

Prestin, a cochlear motor protein, is defective in non-syndromic hearing loss

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Prestin, a membrane protein that is highly and almost exclusively expressed in the outer hair cells (OHCs) of the cochlea, is a motor protein which senses membrane potential and drives rapid length changes in OHCs. Surprisingly, prestin is a member of a gene family, solute carrier (SLC) family 26, that encodes anion transporters and related proteins. Of nine known human genes in this family, three (*SLC26A2*, *SLC26A3* and *SLC26A4*) are associated with different human hereditary diseases. The restricted expression of prestin in OHCs, and its proposed function as a mechanical amplifier, make it a strong candidate gene for human deafness. Here we report the cloning and characterization of four splicing isoforms for the human prestin gene (*SLC26A5a*, *b*, *c* and *d*). *SLC26A5a* is the predominant form of prestin whereas the others showed limited distribution associated with certain developmental stages. Based on the functional importance of prestin we screened for possible mutations involving the prestin gene in a group of deaf probands. We have identified a 5'-UTR splice acceptor mutation (IVS2-2A>G) in exon 3 of the prestin gene, which is responsible for recessive non-syndromic deafness in two unrelated families. In addition, a high frequency of heterozygosity for the same mutation was observed in these subjects, suggesting the possibility of semi-dominant influence of the mutation in causing hearing loss. Finally, the observation of this mutation only in the Caucasian probands indicated an association with a specific ethnic background. This study thereby reveals an essential function of prestin in human auditory processing.

INTRODUCTION

The mammalian inner ear consists of the cochlea (the sense organ of hearing) and vestibule (comprising organs responsible for balance). Within the cochlea are two distinct receptor cell types: the inner hair cells (IHCs) and the outer hair cells (OHCs). IHCs are thought to be the true sensory receptors: they transduce vibration and are the source of essentially all auditory signals

passing to the brain. OHCs are responsible for amplification of cochlear vibrations, possibly through somatic length changes driven by their membrane potentials ('electromotility'). This unique amplification is thought to significantly increase the sensitivity and frequency selectivity of the cochlea (1).

A combination of subtractive PCR hybridization and differential screening from gerbil OHCs has recently identified a protein, prestin, whose cellular distribution, subcellular

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localization and functional properties indicate that it is the electromotility motor of cochlear OHCs (2,3). Prestin is a member of a newly characterized gene family, solute carrier (SLC) family 26, which encodes anion-transporter-related proteins. Like other members in this family, prestin has a highly hydrophobic core of predicted 12 transmembrane domains with the N- and C-terminal located cytoplasmically (3). Although prestin has not been shown to have any anion transport capability, it does appear to share the overall structure and specific protein domains of the anion transporter family SLC26 such as having a conserved sulfate transport motif and STAS (sulfate transporters and antisigma factor antagonists) domain. The nine family members cloned thus far in humans, SLC26A1-9, transport chloride, iodine, bicarbonate, oxalate and hydroxyl anions, with different specificities (4). Mutations in the SLC26A2, SLC26A3 and SLC26A4 genes are responsible for three distinct recessive disorders: diastrophic dysplasia, congenital chloride diarrhea and Pendred syndrome, respectively (5). SLC26A4 encodes a chloride-iodide transport protein expressed in the thyroid, kidney and inner ear. Its different mutations can lead to either syndromic deafness (Pendred syndrome) (6) or non-syndromic recessive deafness (DFNB4) (7).

Dramatic progress has been made in the identification of more than 80 gene loci that can cause non-syndromic deafness, the most common form of genetic hearing loss (<http://dnalab-www.uia.ac.be/dnalab/hhh/>). Additional loci for non-syndromic deafness exist, but the lack of suitable families for linkage analysis has made localization difficult (8). Moreover, only nearly 30 of these genes have been cloned to date (<http://dnalab-www.uia.ac.be/dnalab/hhh/>), owing to the absence of distinguishing clinical signs in deaf individuals, the heterogeneity of genetic deafness and small size of affected human families. With the difficulty of positional cloning, a candidate gene approach—based on the identification of genes uniquely or preferentially expressed in the inner ear—may produce additional human deafness genes more efficiently (9–12). Clearly, the unique function of prestin and its specific expression in OHCs make this gene an excellent candidate for a human non-syndromic deafness. In the present study, we report the cloning and characterization of multiple isoforms of human prestin gene and its genomic structure. Significantly, we identify a splicing junction mutation in the prestin gene, which leads to non-syndromic hearing loss.

RESULTS

Cloning of human prestin gene and its splicing variants

Standard RT-PCR amplified most of the coding region of the human prestin gene from human fetal ear (at 10, 20 and 22 weeks' gestation) cDNA pools, using the oligonucleotides derived from sequence comparisons between the mouse prestin cDNA and human genome database (Table 1). To identify 5' and 3' ends of human prestin cDNA, RACE amplification was done using the RACE cDNA generated from human fetal cochlear RNA. The 5' RACE produced a single PCR product with primer pair of 3' primer (Invitrogen) and Hu-pres3r. For 3' RACE multiple bands were generated

