From flies' eyes to our ears: Mutations in a human class III myosin cause progressive nonsyndromic hearing loss DFNB30

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Normal vision in *Drosophila* requires NINAC, a class III myosin. Class III myosins are hybrid motor-signaling molecules, with an N-terminal kinase domain, highly conserved head and neck domains, and a class III-specific tail domain. In *Drosophila* rhabdomeres, NINAC interacts with actin filaments and with a PDZ scaffolding protein to organize the phototransduction machinery into a signaling complex. Recessive null mutations in *Drosophila* NINAC delay termination of the photoreceptor response and lead to progressive retinal degeneration. Here, we show that normal hearing in humans requires myosin IIIA, the human homolog of NINAC. In an extended Israeli family, nonsyndromic progressive hearing loss is caused by three different recessive, loss-of-function mutations in myosin IIIA. Of 18 affected relatives in Family N, 7 are homozygous and 11 are compound heterozygous for pairs of mutant alleles. Expression of mammalian myosin IIIA is highly restricted, with the strongest expression in retina and cochlea. The involvement of homologous class III myosins in both *Drosophila* vision and human hearing is an evolutionary link between these sensory systems.

Genetic analysis of naturally occurring mutations leading to hearing loss is a powerful tool for revealing the molecular mechanisms that control the development and maintenance of normal hearing (1). Mutations that occur naturally in human families have led thus far to the mapping of more than 70 loci and the discovery of 24 genes that control human hearing (more on the Hereditary Hearing Loss web site at: http://dnalab-www.uia.ac.be/dnalab/hhh/). Extended families from endogamous communities are ideally suited for these analyses. The unique demographic history of the Middle East has led to many such communities. Family N, the subject of this study, traces its ancestry to the Jewish community of Mosul, Iraq. This community dates to 586 B.C. and was highly endogamous, with considerable emigration but little immigration, for more than 2,500 years. Most remaining Jewish residents of Mosul, including Family N, migrated to Israel in 1950–51.

Members of three generations of Family N have experienced bilateral progressive hearing loss, which first affects the high frequencies. Hearing loss begins in the second decade, and by age 50 is severe in high and middle frequencies and moderate at low frequencies (Fig. 1). Vision and balance of all affected individuals are normal. The purpose of this project was to identify the gene and mutant allele(s) responsible for inherited hearing loss in Family N.

Methods

Clinical Assessment of Family N. Hearing of both unaffected and affected participants in Family N was measured by pure-tone audiometry at audiological centers in Israel. Blood samples were drawn be venipuncture after obtaining informed consent in accordance with guidelines of the Tel Aviv University Helsinki Committee and University of Washington Human Subjects Division (approval #99–1150).

Gene Mapping. Genome-wide linkage analysis was carried out by using 379 markers from ABI PRISM Linkage Mapping Set 2 (Applied Biosystems) genotyped on an ABI 377 (Applied Biosystems). Allele sizes were determined by using ABI GENESCAN V.2.02 software. Software developed in our lab (available on request from M.K.L.) displays genotypes on pedigrees and collects data for linkage analysis by FASTLINK V.3.0 (2) and LINKAGE V.5.1 (3).

Sequence Analysis of Candidate Genes. Primer pairs were designed to amplify and sequence coding regions and splice sites of all candidate genes. Primer pairs developed for sequencing human myosin IIIA from genomic DNA are provided in Table 1, which is published as supporting information on the PNAS web site, www.pnas.org. Sequencing was carried out by using BigDye primer cycle-sequencing chemistry (Applied Biosystems) and was then visualized on ABI 377 sequencers. Sequences were aligned and compared by the use of SEQHELP (4). Human myosin IIIA is currently represented in GenBank by entries NM 017433 and XM011851. These entries differ from each other, but both include errors. Corrected sequence of human myosin IIIA, as used in this project, has been submitted to GenBank (accession no. AY101367).

Myosin IIIA Genotypes of Hearing Controls. DNA was obtained from normal hearing controls, including 172 Israelis of Iraqi Jewish ancestry (5), 96 Israelis of Ashkenazi Jewish ancestry, and 96 Palestinians of Arabic ancestry. The three myosin IIIA mutations were genotyped in genomic DNA by using exon 28 primers and *Xcm*I to detect 3126 T->G, exon 18 primers and *Bsr*I to detect 1777(-12)G->A, and exon 9 primers and *Psi*I to detect 732(-2)A->G.

