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Report

Multiple Mutations of *MYO1A,* a Cochlear-Expressed Gene, in Sensorineural Hearing Loss

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Myosin I isozymes have been implicated in various motile processes, including organelle translocation, ion-channel gating, and cytoskeleton reorganization. Unconventional myosins were among the first family of proteins found to be associated with hearing loss in both humans and mice. Here, we report the identification of a nonsense mutation, of a trinucleotide insertion leading to an addition of an amino acid, and of six missense mutations in *MYO1A* cDNA sequence in a group of hearing-impaired patients from Italy. *MYO1A,* which is located within the DFNA48 locus, is the first myosin I family member found to be involved in causing deafness and may be a major contributor to autosomal dominant–hearing loss.

Nonsyndromic hearing loss is the most common form of genetic deafness. Approximately 15%–20% of cases are transmitted as nonsyndromic autosomal dominant defects (NSAD), for which >40 different loci have been described and 17 genes identified (Hereditary Hearing Loss Homepage; Petersen 2002). Recently, we reported the mapping of a novel NSAD locus, DFNA48, to chromosome 12q13-q14 in a large multigenerational Italian family (D'Adamo et al. 2003).

The DFNA48 region contains, among others, integrin genes, the retinoic acid receptor gamma gene (*RARG* [MIM 180190]), the prefoldin 5-chaperone gene (*PFDN5* [MIM 604899]), the class I myosin *MYO1A* (MIM 601478; GenBank accession number Q9UBC5), the myosin light-chain genes (*MLC1SA* [MIM 160781]), and *MYL6,* all of which were considered good candidates for hearing loss. MYO1A, also known as "brush border myosin-I," is a particularly strong candidate, as it is a member of the myosin superfamily, which was among the first family of proteins found to be associated with hearing loss and is considered to play a major role in hair-cell function (Friedman et al. 1999). Mutations in shaker1 mutant mice and human USH1B (MIM 276903) were identified in the myosin VIIA (*MYO7A* [MIM 276903]) gene simultaneously; not long afterward, forms of both dominant and recessive hearing loss were found with *MYO7A* mutations (Gibson et al. 1995; Weil et al. 1995; Liu et al. 1997*a*, 1997*b*). Mutations in the unconventional myosin VI gene, *Myo6,* were found to be associated with deafness and vestibular dysfunction in the Snell's waltzer (*sv*) mouse (Avraham et al. 1995). Subsequently, the myosin VI (*MYO6* [MIM 606346]) gene was found to be mutated in a large Italian family affected by NSAD (Melchionda et al. 2001). A third unconventional myosin, myosin XVA (*MYO15A* [MIM 602666]), is associated with the DFNB3 locus on chromosome 17 and the shaker2 mutant mouse (Probst et al. 1998; Wang et al. 1998). Finally, most recently, it has been shown that normal hearing in humans requires myosin IIIA (*MYO3A* [MIM 606808]) (Walsh et al. 2002).

For these reasons, we decided to perform mutation screening of the *MYO1A* gene not only in patients from the large Italian kindred mapping to the DFNA48 locus but also in a large series of 230 hearing-impaired patients negative for the presence of connexin 26 (*GJB2*) gene