Table 1. Primers used for RACE analysis of prestin isoforms

Primers	Sequences
Hu-pres1f	AGTGACACTCAGGAAATGCTTGTTC
Hu-pres2f	AGGGGATAACGTCACACTGTGCAT
Hu-pres3f	TGTGGCCATATATCTCACAGAGCC
Hu-pres4f	CTCTTCCACCTTGTGTACCCTAGA
Hu-pres5f	TTTGAATCATTGCCCCAGGCTGTG
Hu-pres6f	AAAGACTGGAGTGAACCCAGCAGT
Hu-pres8f	GACTTGGTCTCAGGCATAAGCACA
Hu-pres1r	GGAGTACATGTGAATGCCTGTTC
Hu-pres2r	TGAATTTGTATGCTGGCAGCCATT
Hu-pres3r	TGTGCTTATGCCTGAGACCAAGTC
Hu-pres4r	AGTTGGTCTCAGGCATAAGCACA
Hu-pres6r	CAGAGGCTCTGTGAGATATATGGC
Hu-pres7r	CCTAGAGGAAGTGTTCACACGACA
Hu-pres8r	CACAGCCTGGGGCAATGATTCAAA
Hu-pres9r	ACTGTGGGTTCACTCCAGTCCTTT
Hu-pres17rev	AGCTGGCATTCAAACCTGTCCAC
Hu-pres18rev	TAAGTGACTTGGCTGAGGTCAACC

from nested PCR with human ear cDNA, with the first round using primer pair Hu-pres1f/3' primer (Invitrogen) and the second round with Hu-pres3f/3'-nest (Invitrogen). Both 5' and 3' RACE PCR products were gel purified and sequenced. The sequencing data from overlapping PCR products was assembled using Sequencher V4.0 (GeneCodes Corp., Ann Arbor, MI, USA).

The single 5' RACE fragment indicated that there are no alternative splicing variants at the 5' portion of prestin, which was confirmed by the identical sequences of this segment from human ear cDNAs. Sequencing the four bands of 3' RACE derived from primer pair Hu-pres3f/3'-nest showed that they were alternative splicing variants which differed at their 3' portion. The assembly of the RT-PCR, 5'- and 3'-RACE products revealed four prestin cDNAs which were 2671, 2492, 1960 and 1416 bp in length and encoded predicted ORFs of 744, 685, 516 and 335 amino acids. We have designated them *SLC26A5a*, *SLC26A5b*, *SLC26A5c* and *SLC26A5d*, respectively. *SLC26A5b*, *SLC26A5c* and *SLC26A5d* all share the same terminal 3' exon, but differ in their intervening cDNA sequences (Fig. 1). *SLC26A5a* and *SLC26A5b* share the majority of the sequence, and differ only at the terminal 3' exon (see genomic structure, Fig. 1). *SLC26A5a-c* contain the complete set of predicted transmembrane domains, whereas *SLC26A5d* has only seven of the 12 predicted transmembrane domains. All four prestin isoforms preserve the sulfate transport motif (second transmembrane domain), but not the STAS domain (sulfate transporters and antisigma-factor antagonists). A consensus polyadenylation signal (AATAAA) was present in the 3'-UTR of *SLC26A5b*, *SLC26A5c* and *SLC26A5d*, but not in the 3'-UTR of *SLC26A5a*.

To further characterize the origin and distribution of the prestin isoforms, RT-PCR was performed using human fetal ear cDNA obtained at different stages of development. As shown in Figure 2 one round of RT-PCR (30 cycles) was sufficient to amplify the 3' portion of *SLC26A5a* in all ear samples tested. However nested RT-PCR was required to amplify any of the remaining isoforms. Interestingly, only one of the three remaining prestin isoforms was detected in any