RNA Expression from Mutant Alleles. Total RNA was prepared from EBV-transformed lymphocytes with RNeasy (Qiagen). Myosin IIIA gene-specific cDNA was primed from an oligonucleotide in the 3′-UTR, 5′-TTGAACGGTTAGGACTGCTGGACG-3′ with 5 μg of total RNA and was reverse transcribed with Superscript II (Invitrogen). The effect on the myosin IIIA message of allele 1777(-12) G->A was determined by amplifying

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Abbreviation: P*n*, postnatal day *n*.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY101367 and AY101368).

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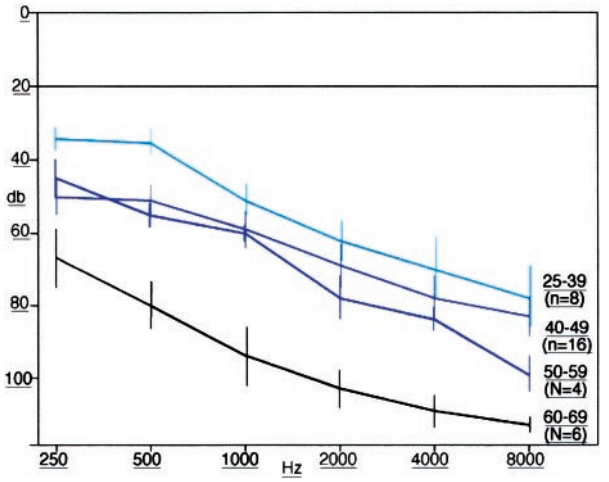

Fig. 1. Progressive hearing loss in Family N. Hearing thresholds in decibels (dB), measured by pure-tone audiometry, are shown for sound frequencies ranging from very low (250 Hz) to very high (8000 Hz). Hearing thresholds (with standard errors) are shown for affected relatives of Family N. The number of audiograms (N) included for relatives aged 25–39, 40–49, 50–59, and 60+ years are indicated.

myosin IIIA gene-specific cDNA flanking exons 16 and 19 in a nested PCR by using outer primers (F) 5′-GCCCATTACAAACTGCCTG-3′ and (R) 5′-TCCTCTAGTGACCACACAGTGG-3′ and inner primers (F) 5′-CAAACTGCCTGAAAATAAGCC-3′ and (R) 5′-CTTCTTGTAGCTCATCTGCCC-3′. Message stability of the myosin IIIA allele 732(-2)A->G was determined by comparing the heterozygosity of the single nucleotide polymorphism(SNP) at bp 2496 in exon 22 in genomic DNA vs. cDNA. Myosin IIIA gene-specific cDNA was amplified with two sets of primers flanking exons 21 and 23 in a nested PCR by using outer primers (F) 5′-GGGAACAGAATGAATACCTAAATG-3′ and (R) 5′-TTTGGTCAGAGGGTGGTTG-3′ and inner primers (F) 5′-CCTAAATGAAGATGTGGATGC-3′ and (R) 5′-GGATGACCTCAAAAGTAGCAC-3′.

Determination of the Mouse Myosin IIIA Sequence. Publicly available mouse sequence traces (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi) were aligned to human myosin IIIA by BLAST (6). Primers defined from the putative mouse sequence were used to amplify and sequence mouse eye total RNA (CLONTECH). The complete mouse sequence was determined and confirmed by sequencing overlapping fragments. Genomic sequence flanking mouse exons was determined by aligning confirmed exons to publicly available traces by BLAST and then identifying primers and sequencing mouse genomic DNA. Mouse Myosin IIIA coding sequence, genomic structure, and predicted protein sequence are shown in Figs. 7 and 8, which are published as supporting information on the PNAS web site and have been submitted to GenBank.