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mutations and attending the Medical Genetics Service of IRCCS-CSS Hospital (San Giovanni Rotondo, Italy). Inclusion criteria were: (*a*) absence of the most common mutations within the *GJB2* gene after a molecular screening performed at the Medical Genetics Service of IRCCS-CSS Hospital, (*b*) sensorineural hearing loss, and (*c*) normal tympanometric evaluation. The series includes case subjects with a variable degree of hearing loss, ranging from mild to profound, and with a variable age of onset, from congenital to late onset. Familial records were not obtained in most cases. The majority of patients came from central and southern Italy. After obtaining informed consent, peripheral blood was obtained from all subjects, and DNA was isolated from blood leukocytes according to standard methods. An overall number of 23 primer pairs were designed to amplify the 27 coding exons of the *MYO1A* gene, including the splice sites (PCR primer sequences and conditions available upon request). All amplicons were screened by denaturing high-performance liquid chromatography (DHPLC), and those showing an abnormal chromatographic profile were subsequently sequenced. DHPLC was performed on a WAVE Nucleic Acid Fragment Analysis System HSM (Transgenomic), according to supplier protocols. DHPLC data analysis was based on a subjective comparison of sample and reference chromatograms. PCR products that showed an abnormal chromatographic profile on DHPLC analysis were sequenced directly on an automated sequencer (ABI 377 and 3100; Perkin Elmer) by use of the ABI-PRISM big-dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). For evolutionary analysis, a multiple-sequence alignment (MSA) of 42 homologous myosins, obtained from the HOMSTRAD database (Mizuguchi et al. 1998), was used (available upon request). The evolutionary analysis was carried out by use of the ConSurf Web server, which uses the Rate4Site algorithm for estimating the evolutionary rates at each amino acid site (Pupko et al. 2002; Glaser et al. 2003). Rate4Site uses the maximum likelihood (ML) principle, using as input a neighbor-joining phylogenetic tree (Saitou and Nei 1987) constructed from the MSA. The ML method considers the tree topology and branch lengths, as well as the underlying stochastic process of the evolution of the sequences. For proteins with a known three-dimensional (3D) structure, the ConSurf server is used (Glaser et al. 2003). The rates of evolution among homologous proteins are mapped onto the 3D structure of one of the homologous proteins. Surface patches of slowly evolving—also referred to as "evolutionarily conserved" or simply "conserved"—residues are occasionally functionally important. Similarly, slowly evolving buried residues are usually important for maintaining the protein's 3D structure. The ConSurf scores range 1–9: 1 is for rapidly evolving (variable) sites, color coded in turquoise; 5 is the average, color coded as white; and 9 is for slowly evolving (evolutionarily conserved) sites, color coded in maroon.

After the mutational screening, we were able to identify a nonsense mutation, a trinucleotide insertion, and six missense mutations within the *MYO1A* gene, as summarized in table 1.

The nonsense mutation is due to a C→T nucleotide change at position 277 of the cDNA (relative to the ATG, designated "+1") (fig. 1*A*). This substitution changes the residue arginine to a stop signal at position 93 of the protein (R93X) in the motor domain and can be easily detected by PCR digestion, since it destroys an *Ava*II restriction site. (fig. 1*B*). This mutation was present in a male patient from southern Italy affected by a moderate-to-severe bilateral sensorineural hearing impairment, who received this mutant allele from his mother. She states she has normal hearing, but no audiological evaluation has been carried out or is currently obtainable. The healthy brother of the proband does not carry the R93X mutant allele.

A CTT insertion was detected between nucleotides 349 and 350 (349-350insCTT) of the *MYO1A* cDNA sequence. This insertion results in the addition of a serine residue after position 116 of the protein between a serine residue and a tyrosine in a region of the protein that is conserved across species (fig. 1*C*). According to the 3D model, this mutation adds a residue in the middle of a buried α-helix, which may cause structural damage (fig. 2). This mutation was found in a female patient from Sicily who is affected by a moderate-to-severe bilateral sensorineural hearing loss, particularly in the high-frequency range. Family history is positive in the maternal branch, with a dominant pattern of transmission with variable penetrance and/or expressivity.

A G→A nucleotide change was detected at position 916 of the cDNA. This change leads to an amino acid valine→methionine substitution at position 306 of the protein (V306M) and was detected in a male patient, age 40 years, who was affected by bilateral severe hearing loss, involving mainly high frequencies. We analyzed this and other missense mutations with ConSurf, an algorithmic tool that provides an estimation of the degree of con-

Table 1

***MYO1A* Mutations Identified in Italian Probands**

Exon	Nucleotide	Amino Acid
3	277C/T	R93X
4	349-350A	349-350insCTT
10	916G/A	V306M
12	1155G/T	E385D
18	1985G/A	G662E
18	2021G/A	G674D
22	2390C/T	S797F
25	2728T/C	S910P