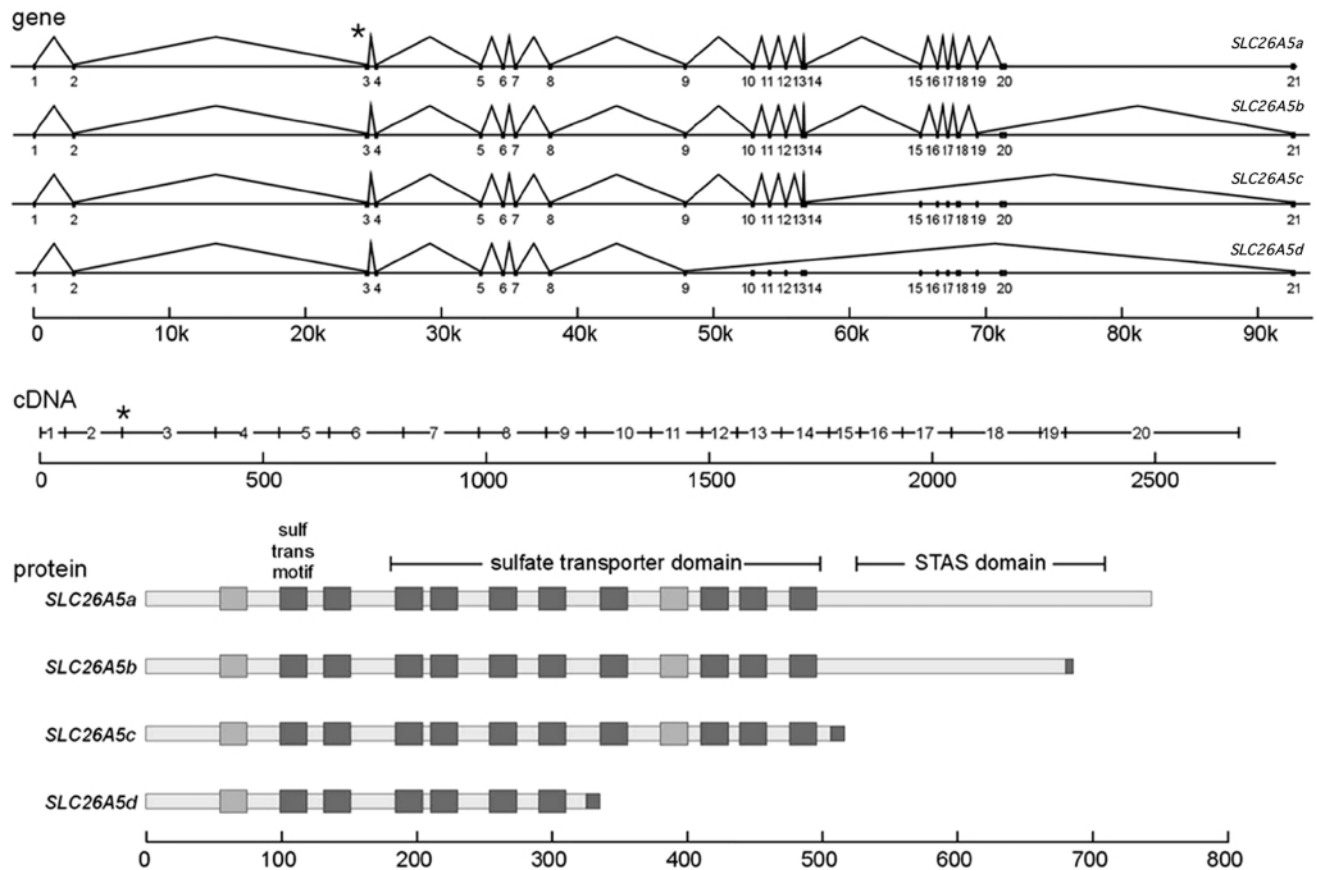


Figure 1. Exon structure and alternative splice forms of human prestin. Exons are shown relative to the gene (top) and cDNA (middle). Four splice forms vary in the last exon, and in the number of exons skipped before the last exon. The predicted protein structures of the four splice forms are shown relative to the cDNA. Strongly predicted (dark gray) and probable (light gray) transmembrane domains are indicated; also shown are the STAS domain and the sulfate transporter domain. A sulfate transport motif is present in the second transmembrane segment, while the STAS domain is in the c-terminus. The location of the IVS2-2A>G mutation, shortly before the translation start, is marked by an asterisk.

given cochlea sample. The three cochlear cDNAs (with the isoforms of *SLC26A5b*, *c* and *d*) were derived from 22, 20 and 19 weeks gestational age, respectively. Based on the results, we conclude that *SLC26A5a* is the most abundant, adult form of prestin in the cochlea, and that the other three isoforms are expressed at a lower level and appear to show different expression during development. However we could not rule out the possibility that *SLC26A5b* may be expressed at very low level at 19 and 20 weeks' gestation stages which was not detected due to potential preferential amplification of smaller PCR products (*SLC26A5c* and *SLC26A5d*) at the stages.

Expression of prestin

Using a prestin PCR product as the probe (H-pres3f/H-pres8r, covers 720 bp between exons 7 and 12) for northern analysis we did not detect expression in human brain, colon, heart, kidney, liver, lung, muscle, placenta, small intestine, spleen, stomach or testis (data not shown), demonstrating the very limited extent of prestin expression. As described above, RT-PCR confirmed prestin expression in human cochlea.

Genomic structure of human prestin gene

The human prestin cDNA sequences were used to search NCBI and Celera human data banks. The inferred exons were determined by a perfect match between the cDNA and genomic sequences, bounded by consensus splice donor and acceptor sites (Table 2). A high degree of similarity between the human and mouse *SLC26A5* coding regions (13) also helped to determine exon-intron boundaries. The human prestin gene spans over 90 kb on chromosome 7, at location 7q22.1; the distribution of exons is shown in Figure 2. The last exon of isoforms *SLC26A5b-d* is located 20 kb downstream of the last exon of *SLC26A5a* and has a perfect acceptor site, demonstrating that it is a bona fide exon.

Mutation analysis of *SLC26A5*

Because prestin is essential for normal hearing in mice (14), we asked whether mutations in human prestin caused hearing loss. In total, 220 hearing impaired (greater than 20 dB hearing loss across 500, 1000, 2000 and 4000 Hz as determined by pure tone audiometry and presence of normal middle ear function) Caucasian probands were screened for *SLC26A5* mutations. In