Myosin IIIA Expression in Mouse Cochlea. All experiments with mice were carried out with the approval of the Tel Aviv University Animal Care and Use Committee (11-00-65). Cochleae were dissected out of the temporal bones of mice at postnatal days (P*n*) P0, P5, and P10. Total RNA was prepared from cochleae with Tri reagent (Sigma). Genomic DNA was removed from all RNA samples with DNA-Free (Ambion). Total RNA was purified by using phenol-chloroform extraction (GIBCO) and phase-lock gel tubes (Eppendorf), followed by an isopropanol precipitation. Reverse transcription (RT) reactions were performed by using Expand Reverse Transcriptase (Roche Molec-

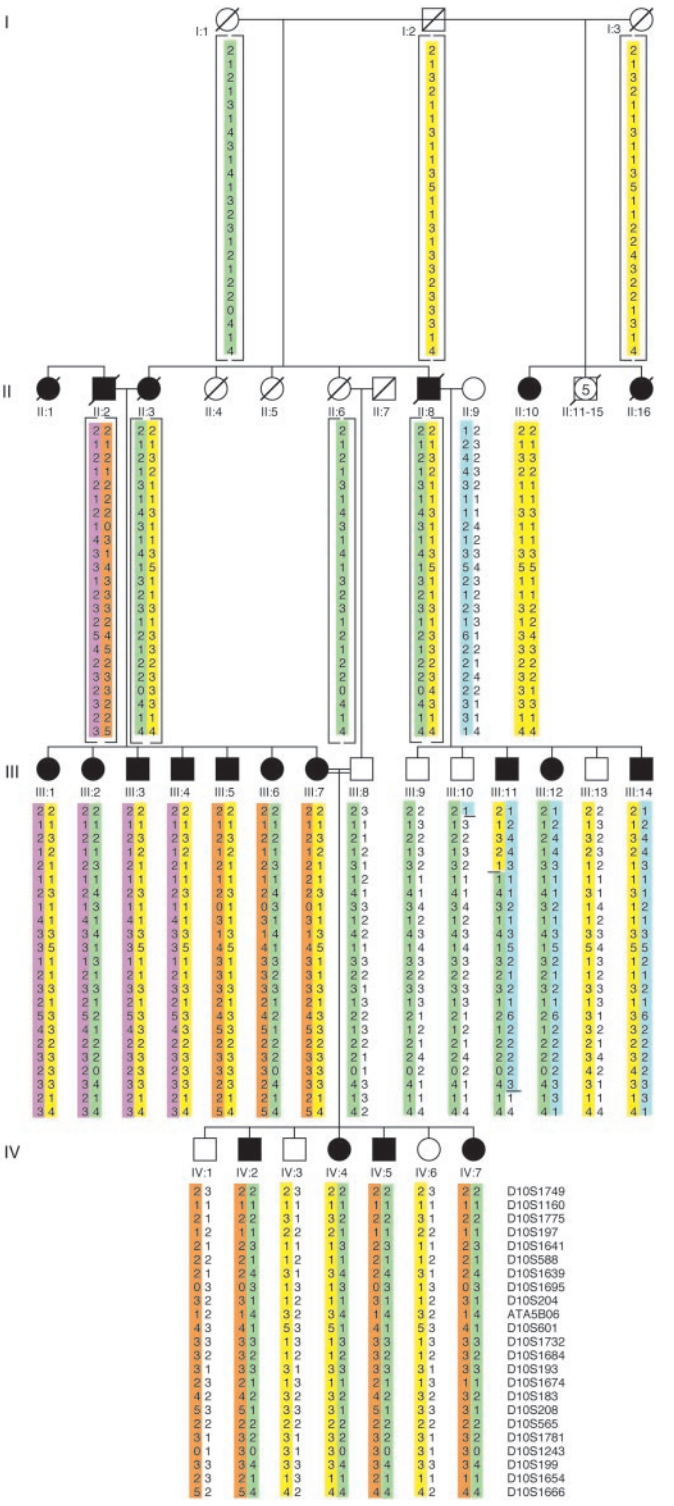

Fig. 2. Chromosome 10p haplotypes linked to deafness in Family N. Deaf individuals are indicated by black symbols. All individuals included in the pedigree are more than 30 years of age, older than onset of hearing loss in the family. Under a recessive model with age-dependent expression, a 13-cM region of chromosome 10p12-p11 bounded by D10S1749 and D10S1654 is linked to hearing loss. Deaf individuals are homozygous or compound heterozygous for any of five different extended haplotypes, indicated in orange, green, pink, yellow, and blue. Homozygosity mapping did not reveal any regions of homozygosity shared by all deaf individuals, suggesting multiple mutant alleles leading to deafness in the family.