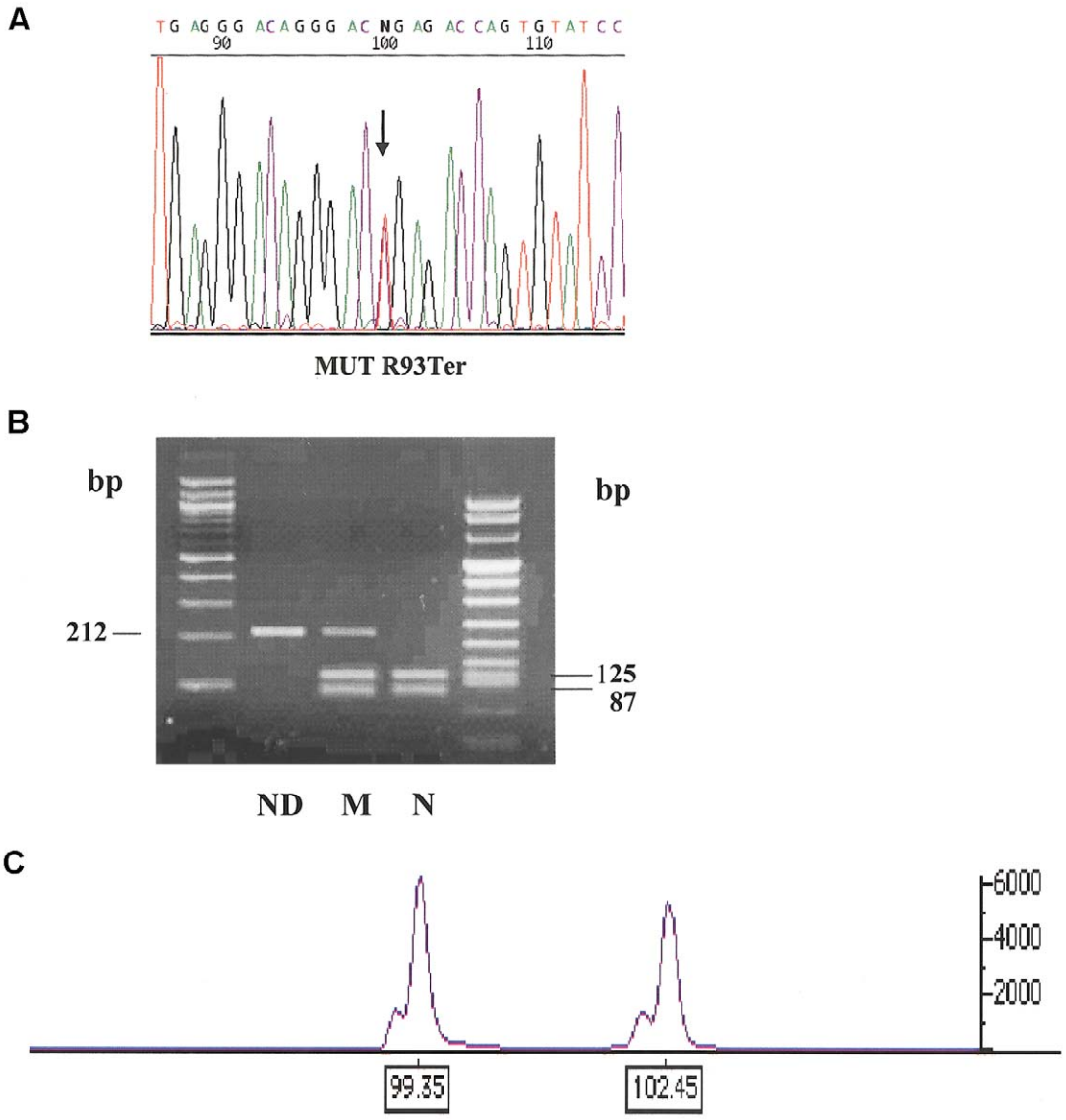

Figure 1 Nonsense and frameshift mutations. *A*, Sequence data of the nonsense R93X mutation. *B*, Results showing the presence of R93X after *Ava*II restriction enzyme digestion. The presence of the mutation destroys a restriction site. ND = not digested; M = mutated; N = normal. *C*, Electrophoretic results showing the presence of the 349-350insCTT mutation. Fluorescent primers were designed and used for detecting the presence of the insertion in a 3100 ABI Prism Capillary Electrophoresis.

servation of each residue relative to its homologues (Glaser et al. 2003). The V306M replacement affects a residue of average conservation, which suggests that this mutation may not be harmful (fig. 2). However, additional functional data are required to determine if this mutation is truly causative or if it is a rare variant.

