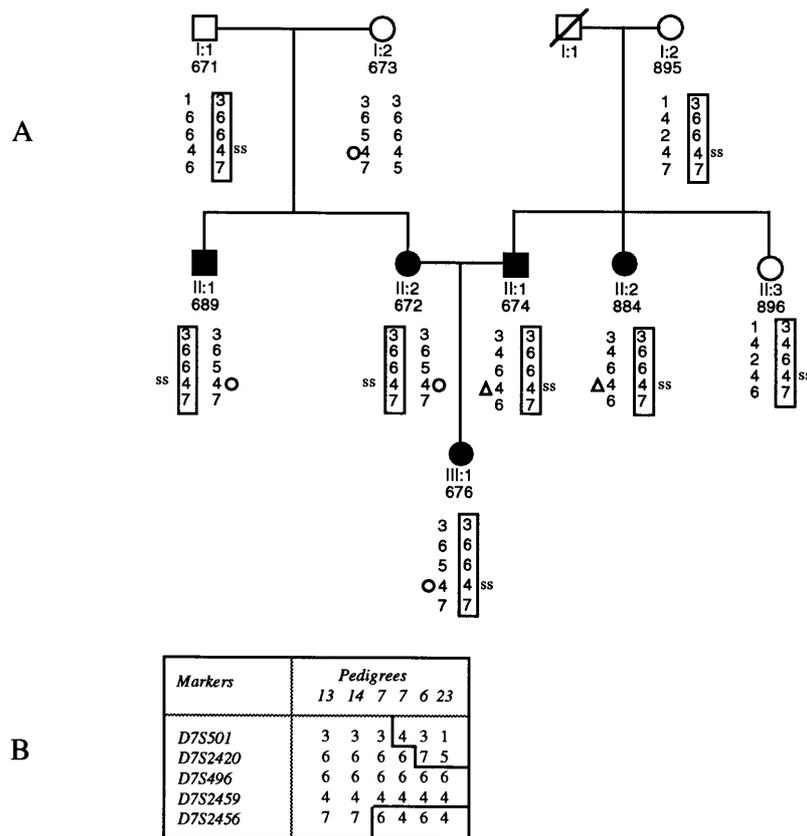erved amino acid. The identical sequence change 1151A→G was observed in two independently ascertained families and four isolated patients with Pendred syndrome. Two further missense mutations were identified in more than one proband with Pendred syndrome, a T–C transition at position 707 of exon 6 observed in 10, and an A–C substitution at 1246 of exon 10, which was detected as a heterozygous mutation, compound with the E384G missense mutation in two of the seven probands with this mutation.

Table 2. Characterization of mutations

Pedigree no.	Exon	Nucleotide change	Amino acid change		Detection method
(A) Linked families					
1~	15	1667A→G	Y556C		GE
13	8	1001+1G→A	donor splice site		*Fok*I
	17	2005G→A	G672E		*Mme*I*
14	8	1001+1G→A	donor splice site		*Fok*I
	19	2127delT	frameshift, stop at 719		GE
18~	10	1226G→A	R409H		*Rle*AI
27 a	6	707 T→C	L236P		*Cje*I
	6	753–756 del CTCT	frameshift, stop at 286		GE
27 b	12	1334–1335insAGTC	frameshift, stop at 467		GE
30~	10	1229C→T	T410M		*Nla*III
8~+24~	not detected				
(B) Consistent cases of Pendred syndrome					
4	10	1151A→G	E384G		*Hin*fI
	10	1246A→C	T416P		*Ban*II
7	8	1001+1G→A	donor splice site		*Fok*I
23	8	1001+1G→A	donor splice site		*Fok*I
	6	707 T→C	L236P		*Cje*I
25	14	1558T→C	Y530H		GE
	6	707 T→C	L236P		*Cje*I
28	13	1536–1538delAG	frameshift stop at 524		GE
	10	1246A→C	T416P		*Ban*II
3	10	1151A→G	E384G		*Hin*fI
5	6	707 T→C	L236P		*Cje*I
6	8	1001+1G→A	donor splice site		*Fok*I
16	10	1246A→C	T416P		*Ban*II
19	4	336–377insT	frameshift stop at 180		GE
32	11	1284–1286delTGC	–A 429		*Tse*I
15		not detected			
(C) Isolated patients with a suspected diagnosis of Pendred syndrome					
21	8	1001+1G→A	donor splice site		*Fok*I
	10	1151A→G	E384G		*Hin*fI
26	4	412G→T	V138F		*Dde*I*
	6	707 T→C	L236P		*Cje*I
40	10	1151A→G	E384G		*Hin*fI
	10	1246A→C	T416P		*Ban*II
41	6	707 T→C	L236P		*Cje*I
42	6	707 T→C	L236P		*Cje*I
44	10	1246A→C	T416P		*Ban*II
	6	707 T→C	L236P		*Cje*I
48	6	707 T→C	L236P		*Cje*I
	8	1001+1G→A	donor splice site		*Fok*I
17	10	1151A→G	E384G		*Hin*fI
20	10	1246A→C	T416P		*Ban*II
34	8	1001+1G→A	donor splice site		*Fok*I
43	10	1151A→G	E384G		*Hin*fI
45	8	1001+1G→A	Donor splice site		*Fok*I
46	6	707 T→C	L236P		*Cje*I
47	10	1246A→C	T416P		*Ban*II