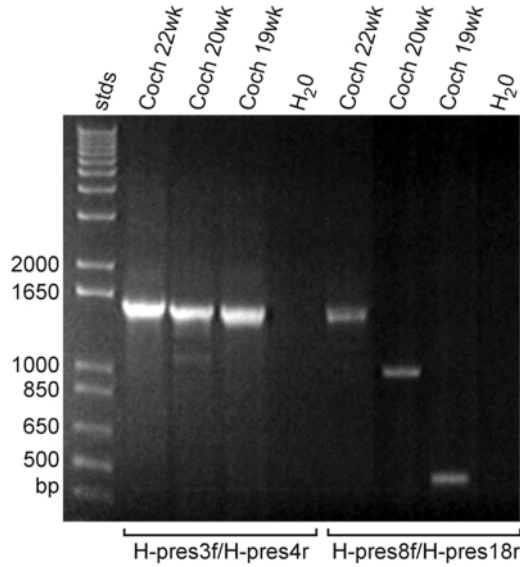


Figure 2. Identification of multiple splicing isoforms of the prestin gene. One round of RT-PCR with the primer pair H-pres3f/H-pres4r amplified a SLC26A5a product of 1.54 kb in all cochlear samples, whereas a nested RT-PCR (used the primer pair H-pres3f/H-pres17r followed by H-pres8f/H-pres18r) was required to amplify SLC26A5b (1.58 kb in Coch 22 weeks), SLC26A5c (1056 bp in Coch 20 weeks) or SLC26A5d (512 bp in Coch 19 weeks) isoforms. For the primer pair H-pres8f/H-pres18r, only one product was amplified in each of the samples.

addition, screening was performed on several control groups including 150 Caucasian subjects with reported normal hearing. Their ages ranged from 10 to 67 years. Following initial testing for mutations in *GJB2* and *GJB6* with negative results (data not shown), the DNA samples from probands were screened for *SLC26A5* mutations by an SSCP-sequencing approach, using 21 pairs of primers covering all exons of *SLC26A5* isoforms and their flanking splice sites.

A homozygous variant SSCP pattern for exon 3 using primers 5'FR (Table 3) was observed in two probands (data not shown). Sequence analysis of the SSCP variant demonstrated that the two deaf individuals were homozygous for an A-G transition at the intron 2/exon 3 junction (Fig. 3C). Since this IVS2-2A>G mutation affects the invariant A of the acceptor splice site AG dinucleotide (Fig. 3D and E), the mutation would be expected to lead to aberrant splicing. Computer-assisted analysis by Neural Network at Berkeley Drosophila Genome Project (BDGP; www.fruitfly.org/seq_tools/splice.html) predicted a splice score of 0.98 of a maximum possible score of 1.00 for the wild-type acceptor site of exon 3, whereas the mutant sequence was not recognized as a splice site (a score of zero).

One of the two deaf probands was born to consanguineous parents in a multiplex sibship (Fig. 3A), while the other was born to non-consanguineous parents in a simplex sibship. Clinical and audiological evaluation showed that both homozygous probands had a severe-profound bilateral non-syndromic sensorineural loss that appeared to have been congenital with no evidence for progression (Fig. 3B). Physical examination of the two probands showed no dysmorphic features of the face, jaw, palate, or external ears, and no history of vertigo or

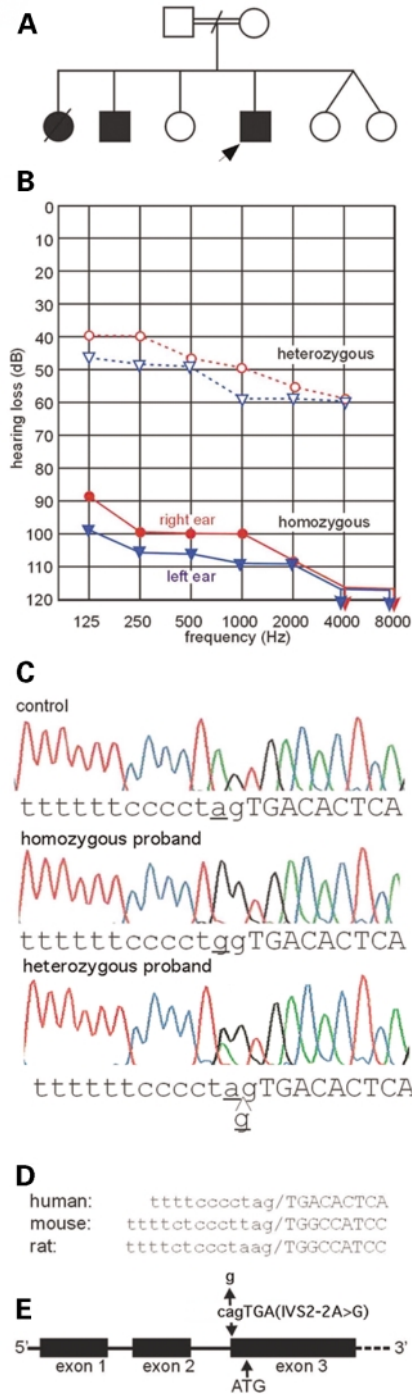


Figure 3. Detection of the IVS2-2A>G mutation. (A) Pedigree of the multiplex family. The proband is indicated (arrowheads). (B) Audiograms of the proband with the homozygous IVS2-2A>G mutation at age 15 and a proband carrying the heterozygous mutation at age 29. Red and blue indicate the left and right ear, respectively. (C) Direct sequence analysis of the intron 2 and exon 3 junction of *SLC26A5* in a normal control as well as deaf homozygous and heterozygous probands. The exonic sequence is represented in upper-case letters, intronic sequence by lower-case letters. The proband from the multiplex family shows a homozygous A-G transition (underlined), as was the proband of the simplex family (data not shown). A proband with a partial hearing loss (B) is heterozygous for the mutation. The sequence of normal control is also shown. (D) Comparison among species of the splice acceptor for exon 3. (E) Schematic representation of the first three exons of *SLC26A5*, with the locations of the IVS2-2A>G mutation and the start codon indicated.