A missense mutation (E385D) due to a G→T nucleotide change at position 1155 of *MYO1A* cDNA sequence was detected in a 10-year-old female patient from central Italy. In this case, the patient exhibits sensorineural hearing loss with an early onset (age 2 years); the degree of hearing loss was moderate to severe in the left ear but mild in the right one, and the patient does not use hearing aids. A family history of moderate-to-severe progressive hearing loss was present in the maternal branch, transmitted as an autosomal dominant trait with variable penetrance and/or expressivity. The nucleotide change leads to an amino acid substitution from glutamic acid to aspartic acid, both negatively charged residues that differ from each other by one methylene group (CH_2). However, glutamic acid at position 385 is highly conserved according to the ConSurf analysis and, therefore, is predicted to play an essential functional role in MYO1A (fig. 2). Furthermore, the 3D model demonstrates that

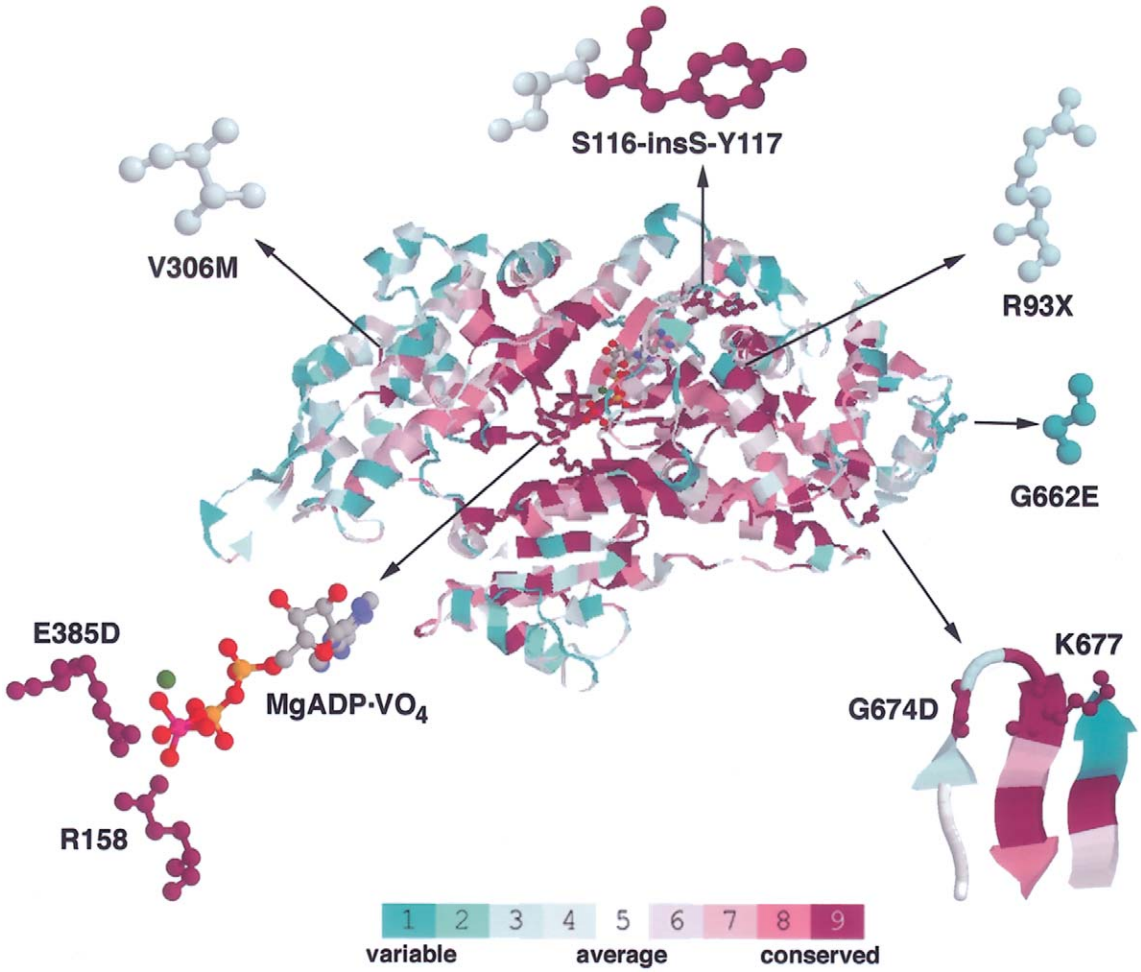

Figure 2 Ribbon representation of a 3D model of the MYO1A motor domain. The model was obtained using the experimentally determined structure of *Dictyostelium discoideum* myosin-IE as a template (Kollmar et al. 2002). The two motor domains share 45% sequence identity with each other. The homology-model building was carried out using a pairwise alignment of these two proteins and the Nest facility of the Jackal protein structure modeling package. The degree of conservation of the residues in MYO1A was analyzed using ConSurf (Glaser et al. 2003); residue conservation scores, calculated using 42 homologous sequences, are color coded onto the structure of MYO1A (see key). ConSurf assigns amino acid conservation grades in range 1–9, where 9 is maximal conservation and 1 is minimal conservation—that is, highly variable. The residues mutated in the MYO1A motor domain are shown using ball-and-stick representation (insets).

the highly conserved E385 residue lies in close proximity to the transition state analog MgADP•VO_4 in the ATP-binding site. Its negatively charged carboxyl end may form a hydrogen bond with a highly conserved arginine 158 (R158). Therefore, even a conservative replacement to an aspartic acid may destabilize the electrostatic interactions within the ATP-binding site and is likely to affect ATPase activity.