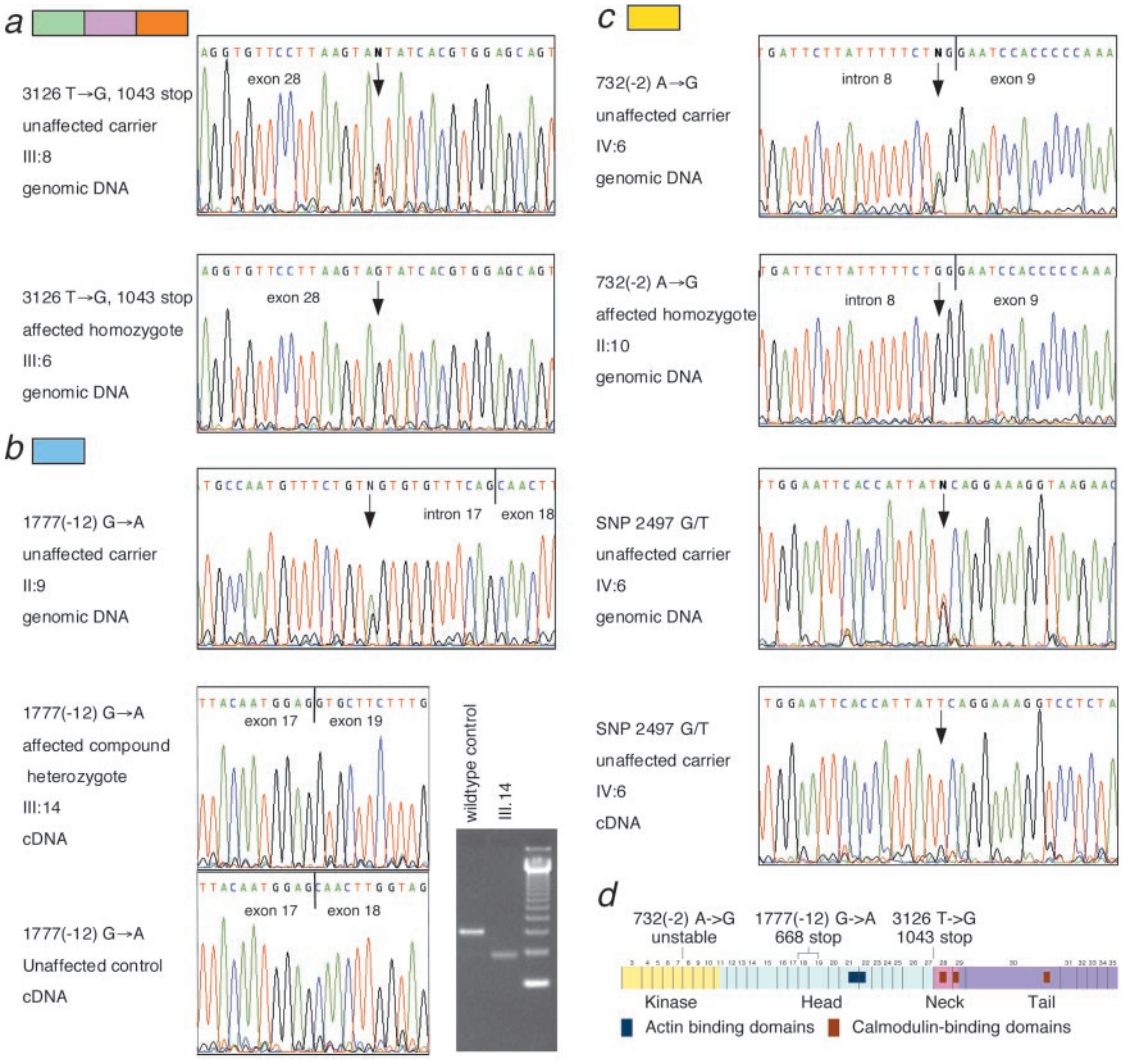

Fig. 3. Three different mutations of myosin IIIA are associated with deafness in Family N. (*a*) 3126 T->G in exon 28 changes TAT to TAG and hence creates a stop at codon 1043. This mutation is illustrated by genomic DNA sequences of unaffected heterozygote III:8 and affected homozygote III:6. All haplotypes shown in green, orange, and pink in Fig. 2 carry this nonsense mutation. (*b*) 1777(-12) G->A in intron 17 causes a splicing error that leads to deletion of exon 18 and a stop at codon 668. This mutation is illustrated by genomic sequence of unaffected heterozygote II:9. The consequence of this mutation is illustrated by cDNA sequences of compound heterozygote III:14 compared with a wild-type control. Furthermore, amplification of exons 16–19 from cDNA of III:14 yields a 183-bp fragment, as expected if exon 18 is deleted from the message. All haplotypes shown in blue in Fig. 2 carry this mutation. (*c*) 732(-2) A->G in intron 7 destroys the canonical (AG) splice acceptor of exon 8 and seems to lead to an unstable message. This mutation is illustrated by genomic sequence of unaffected heterozygote IV:6 and affected homozygote II:10. The absence of stable message made from this allele is illustrated by comparing SNP genotypes in genomic DNA vs. cDNA of a heterozygous carrier of the mutation, IV:6. The mutation at 732(-2) A->G is in disequilibrium with allele G of the SNP at myosin IIIA bp 2496. Genomic DNA of IV:6 is heterozygous for 2496 G/T, whereas cDNA of IV:6 is hemizygous for 2496 T. Similarly, amplification of exons 16–19 from cDNA of III:14 (*b*, above) yields the 