Families were categorized as described in Materials and Methods. Shown in bold are all the families in which both mutations were identified. *PDS* 27 contains affected first cousins who inherit a common mutation (753–756 del CTCT), and additional mutations denoted a and b. Nucleotide numbers refer to the cDNA where the A of the ATG initiator codon is denoted nucleotide +1, and are in accordance with recent nomenclature recommendations (28). Consanguineous families are denoted by the symbol ~. All subjects are of North European origin with the exception of pedigrees 8 (Israel) and 30 (Pakistan). All mutations were confirmed by restriction enzyme digestion.
*Loss of a site mutation; all others are gains, or segregation by polyacrylamide gel electrophoresis (GE). All missense mutations result in substitutions of evolutionarily conserved amino acids.

Markers	Pedigrees					
	13	*14*	*7*	*7*	*6*	*23*
D7S501	3	3	3	4	3	1
D7S2420	6	6	6	6	7	5
D7S496	6	6	6	6	6	6
D7S2459	4	4	4	4	4	4
D7S2456	7	7	6	4	6	4

Figure 2. Segregation of a recurrent mutation and associated haplotypes. (**A**) Large extended family formed by the combination of pedigrees 13 and 14. Three mutations are shown segregating in this family: ss, mutation 1101+G→A which affects the donor splice site; Δ, 2127delT; ○, 2005G→A. The splice site mutation is inherited in both branches of this family, on each occasion on a common haplotype (boxed). (**B**) Markers given in (A), the physical localization of which are shown in Figure 1. The table demonstrates conservation of the 1101+G→A disease haplotype in all six families analysed.

A 4 bp deletion at position 753 in exon 6 and an insertion at 1334 of exon 12 predict frameshifts and the creation of premature stop codons at 286 and 467, respectively, of the 780 amino acid pendrin polypeptide. These truncated proteins would be deleted for the putative extracellular C-terminus and a variable number of the transmembrane spanning domains of pendrin and are likely to be null alleles. One patient (*PDS* 27; Table 2), the offspring of unrelated and unaffected parents with Pendred syndrome, was found to be a compound heterozygote for these two frameshift mutations. This 26-year-old man presented with profound congenital hearing loss, had a 49% discharge of radiolabelled iodide following perchlorate challenge, is euthyroid and has a large goitre. Of some interest, his cousin is also affected, sharing the common maternally inherited mutation but with a further novel missense mutation L236P, inherited or 'married in' via the unrelated father.

Detection of mutations in thyroid cDNA

The expression profile of pendrin appears restricted: we have identified transcripts from thyroid, brain and kidney (data not shown) but have failed to detect a product from cDNA generated from immortalized lymphoblastoid cells. Hence, confirmation of the effect of the genomic sequence alterations identified in Pendred syndrome patients has been confined to three subjects who underwent partial thyroidectomy for the clinical management of their goitre. Direct sequencing of amplified *PDS* cDNA covering exons 5–21 revealed missense mutations, 707T→C in exon 6, 1151A→G in exon 10 and in the proband from *PDS*, this mutation and a further variant, 1246A→C (Fig. 3). Each mutation was confirmed through analysis of genomic DNA from probands and relatives. In no patient with the recurrent donor splice mutation at exon 8 or any of the frameshift mutations was thyroid tissue available for analysis.