Another G→A change affecting nucleotide 1985 was detected in a 5-year-old male patient affected by a mild sensorineural hearing loss. This change leads to a substitution of an exposed glycine residue with glutamic acid at position 662 (G662E) that appears to be variable in nature (fig. 2). Thus, functional assays must be performed to determine whether this change is a mutation or polymorphism.

We also detected a G→A transition at position 2021 of the cDNA sequence, which replaces a glycine (GGC) with an aspartic acid (GAC) at residue 674 of the protein (G674D). According to the ConSurf analysis, this change affects a residue that is exclusively conserved among all the homologues (fig. 2). In the 3D model, G674 forms a β-hairpin through main-chain interactions with lysine 677 (K677). A small amino acid, such as glycine, is crucial for forming this β-turn. Replacement by a large amino acid, such as an aspartic acid, is likely to cause steric hindrance that can destabilize the β-core and the 3D-fold of the protein. Therefore, this mutation may cause

structural, rather than functional, damage. This mutation has been detected in a male patient from southern Italy, age 13 years, who is affected by a moderate-to-severe bilateral hearing loss. No family records are available.

A missense mutation (S797F) due to a C→T nucleotide change at position 2390 of the *MYO1A* cDNA sequence was detected in a patient affected by a moderate sensorineural hearing loss with an early onset (age 6 years). The patient inherited the mutation from his hearing-impaired father, with a classical autosomal dominant pattern of transmission. The nucleotide change leads to an amino acid substitution of a serine residue with a phenylalanine (fig. 2) in a region that is unique; therefore, too few homologues can be collected to perform a reliable evolutionary analysis.

Finally, a T→C change affecting nucleotide 2728 was detected in a male patient from southern Italy, affected by a severe bilateral sensorineural hearing loss. Family records are negative. This change leads to a substitution to a proline at position 910 (S910P) in the myosin tail domain (fig. 2) in a region with few homologues. Nevertheless, this change can create a "kink" that may destabilize the tail domain.