Table 2. Inferred genomic structure of human prestin gene. The sequences of exons are underlined. Exons 20 and 21 are the 3' end of exons in different splicing variants (see text for description of splicing isoforms)

<u>CACCTGGAGGCAGCGCGCGTGAAGAGGCAGCGGCTGTG</u>	exon 1 (54 bp)	<u>GAGTCGCGGGGCGCGGCCGCGCTGCCCGCGGCCCTCCTCCTA</u>
<u>TTAACTTCTTTTCTTCTTCTATACATAGGGCCAAGGAGCGAG</u>	exon 2 (129 bp)	<u>GGGCAGCCCGGGCTGGTGAAGTGCCTCGGGCGCCGCGGGCC</u>
<u>AAAATGGAGTTTTCTTTTCCCTAGTGACACTCAGGAAAT</u>	exon 3 (205 bp)	<u>TGCACATTTGGATCTGGGTAAGTAGACGGCCCCCTGGAACCTAGG</u>
<u>TTTATCTCTGTCTGTCTTTGTCCTTTCAGATGTAAGTCTCTAAAA</u>	exon 4 (140 bp)	<u>TGAAACAGGCATTCACGTACGTTGCCTTTAACCTGTTTCTGTTATT</u>
<u>CATTTCTGTTTGGTTTTTAATCCACAGGCTTAGCCTTTGCAA</u>	exon 5 (111 bp)	<u>CTTCAGATCTCCTCAAGGTCAAGTACGATCTTTCTTTTCCCCCTC</u>
<u>ACAGGGATCTTCATGCTTCATTTATTCAGGTCCTTTTGTCTGT</u>	exon 6 (167 bp)	<u>AGACACATATCCATAGGTAAAAGCTACAACTTGATATTCTATTAC</u>
<u>TTGTTTTCTTTGTCATTAATAAAAAAATAGTTTTGCTTAGGTGT</u>	exon 7 (166 bp)	<u>TTTCAGGAATCATTTCAGGTAAGGCTCAAAGATGGGGAAATGGACTG</u>
<u>TAAACATCCAGCATTCACTGTGTACAGAGTACAGTTGCTGT</u>	exon 8 (153 bp)	<u>ATCTTTTCCGTGGTGTATGTAAGTAAGAACTACCTGACGTTCTGGT</u>
<u>GGTGCATTTGGATTTGTCTTTTGTAGTTCGTAATGGGAAC</u>	exon 9 (83 bp)	<u>CCTTTAGAGTTCTTTGCGGTAAGTACCTTCGGTCAGTGACCTACTC</u>
<u>CAAGTTAAAGGCAGCTTGAATTTTCAGGCTGCTACCTCCAGC</u>	exon 10 (148 bp)	<u>TGTTTTCATCTCCTTAGGTTAGGAAAGTGGTTTAAAGTAACTGTT</u>
<u>TTGTGTTTTGTTTTCTACATGGGAAAGGAGCTCATGCGCTGG</u>	exon 11 (114 bp)	<u>CAGGTTGACCGCAATCAGGTAAGATAATCAGTTTCTGTCACATGT</u>
<u>CCCTTCCATGTCTTCTCTCATTCAGCTTGCAGGTTGTTGG</u>	exon 12 (78 bp)	<u>CCCGTGGGAAAGACACAGGTATGTACATAACAGGCCTCTAAGACCT</u>
<u>GGTGCATTTGGATTTGTCTTTTGTAGTTCGTAATGGGAAC</u>	exon 13 (96 bp)	<u>TTTGAATCAATTGCCCCAGGTGTGTGTAATGTGGACTTGGGTGAAGT</u>
<u>CTCCATCAAGTTTTCTGTCTTTTGTCTTAGACCATCTGGCTTACC</u>	exon 14 (107 bp)	<u>ACGACCAAAATAGAGCTGGTGAAGTAAAGTGGTTTAAAGTAACTGTT</u>
<u>ATGGTATTGGACTCTTTATCGTCAGTCCAAGCTACTAAGTC</u>	exon 15 (70 bp)	<u>ATTTACAGAACACAGAGGTGAGTGCCAGATTGGAATGGGTGTGA</u>
<u>TTAAATTCAGCAATATTTACTCTTCAGGTGAAAGAAATTCCT</u>	exon 16 (93 bp)	<u>TAGATGCATATGAGGAGGTAGGACCTTTTTCTATCACTTAAAAAG</u>
<u>ATTTGTCATGAGTGTCTTCTGTAAAGACTGGAGTGAACCCA</u>	exon 17 (108 bp)	<u>ATGCATTAACCGAAAGGTGAGTCATGAGAAATGATTATTTCTGA</u>
<u>TTCTGGTTTGGTTAATTTTGTACACAGGATGCAGAAGTAGAT</u>	exon 18 (201 bp)	<u>GCAACTGTTGTCAAAGCAGTGAAGTGGTCTTTTCATTGAAATAATCC</u>
<u>ACTAAATTTTATTTCAACATATTTACAGATTTGTAAGAAATAT</u>	exon 19 (55 bp)	<u>TGAAAACCTCTGGCAGGGGTAAGCATCCTCTTGAACGAGTCATTTAA</u>
<u>TGGACTAATTTCTTTCTTTTAAATAGCACAAAGTTGTGAA</u>	exon 20 (386 bp)	<u>TTAGCAGGATGCAGTGGTGAATATACCTTAAGATGGGGTGAAGC</u>
<u>ACAAGTCACTACCCCTCTGTTTCAGCTTTTCATACAGAGATG</u>	exon 21 (200 bp)	<u>TTGTAATTTGTTTTTTTAAATGAACTTCAAAGATAAAAAAA</u>
		<u>ATTGTTTTCTCTTTTGTGAAATAAAGAAAAAATGAATCTACAAA</u>

evidence for vestibular dysfunction. No other clinical abnormalities were noted. In the multiplex family (Fig. 3A), there is a history of a sister and a brother who are also reported deaf, but were not available for testing.