Recurrent mutations and haplotype analysis

Examination of the genomic sequence within and around the four recurrent mutations, (three missense, 1151A→G, 707T→C and 1246 A→C) and a splice site mutation at exon 8 (1001+1G→A) shows no obvious features likely to account for their elevated incidence in the cohort of Pendred patients studied. To discriminate between founder effects and *de novo* recurrence, disease-associated haplotypes across the Pendred gene region at chromosome 7q31 were determined by typing five microsatellites (Fig. 2). Each of the recurrent mutations occurred on distinct but common haplotypes supporting the notion for common founders occurring in independently ascertained families. Of interest, the donor splice site mutation 1001 +1 G→A observed in 10 families, in five of which descendants could be traced to the north-east of
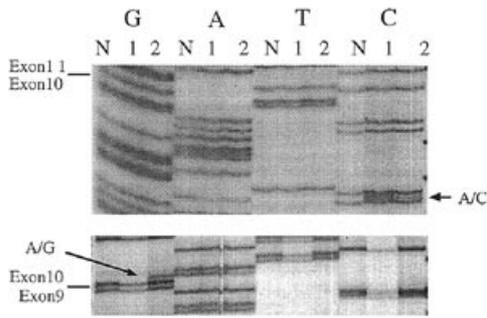
Figure 3. RT-PCR. Sequence analysis of RT-PCR generated thyroid cDNA from a normal (N) and two Pendred syndrome patients. Products were amplified using primers 3F and 3R (Fig. 4) then sequenced using each primer. Shown here are long (top) and short (bottom) gel runs of reactions sequenced with primer 3f, which cover the 5′ and 3′ ends of exon 10. In sample 2, both mutations occur in exon 10, 1151A→G is inherited from the deceased mother and was not detected by SSCP. The other mutation is 1246A→C and is also observed in sample 1.

Figure 4. Mutation compilation. Distribution of mutations along the open reading frame of the *PDS* transcript. All exons apart from 21, which includes the sizeable 3′ UTR, are drawn to scale. Each symbol represents a different mutation, although several have been observed in multiple families (Table 2). Also shown as filled-in symbols are the previously identified mutations (14).
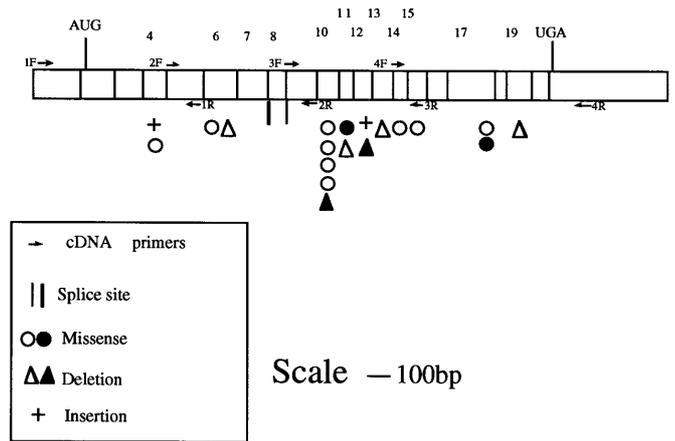
England, the region from which the family described in 1896 by Pendred resided (10). Limited genealogical studies have not identified known relations between the families reported here and those investigated at the turn of the century. Analysis of parental DNA failed to reveal any *de novo* mutations in this study cohort, either within the familial or isolated cases of Pendred syndrome.

DISCUSSION

Our observations and the identification of 16 novel mutations within the *PDS* gene confirms the initial report of Everett *et al.* (14) and establishes this gene as the cause of the majority of, if not all, cases of Pendred syndrome. However, detailed analysis including direct sequencing of the entire coding sequence of the *PDS* gene failed to identify the causative mutation in two consanguineous kinships linked to chromosome 7q31. Future studies in these families should include analysis of the regulatory and intronic sequence of the *PDS* gene, but the existence of a second disease gene for Pendred syndrome within this interval has not been excluded.

Despite the number of *PDS* mutations identified, their distribution appears restricted to only 12 of the 21 exons of the large *PDS* gene (Fig. 4) and the recurrence of a number in several families. The majority of disease alleles, 9/16 (62%), were missense mutations localized to the region of pendrin's putative transmembrane spanning domains and hence may impact on the stability of the protein within cell membranes.