In addition, the analysis of the coding region and exon-intron boundaries of the *MYO1A* gene identified five intronic nucleotide variants and one third-base change in the coding region that either do not affect the MYO1A-predicted protein or did not segregate with deafness (data not shown). No mutation has been thus far identified in the coding region or exon-intron boundaries of the *MYO1A* gene in the large multigenerational Italian family mapping to DFNA48. We are now working to rule out the possible presence of a deletion or of a mutated allele in the genomic regions not yet analyzed, such as introns or promoters. Alternatively, one of the other candidate genes in the region may lead to deafness in the large family.

The presence of the eight above-mentioned mutations was tested for on 200 normal chromosomes of individuals coming from the same geographical area of patients. None of them were detected in any normal chromosome.

To evaluate *Myo1a* (GenBank accession number AF009960) expression in the inner ear, we used RT-PCR analysis of RNA extracted from mouse inner ears and cochlea. The predicted genomic structure of the *Myo1a* gene was obtained from the Ensembl Mouse Genomic Browser. Primers spanning three introns and covering a total of 1.7 kb of genomic sequence were chosen so that the 417-bp cDNA PCR product could be distinguished from genomic DNA (primers available on request). A strong band was obtained in cDNA derived from E14, E16, E18, and P0 inner ears (fig. 3) and confirmed by direct sequencing. Further dissection of cochlea from P0 revealed the same transcript as well (data not shown).

In conclusion, the human *MYO1A* gene maps within

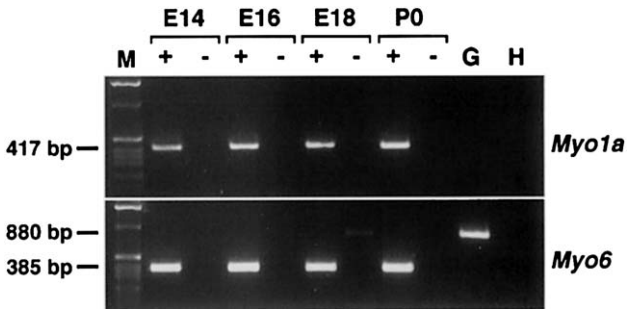

Figure 3 *Myo1a* and *Myo6* in mouse inner ear. At each of E14, E16, E18, and P0, one RNA preparation was used to amplify the two genes by RT-PCR. Amplifications were carried out with (+) and without (−) reverse transcriptase, by use of inner-ear RNA, genomic DNA (G), and H_20 control (H). The *Myo1a* fragment of 417 bp is the predicted size of the amplicons spanning *Myo1a* exons 13–16. Predictions were verified by sequencing the products. The *Myo6* amplification (Ahituv et al. 2000) was used as a positive control for inner-ear expression. Fragment of 385 bp and 880 bp represents the predicted sizes of these amplicons from cDNA and genomic DNA, respectively.

the DFNA48 region and was an excellent candidate because of its chromosomal location, cochlear expression, and function. A nonsense mutation, a trinucleotide insertion leading to an additional amino acid, and six missense mutations have been identified in eight unrelated patients coming from central and southern Italy and affected by sensorineural bilateral hearing loss of variable degree, usually ranging from moderate to severe but never profound. In some cases, it was possible to trace a clear family history of hearing loss, confirmed also by audiometric evaluations. In contrast, for most of our patients, it was not possible to access clinical information and/or perform any audiological evaluation of the patients' relatives; therefore, we have a lack of accurate information on the audiological status of parents and relatives. In the case of our patient with the R93X nonsense mutation, such an evaluation of his relatives may have important relevance. The proband inherited the mutant allele from his mother, who claims she has normal hearing. So far, we do not have any audiological examination of the mother, and she is not available for future investigations. Very frequently, with progressive forms of hearing loss, individuals refer to themselves as having normal hearing even if audiological evaluations demonstrate that their thresholds are not normal. Thus, in this case of the patient with the R93X allele, the "normal hearing" claimed by the proband's mother could be explained by a lower threshold considered normal by the mother herself. However, if the mother were tested and an audiological examination revealed normal hearing, it would suggest reduced penetrance, as is known to occur in other forms of hereditary hearing loss or other genetic diseases transmitted as an autosomal dominant trait.