In addition to the two individuals with homozygous mutations, seven (3.2%) out of 220 white deaf probands were found to be heterozygous for the same mutation. The severity of hearing loss in these heterozygous individuals ranged from mild to profound (Fig. 3B). Their ages of onset of hearing loss ranged from birth to 35 years. None of 250 control subjects were found to be homozygous for this mutation. In the 150 white control subjects with no known hearing impairment, one was a heterozygote of the mutation IVS2-2A>G, giving a carrier frequency of 0.6%. However, this individual was not audiotically tested and could have had a mild degree of hearing loss. No examples of the variant were found in the other 100 additional non-Caucasian controls.

To search for *SLC26A5* mutations in deafness families showing a possible linkage to the *SLC26A5* locus and other racial groups, we screened patients from DFN14 families (15) from Lebanon and DFN17 (16) families from India, as well as 150 deaf probands from other ethnic backgrounds. None of them carried any *SLC26A5* mutation including the mutation found in our patients. These data provide further evidence of the apparent restriction of occurrence of this form of deafness to the Caucasian population.

DISCUSSION

Cloning of the human prestin cDNA revealed a gene with 21 exons and at least four splice isoforms. Two untranslated exons occur upstream of the exon containing the ATG codon (Fig. 2). A similar configuration of the first three exons was found in all known prestin genes, and in some other members of the *SLC26* family. Without exons 1 and 2 and the non-coding region of

exon 3, prestin expression was not observed in transiently transfected TSA cells, suggesting that the 5'-UTR region has an important role in allowing for optimal protein expression in the TSA201 cells, perhaps via mRNA stability or translational efficiency (13).

Alternative splicing generates at least four isoforms of prestin, varying at the C-terminal. However, RT-PCR showed that *SLC26A5a* is the most common form of prestin in human fetal cochlea. The other three isoforms were detected in only one developmental stage each, suggesting that their expression may be developmentally regulated. The functional significance of the isoforms is not understood: while all isoforms retain the sulfate transporter motif, STAS domain is only partially present in *SLC26A5c* and completely absent in *SLC26A5d*. Also isoforms *SLC26A5c* and *SLC26A5d* are without cytoplasmic chain in the C-terminus, which may affect their distribution within the hair cells.

The mutation occurs in a highly conserved nucleotide of the splice acceptor for exon 3 (Fig. 3D and E), the first coding exon, which is conserved not only in *SLC26A5* (prestin) but also in most members of the *SLC26* transporter family. A mutation at this point would very likely affect mRNA stability or translational efficiency of *SLC26A5*, and would do so for all four splice forms. Other possible consequences include exon skipping, activation of cryptic splice sites, creation of pseudo-exons within introns, and intron retention (17,18). However, as the *SLC26A5* gene is almost exclusively expressed in outer hair cells of the cochlea, we were, of course, unable to obtain tissue from affected individuals to determine how the mutation actually influences the mRNA splicing and levels.

A loss of prestin in humans is very likely to cause a loss in cochlear function. Prestin is highly expressed in outer hair cells, with tens of millions of molecules per cell, and lines the lateral wall of these cells in a close-packed array (3). It is necessary for fast electromotility, and hypothesized to mediate the active amplification that boosts cochlear sensitivity by 100–