183-bp message associated with the 1777(-12) G->A allele but not the 310-bp transcript that would be associated with the 732(-2) A->G allele. All haplotypes shown in yellow in Fig. 2 carry the 732(-2) G->A mutation. (*d*) Sketch of the functional domains of myosin IIIA with sites of the Family N mutations indicated.

ular Biochemicals) with Homo-Oligomeric DNA d(T)12–18 and Random Hexamer (Amersham Pharmacia). cDNA was amplified from the RT reactions by using primers designed to exons 30 and 35 of mouse myosin IIIA, (F) 5′-CCCAGCAACTGAAGCCATTTTATC-3′ and (R) 5′-ACTTTCTGAAGCTCCGTAGGTGA-3′, primers spanning the last intron of *InaD*, (F) 5′-AGCTGCCAGAGATGGAAGATTATG-3′ and (R) 5′-AACACCTCCAAGTTCTCCTCGTC-3′, and primers spanning the last intron of *Myo6* (F) 5′-CTGGTGGTATGCCCATTTTGA-3′ and (R) 5′-TCGCTTTGCATAAGGCATTTCTA-3′.

***In Situ* Hybridization on Cochleae.** Cochleae were dissected from surrounding tissue and fixed by immersion in 4% (wt/vol) paraformaldehyde in PBS for 12–16 h at 4°C. We performed whole-mount *in situ* hybridization as described (7), with modifications. *In situ* reaction products were visualized by cryoprotecting whole cochleae with sucrose, embedding the tissue in OCT, and cryostat sectioning (10 μm). To generate the RNA probe, a 736-bp fragment of the tail region of *Myo3a* was subcloned in pPCR-Script Amp SK(+) cloning vector (Stratagene). Sense and antisense probes were made by *in vitro* transcription and labeling with digoxigenin (Roche Molecular Biochemicals).

Results

The best fitting likelihood model for inheritance of deafness in Family N was recessive with age-dependent penetrance, al-