The identification of a nonsense mutation, an in-frame insertion, and several missense mutations in the *MYO1A* gene associated with hearing loss suggests a crucial role for the MYO1A protein in hearing. For the six missense mutations found, we used ConSurf to analyze the importance of each mutated residue for maintaining the structure or function of the protein. This algorithmic tool provides an estimate of the evolutionary rate at each amino acid site, using a statistically rigorous approach that takes into account the phylogenetic relationships within the protein family. Two missense mutations described here, E385D and G674D, affect residues that are slowly evolving and, therefore, may play a fundamental role for the proper function or structure of the protein. On the basis of our analysis, we predict that four of six missense mutations found contribute to *MYO1A*-associated hearing loss. A functional analysis of all missense mutations is required to further validate their role in deafness. In addition, none of the missense mutations reported here have been detected in normal chromosomes.

The myosin I isozymes were the first unconventional myosins to be purified and have been implicated in various motile processes, including organelle translocation, ion-channel gating, and cytoskeletal reorganization, but their exact cellular functions are still unclear. All members of the myosin I family, from yeast to man, have three structural domains: a catalytic head domain that binds ATP and actin, a tail domain believed to be involved in targeting the myosins to specific subcellular locations, and a junction or neck domain that connects them and interacts with light chains. Brush border myosin-I (MYO1A) is a major component of the actin-rich cytoskeleton of the brush border surface in intestinal epithelial cells and is strongly expressed in the large and small intestine (Skowron et al. 1998). The expression of *Myo1a* was barely detected by RT-PCR in RNA extracted from neonatal mouse utricles, a component of the vestibular system (Dumont et al. 2002). We were successful in amplifying *Myo1a* from mouse inner-ear cDNA at several developmental stages and from cochlea at P0. A role for MYO1A in the inner ear has not been established, although the actin-rich cytoskeletal structure of intestinal and inner-ear cells, including hair cells and supporting cells, are similar. In the intestine, MYO1A may play a role in membrane trafficking (Skowron and Mooseker 1999). As myosin VI is predicted to have similar roles of transport in the kidney and inner ear (Tuxworth and Titus 2000), the same may hold true for MYO1A. Additional studies are required to better understand the pathogenic mechanisms leading to hearing loss as a consequence of an *MYO1A* mutant allele.

MYO1A is the first myosin I family member found to be linked to human hereditary deafness, now joining another class I myosin that was found to play an essential role in the auditory transduction mechanism, myosin-1c (MYO1C [MIM 606538]) (Holt et al. 2002). It will be interesting to investigate the potential role of other cochlear-expressing myosin I genes, such as *MYO1C* and *MYO1E* (MIM 601479), in hearing loss (Dumont et al. 2002).

Finally, the identification of at least six different mutations suggests that this gene may be a significant contributor to autosomal dominant cases. In fact, only a few genes among those so far described present with mutations in more than a single family (Petersen 2002). Additional studies are required to definitively prove the real contribution of *MYO1A* to deafness in a population base.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

ConSurf Server, http://consurf.tau.ac.il/ (for estimating the evolutionary rates at amino acid sites)

Ensembl Genome Browser, http://www.ensembl.org/Mus_musculus/ (for mouse genome)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for human MYO1A [accession number Q9UBC5] and mouse Myo1a [accession number AF009960])

Hereditary Hearing Loss Homepage, http://www.uia.ac.be/dnalab/hhh/ (for the genetics of hereditary hearing impairment)

Homologous Structure Sequence Alignment (HOMSTRAD), http://www-cryst.bioc.cam.ac.uk/~homstrad/

Jackal, http://trantor.bioc.columbia.edu/~xiang/jackal/#nest (for protein structure modeling)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *RARG, PFDN5,* MYO1A, *MLC1SA, MYL6,* USH1B, *MYO7A, MYO6, MYO15A, MYO3A, MYO1C,* and *MYO1E*)

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