The diagnosis of Pendred syndrome has traditionally relied upon the combination of clinical features and the use of perchlorate discharge tests, a situation that has led to under-diagnosis of the disorder (10). In this study, we have applied diagnostic criteria that have included linkage analysis for the *PDS* linkage interval at 7q31. Using this approach, SSCP analysis of the entire coding sequence of the *PDS* gene detected 26/36 (72%) Pendred disease alleles from a cohort of familial cases either known to be linked (group A) or in whom haplotype analysis supported linkage to chromosome 7q31 (group B). Investigation of singletons (group C) with a putative diagnosis of Pendred syndrome was of interest, as mutant alleles were identified in

several patients who hitherto would not have been considered to fulfil widely used clinical criteria for the diagnosis of Pendred syndrome. As only a single disease allele was detected in six families, future studies should focus on analysis of regulatory and non-coding sequences of the *PDS* gene. Of further clinical significance, four of the mutations observed in the study, three missense and a donor splice site mutation, were identified in more than one independently ascertained kindred, and DNA diagnostic assays for a combination of these sequence variants would detect 58% of the Pendred disease alleles in subjects with a confirmed clinical diagnosis of Pendred syndrome.

This study cohort does not allow us to estimate the prevalence of *PDS* mutations in the population and, given the detection of recurrent founder mutations, the frequency is likely to show geographical variation. However, this study serves to emphasize that *PDS* mutations may not be uncommon, with the detection of kindreds in which multiple disease alleles are segregating.

The precise function of pendrin remains unclear and in particular whether it serves as an ionic transporter both in the thyroid gland and the inner ear. In support of a direct role in regulation of transmembrane transport of ions, we have recently demonstrated gross dilatation of the endolymphatic sac in the majority of patients with Pendred syndrome (11). Hence, by analogy with the defect consequent on mutation of DRA (15), the developmental bony defects frequently observed in this condition may be secondary to a form of endolymphatic secretary diarrhoea.

In conclusion, we have demonstrated that the *PDS* gene accounts for the majority of Pendred syndrome disease alleles and that a high proportion of familial cases can be accounted for by a limited number of recurrent mutations likely to reflect the dispersal of a limited number of founder mutations. These findings have important implications for the clinical utility of molecular genetic analysis as an aid in diagnosing a disorder that has hitherto been under-diagnosed (10), leading to possible inaccurate counselling for a condition that has a high risk of recurrence.

MATERIALS AND METHODS

Patient selection and diagnosis of Pendred syndrome

The clinical diagnosis of Pendred syndrome was based on characteristic clinical features as previously described (12). Briefly, audiological investigations confirmed severe sensorineural hearing loss and physical examination revealed a palpable goitre. This diagnosis was supported by demonstration of a perchlorate-induced discharge of >10% of radioiodine from the thyroid gland of all probands (8). Linkage analysis for the *PDS* region at chromosome 7q31 was used to group subjects. Group A consisted of eight families definitely linked to the *PDS* region, and group B contained 12 kindreds in whom haplotype segregation was compatible with involvement of the *PDS* locus. Group C consisted of 12 isolated subjects with features suggestive of a diagnosis of Pendred syndrome, specifically (i) sensorineural hearing loss and abnormal inner ear radiology or (ii) sensorineural hearing loss with goitre, but normal perchlorate discharge.

Genomic DNA analysis

DNA was extracted from EDTA whole blood by standard methods. DNA sequence variation from wild-type was identified by a combination of SSCP and direct sequencing. For SSCP PCR products were amplified from 40 ng of genomic DNA using 12.5 ng [γ-^{33}P]-radiolabelled primers (26) in a 10 μl reaction with 0.5 U Taq polymerase (Applied Biosystems) and 200 μM each of dATP, dCTP, dGTP and dTTP. This was buffered by 100 mM Tris–HCl, 100 mM KCl, 1.25–1.75 mM MgCl$_2$, and 0.2% gelatin. Reactions were carried out on an MJ Research Peltier thermal cycler for 28 cycles of 94°C for 40 s, optimal annealing temperature for 30 s (Table 1) and 72°C for 1 min. Three microlitres of each reaction was combined with 8 μl 95% formamide loading dye denatured for 5 min at 98°C then run on a 4.5% non-denaturing polyacrylamide gel, containing 10% glycerol, at a constant 6–12 W at room temperature, overnight.

All band shifts were characterized by radioactive cycle sequencing using the Amersham Life Science Thermosequenase Kit of Qiagen (QIAquick PCR purification kit) purified exon-specific PCR products, with all ambiguous variants being sequenced in both directions.

Microsatellite markers from the 1.4 Mb region containing the *PDS* gene at chromosome 7q31 were typed as previously described (27).

RNA extraction and analysis of reverse transcription products

Total RNA was isolated from human thyroid tissue using RNAzolB (Tel Test). RT products were prepared from 200 ng of total RNA using MMLV reverse transcriptase (Gibco BRL) according to the manufacturer's protocol. One microlitre of the RT product was then amplified by the same PCR conditions as described above using exon-specific primers (Fig. 4).