Table 3. *SLC26A5* PCR primer sequences for SSCP screening

Primers	Primer sequences	Product size (bp)
Prestin-5'F	5'-GCC TTG CAC TTA ATA TGA GCT TTT-3'	349
Prestin-5'R	5'-TGC TGC AAG GAT TTC ATT TTC-3'	
Prestin-1F	5'-CTC GGG TTA CCG GGA GTC-3'	210
Prestin-1R	5'-GCC TCA GCT CAG TCC CCT-3'	
Prestin-2F	5'-AAA CAA ACC AAC AAC TGC TGA A-3'	265
Prestin-2R	5'-TGA GGA ATC CTT TGA TCT GGA-3'	
Prestin-3F	5'-CTT GTC TCC GGC TGT TAA GG-3'	250
Prestin-3R	5'-GAC CTT GCG CCT CTC ATA AC-3'	
Prestin-4F	5'-TGG TTT GAA TTC TAC AGA GAT GTT TC-3'	250
Prestin-4R	5'-TGT TTG AGG ACA GCA AGG G-3'	
Prestin-5F	5'-ATC CCC CAT TTC TGT TTG GT-3'	227
Prestin-5R	5'-TGA CCA TGC TAT TTC TTT GGC-3'	
Prestin-6F	5'-CCC TGA ACA GGG ATC TTC AT-3'	238
Prestin-6R	5'-AAC GAG ACA GTC CAT TTC CC-3'	
Prestin-7F	5'-TGT GAT TCA TTT GTT TCT TTG TCA-3'	232
Prestin-7R	5'-CCA GAA CGT CAG GTA GTT TCT T-3'	
Prestin-8F	5'-AAC ATC CAG CAT TCA CTG TGT T-3'	227
Prestin-8R	5'-AAA TGC AGT TGT AGA AGC CGA-3'	
Prestin-9F	5'-TTT GAA GCT GAT TTT TGC AGT-3'	199
Prestin-9R	5'-TCC ATT GAT TAT TTC AGT CAC ACA-3'	
Prestin-10F	5'-GCT TGA CTT TTC AGG CTG CT-3'	195
Prestin-10R	5'-TGA AAC ATG TGC AGG AAA CTG-3'	
Prestin-11F	5'-TGT TTT GTT TCT ACA TGG GAA AGG-3'	175
Prestin-11R	5'-CCA TAT CAT CAG GTC TTA GAG GC-3'	
Prestin-12F	5'-TTC AAG TCT CCT AAA AGC CCC-3'	181
Prestin-12R	5'-AAA ATA GCA AAA TCT CTA CAC ATG AAA-3'	
Prestin-13F	5'-GGA ACC AGC AAA GGA CAC TC-3'	200
Prestin-13R	5'-TCT ATG ACA TAT TAA CAG AGC CAT CA-3'	
Prestin-14F	5'-TGT TTA ACA TGG TTC TCC ATC AA-3'	183
Prestin-14R	5'-GAC ATT CAC ACC CAT TCC AA-3'	
Prestin-15F	5'-TCA ACA CAA TGG TAT TGG ACA-3'	179
Prestin-15R	5'-AAA AAC AGT ACT TAC CCA AGC ACA-3'	
Prestin-16F	5'-AAT ATT TCC ATG TGT TAA ATT CAG-3'	200
Prestin-16R	5'-GAT CCT CAT ATC CCT GTC AA-3'	
Prestin-17F	5'-GTC CCT GAG AGC CAT GAA GT-3'	204
Prestin-17R	5'-TGC AGA ACA AGA CAG AAC GG-3'	
Prestin-18F	5'-CAA CCA GAA ATG TGA GTT GTT CA-3'	282
Prestin-18R	5'-TGA CTC GTT CAA GAG GAT GC-3'	
Prestin-19F	5'-AAG AGA CTT TCA TCT GGC ACA-3'	206
Prestin-19R	5'-AAA ATT AAG GAC ATT TCC AAG TGC-3'	
Prestin-3'F	5'-TTG TTG AAG ACT GAT ATC CAG AGT G-3'	285
Prestin-3'R	5'-GAG GCT TAT AAC CCC ATC CT-3'	

1000-fold (40–60 dB). Chemical ablation of outer hair cells causes moderate-to-severe hearing loss and deterioration of frequency selectivity (19,20), and a loss of prestin is expected to have the same effect. Indeed, a recent study of prestin knockout mice revealed hearing sensitivity that was reduced by 45–60 dB (14). In the present study, the two homozygous patients displayed a hearing loss more severe than that expected from only the loss of amplification. In addition to the loss of electromotility in outer hair cells, the knockout mice had a progressive loss of both outer and inner hair cells in the basal region of their cochlea (14). If human patients also have progressive hair-cell loss, it could account for the more severe phenotype.

The relatively high frequency of heterozygous IVS2-2A>G mutations in probands with moderate-to-profound hearing loss suggests that non-syndromic hearing loss may be caused semi-dominantly through haploinsufficiency. Because each cell makes millions of prestin molecules, one normal allele may not be able to produce enough prestin for normal function.

Heterozygous knockout mice also exhibit hearing loss, although it is milder than in most human heterozygotes (5–20 dB) (14). However, until the range of phenotypic effects in heterozygous relatives of affected probands is clearly established, the audiologic findings in individuals who were ascertained because they suffer from hearing loss will remain difficult to interpret. It is possible that, similar to the murine model, some heterozygous individuals with *SCL26A5* mutations may exhibit a mild hearing loss predominantly at high frequencies. This type of hearing loss may not be diagnosed without performing a careful audiologic test. Our findings may indicate that humans are more sensitive than mice to gene dosage effects in heterozygotes. Also a more careful audiologic evaluation will be required to ascertain the hearing profile in heterozygous family members as opposed to heterozygous probands with hearing loss. There is still a possible presence of a second mutant allele in the seven cases in the promotor region, the intronic regions and the 3' and 5' untranslated regions, which we did not screen. The variable

degree of hearing loss in heterozygotes, and the presence of the mutation in one individual with no reported hearing loss, suggest an interaction with additional modifier genes. The SLC26A5 locus may be in digenic interactions. In this case, the second mutation might involve a gene whose product interacts with prestin. Clearly, this hypothesis cannot be proven until the other mutant alleles have been found. At the same time, the carrier frequency of 0.6% suggests that prestin mutations may be a relatively common cause of recessive hearing loss in the Caucasian population.