Exons 1–4 1F (CCTGATAGGATCGGTTGGGA) and 1R (TTCCATTGCTGCTGGATACG), exons 4–9 2F (TGGGATCT-GTTGTTCTGAGC) and 2R (GGAAAATGATGCAGCCAGC), exons 9–15 3F (TCCACCTGTGAGCTTGTTCT) and 3R (TTG-CCATAGAAAATAGGACTGG), exons 15–21 4F (GAAG-CATCCCTAGCACAGATA) and 4R (TTTGTCTTGTATTCCT-TGCTA) These primers anneal at 51, 55, 55 and 53°C, respectively

REFERENCES

1. Martin, J.A.M., Bentzen, O., Colley, J.R., Hennebert, D., Holm, C., Iurato, S., de Jonge, G.A., McCullen, O., Meyer, M.L., Moore, W.J. and Morgon, A. (1981) Childhood deafness in the European community. *Scand. Audiol.*, **10**, 165–174

2. Nadol, J.B. Jr (1993) Hearing loss. *New Eng. J. Med.*, **329**, 1092–1102.

3. Cohen, M.M. Jr. and Gorlin, R.J. (1995) Epidemiology, aetiology and genetic patterns. In Gorlin, R.J., Toriello, H.V. and Cohen, M.M. Jr (eds), *Hereditary Hearing Loss and its Syndromes*. Oxford University Press, Oxford, pp. 9–21.

4. Reardon, W. (1992) Genetic deafness. *J. Med. Genet.*, **29**, 21–526.

5. Pennisi, E. (1997) The architecture of hearing. *Science*, **278**, 1223–1224.

6. Reardon, W. (1998) Connexin 26 gene mutation and autosomal recessive deafness. *Lancet*, **351**, 383–384.

7. Fraser, G.R. (1965) Association of congenital deafness with goitre (Pendred's syndrome): a study of 207 families. *Ann. Hum. Genetics*, **28**, 201–249.

8. Reardon, W., Coffey, R., Pembrey, M.E., Rajput, K., Phelps, P.D., Luxon, L.M., Britton, K.E., Grossman, A., Kendall-Taylor, P. and Trembath, R. (1997) Pitfalls in practice—diagnosis and misdiagnosis in Pendred syndrome. *J. Audiol. Med.*, **6**, 1–9.

9. O'Mahoney, C.F., Luxon, L.M., Chew, S.L. and Wass, J.L. (1996) When the triad of congenital hearing loss, goitre and perchlorate positive is not Pendred syndrome. *J. Audiol. Med.*, **5**, 157–165.

10. Reardon, W., Coffey, R., Phelps, P.D., Luxon, L.M., Stephens, D., Kendall-Taylor, P., Britton, K.E., Grossman, A. and Trembath, R. (1997) Pendred syndrome—100 years of underascertainment? *Q. J. Med.*, **90**, 443–447.

11. Phelps, P.D., Coffey, R.A., Trembath, R.C., Luxon, L.M., Grossman, A.B., Britton, K.E., Kendall-Taylor, P., Graham, J., Cadge, B.C., Stephens, S.G.D., Pembrey, M.E. and Reardon, W. (1998) Radiological malformations of the ear in Pendred syndrome. *Clin. Radiol.*, **53**, 268–273.

12. Coyle, B., Coffey, R., Armour, J.A.L., Gausden, E., Hochberg, Z., Grossman, A., Britton, K., Pembrey, M., Reardon, W. and Trembath, R. (1996) Pendred syndrome (goitre and sensorineural hearing loss) maps to chromosome 7 in the region containing the nonsyndromic deafness gene *DFNB4. Nature Genet.*, **12**, 421–423.

13. Sheffield, V.C., Kraiem, Z., Beck, J.C., Nishimura, D., Stone, E.M, Salameh, M., Sadeh, O. and Glaser, B. (1996) Pendred syndrome maps to chromosome 7q21–34 and is caused by an intrinsic defect in thyroid iodine organification. *Nature Genet.*, **12**, 424–426.

14. Everett, L.A., Glaser, B., Beck, J.C., Idol, J.R., Buchs, A., Heyman, M., Adawi, F., Hazani, E., Nassir, E., Baxevanis, A.D., Sheffield, V.C. and Green, E.D. (1997) Pendred syndrome is caused by mutations in a putative sulphate transporter gene (*PDS*). *Nature Genet.*, **17**, 411–422.