SLC26A5 thus becomes the fourth gene in the SLC26 transporter family to be recognized as a disease gene. In SLC26A2, interestingly, a GT→GC transition (*ca* – 26 + 2T > C) in the splice donor site of a 5' UTR exon has been shown to cause disease—diastrophic dysplasia—by severely reducing mRNA levels (21). Three of these four genes reside on chromosome 7q22–7q31.1, with SLC26A3 and SLC26A4 being ~50 kb apart, and SLC26A5 ~4 Mb more proximal. Currently, two other loci for non-syndromic recessive deafness (DFNB14 and DFNB17) have been mapped to 7q31 (15,16), near prestin at 7q22.1. We further mapped prestin to chromosome 7 between 10 149 and 1015 kb, which is actually within the interval of DFNB14 (with flanking markers of D7S527 and D7S3074). However, we found that neither family carries a mutation in the prestin gene, and have thus excluded a causative role of SLC26A5 mutation in these two forms of deafness.

MATERIALS AND METHODS

Cloning of human prestin cDNA

The mouse prestin cDNA sequence was used to search for the exon sequence in the human genome, utilizing the NCBI and Celera databases. Primers were designed from the matched sequences and used to amplify RACE cDNA pool derived from human inner ear. The human inner ear RNA was provided by Dr Cynthia Morton. Human inner ear RACE cDNA was constructed using the GeneRacer kit (Invitrogen) following the manufacturer's instructions. Nested RT-PCRs were performed to amplify the 5' and 3' ends of the human prestin gene. For 5' amplification the primers used were: 3'-primer/h-pres3r for the first round of PCR, followed by 3'-nest/h-pres1r for the second. 5'-Primer and 5'-nest primers were provided by the GeneRacer kit. The conditions for the first amplification were: 94°C, 2 min, 1 cycle; 94°C, 30 s; 72°C, 1 min, 5 cycles; 94°C, 30 s, 70°C, 30 s, 72°C, 1 min, 5 cycles; 94°C, 30 s, 68°C, 30 s, 72°C, 1 min, 20 cycles; 72°C 10 min. For the second round of amplification the conditions were: 94°C, 2 min, one cycle; 94°C, 30 s, 68°C, 30 s, 72°C, 1 min, 30 cycles; 72°C, 10 min one cycle. For 3' amplification the primers used were: h-pre1f/5'-primer for the first round and h-pre3f/5'-nest for the second. 3'-Primer and 3'-nested primers were from the GeneRacer kit. The conditions for the first 3'-end amplification were the same as the 5'-end.

In addition the primers across the entire human prestin gene were designed to amplify the cDNA fragments for sequencing. A list of primer pairs used was as follows: hu-pre1f/hu-pres2r; hu-pres8f/hu-pres7r; hu-pre3f/hu-pre8r; hu-pre5f/hu-pre9r and hu-pre6f/hu-pre4r. The conditions for the amplification were:

94°C, 3 min for one cycle; 94°C, 30 s, 68°C, 30 s, 72°C, 45 s for 30 cycles; 72°C, 10 min for one cycle. The PCR fragments were purified using the PCR purification kit (Qiagen) and sequenced by a core facility. The sequences for primers used are listed in Table 1.

Northern blot analysis

We obtained a human poly (A)+ RNA multiple tissue northern blot from OriGene Technologies, Inc. Each northern blot contains 2 µg of poly (A)+ RNA per lane. The northern blot was hybridized with a random primed, triple-labeled ([³²P]dCTP) 718 bp cDNA probe, which had been PCR-amplified through the use of primer pair Hu-pres3f/Hu-pres8r from the human prestin cDNA fragment. The northern blot was hybridized at 65°C in ExpressHyb hybridization solution (Clontech) for 3 h and was washed at 65°C in 0.1×SSC/0.1% SDS for 1 h. Filters were exposed to Kodak MS film for several hours.

Clinical evaluations of patients

Probands were ascertained through the Genetics Clinic at Virginia Commonwealth University in Richmond and the Genetics Program at Gallaudet University in Washington, DC (22). Informed consent was obtained from all participants. A clinical evaluation and family history was obtained on each proband. The hearing of all affected individuals in the present series was examined using pure tone audiometry. Air conduction thresholds were measured at 250 and 500 Hz and 1, 2, 4, 6 and 8 kHz. Oto-immittance measurements were undertaken on all individuals and all were otoscopically examined to ascertain function of the middle ear. DNA samples were obtained from peripheral blood.

Mutation analysis of prestin

Twenty-one pairs of primers were designed from the flanking intronic and UTR sequence of *SLC26A5* using the Primer 3 program (www-genome.wi.mit.edu/genome-software/other/primer3.html; Table 3). The *SLC26A5* exons were amplified from genomic DNA by PCR using the above primers. The amplification conditions were 95°C for 5 min, then 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension for 5 min at 72°C. For mutation analysis, the PCR products were initially run on a 1 mm thick 8% non-denaturing polyacrylamide gel (acrylamide: *N,N'*-methylene bisacrylamide 49:1) at 4°C. Single-strand conformational polymorphisms (SSCP) were detected using silver staining as previously described (23). Direct sequencing of PCR products from patients with SSCP variants was then performed on both strands using the fluorescent dideoxy terminator method and an ABI 377 DNA sequencer.

GenBank accession numbers

We obtained the genomic sequence spanning SLC26A5 from two genomic contigs (AC005064 and AC093701). The data reported here have been deposited in NCBI databank with the accession numbers AF523354, AY256823, AY256824 and AY256825.

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