15. Hoglund, P., Haila, S, Socha, J., Tomaszewski, L., Saarialho-Kere, U., Karjalainen-Lindsberg, M.-L., Airola, K., Holmberg, C., de la Chapelle, A. and Kere, J. (1996) Mutations of the Down-regulated in adenoma (*DRA*) gene cause congenital chloride diarrhoea. *Nature Genet.*, **14**, 316–319.

16. Hastbacka, J., de la Chapelle, A., Mahtani, M.M., Clines, G., Reeve-Daly, M.P., Daly, M., Hamilton, B.A., Kusumi, K., Trivedi, B., Weaver, A., Coloma, A., Lovett, M., Buckler, A., Kaitila, I. and Lander, E.S. (1994) The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine structure linkage disequilibrium mapping. *Cell*, **78**, 1073–1087.

17. Hoglund, P., Haila, S., Scherer, S.W., Tsui, L.C., Green, E.D., Weissenbach, J., Holmberg, C., de la Chapelle, A. and Kere, J. (1996). Positional candidate genes for congenital chloride diarrhea suggested by high-resolution physical mapping in chromosome 7q31. *Genome Res.*, **6**, 202–210.

18. Lesage, F., Hugnot, J.-P., Amri, E.-Z., Grimaldi, P., Barhanin, J. and Lazdunski, M. (1994). Expression cloning in K⁺ transport defective yeast and distribution of HBP1, a new putative HMG transcriptional regulator. *Nucleic Acids Res*., **22**, 3685–3688.

19. Lavender, P., Vandel, L., Bannister, A.J. and Kouzarides, T. (1997) The HMG-box transcription factor HBP1 is targeted by the pocket proteins and E1A. *Oncogene*, **14**, 2721–2728.

20. Tevosian, S.G., Shih, H.H., Mendelson, K.G., Sheppard, K.-A., Paulson, K.E. and Yee, A.S. (1997) HBP1: a HMG box transcriptional repressor that is targeted by the retinoblastoma family. *Genes Dev.*, **11**, 383–396.

21. Solberg, R., Sistonen, P., Träskelin, A.-L., Bérubé, D., Simard, J., Krajci, P., Jahnsen, T. and de la Chapelle, A. (1992) Mapping of the regulatory subunits RIβ and RIIβ of cAMP-dependent protein kinase genes on human chromosome 7. *Genomics*, **14**, 63–69.

22. O'Dowd, B.F., Nguyen, T., Jung, B.P., Marchese, A., Cheng, R., Heng, H.H.Q., Kolakowski, L.F., Lynch, K.R. and George, S. (1997) Cloning and chromosomal mapping of four putative novel human G-protein-coupled receptor genes. *Gene*, **187**, 75–81.

23. Taguchi, T., Testa, J.R., Papas, T.S. and Schweinfest, C. (1994) Localization of a candidate colon tumor-supressor gene (*DRA*) to 7q22–q31.1 by florescence *in situ* hybridization. *Genomics*, **20**, 146–147.

24. Pikkarainen, T., Eddy, R., Fukushima, Y., Byers, M., Shows, T., Pihlajaniemi, T., Saraste, M., and Tryggvason, K. (1987) Human laminin B1 chain, a multidomain protein with gene (*LAMB1*) locus in the q22 region of chromosome 7. *J. Biol. Chem.*, **262**, 10454–10462.

25. Scherer, S.W., Otulakowski, G., Robinson, B.H. and Tsui, L.-C. (1991) Localization of the human dihydrolipoamide dehydrogenase gene (*DLD*) to 7q31–32. *Cytogenet. Cell Genet.*, **56**, 176–177.

26. Richardson, C.C. (1971) Polynucleotide kinase from *Escherichia coli* infected with bacteriophage T4. In Cantoni, G.L. and Davies, D.R. (eds), *Procedures in Nucleic Acid Research*. Harper and Row, New York, Vol. 2, p. 815.

27. Gausden, E., Coyle, B., Armour, J.A.L., Coffey, R., Grossman, A., Fraser, G., Winter, R., Pembrey, M.E., Kendall-Taylor, P., Stephens, D., Luxon, L.M., Phelps, P.D., Reardon, W. and Trembath, R. (1997) Pendred syndrome: evidence for genetic homogeneity and further refinement of linkage. *J. Med. Genet.*, **34**, 126–129.

28. Antonarakis, S.E. and The Nomenclature Working Group (1998) Recommendations for a nomenclature system for human gene mutations. *Hum. Mutat.*, **11**, 1–3.