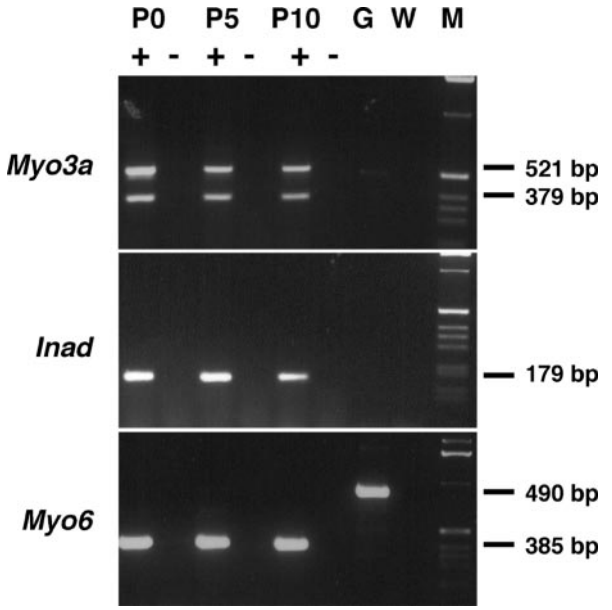

Fig. 4. Expression of *Myo3a, Inad*, and *Myo6* in mouse cochlea. At each of P0, P5, and P10, one RNA preparation was used to amplify the three genes by RT-PCR. Amplifications were carried out with (+) and without (−) reverse transcriptase, by using cochlear RNA, genomic DNA (G), and water control (W). *Myo3a* fragments of 521 bp and 379 bp are the predicted sizes of the amplicons spanning *Myo3a* exons 30–35 and the same amplicon with exon 34 deleted in-frame. Predictions were verified by sequencing the products. The *InaD* fragment of 179 bp and the *Myo6* fragment of 385 bp represent the predicted sizes of these amplicons from cDNA.

though dominant inheritance could not be excluded. To map the hypothetical gene for inherited hearing loss in the family, genome-wide linkage analysis was carried out based on 23 informative relatives (Fig. 2). A model of age-dependent recessive inheritance suggested linkage to markers D10S548 and D10S197. Genotypes of multiple markers on chromosome 10p defined an interval between D10S1749 and D10S1654 linked to deafness with an lod (score) of 4.3. The size of the linked region was 13 cM (information on the genetic map is available at: http://research.marshfieldclinic.org/), corresponding to 10 MB (information on the physical map is available at: http://genome.ucsc.edu/). This deafness locus was assigned the name DFNB30.

Homozygosity mapping was complex in Family N. No microsatellite markers in the DFNB30-linked region were homozygous for all deaf individuals (Fig. 2). Therefore, to identify any subregions that were homozygous among all affected relatives, we genotyped the family for SNPs throughout the 10 MB region. When fine mapping by SNPs revealed no subregion homozygous in all affected relatives, we concluded that multiple alleles (or even multiple genes) were likely to be responsible for deafness in the family. Hence, we focused on portions of the linked region with the greatest identity by descent of marker alleles among affected individuals. Our rationale was to identify regions that hypothetically had the minimum number of different mutant alleles.

To prioritize genes for further analysis both by function and by identity-by-descent of nearby markers in subsets of affected relatives, we reevaluated the transcript map of the 10-MB DFNB30 genomic space, reassembling publicly available sequence data (http://genome.ucsc.edu/) and annotating each clone by using SEQHELP (4). At the time of the study and at the present time, sequence of this region is incomplete. In addition, part of the region is duplicated, leading to ambiguities in assembly. (The duplication also led to our identification of a 13-bp deletion in the apparent coding sequence of an excellent candidate gene that turned out to be a previously unidentified processed pseudogene with genomic structure identical to its functional homologue.)

The region surrounding markers D10S1775 and D10S1160 was identical by descent among individuals with haplotypes indicated in green, pink, and orange in Fig. 2, potentially reducing by two the number of different mutations predicted in the family. Based on the genome draft assembly, these markers are located within introns of myosin IIIA (8), an alternately spliced 6.5-kb gene of 35 exons spanning 308 kb of genomic sequence. Myosin IIIA (*MYO3A*) seemed to be an excellent candidate for DFNB30, because four other myosins are associated with hearing loss (9, 10), and because myosin IIIA is homologous to NINAC, which is critical to a sensory process in flies (11–15).

Based on the combined logic of position and of homology, we sequenced myosin IIIA from genomic DNA of members of Family N representing each of the haplotypes illustrated in Fig. 2. We discovered three different myosin IIIA mutations cosegregating with hearing loss in Family N (Fig. 3; basepairs are counted from +1 at the initial ATG). A nonsense mutation at codon 1043, 3126T->G, caused protein truncation at the junction of the head and neck domains and was associated with three different extended haplotypes in the family (Fig. 3*a*). A mutation in the splice acceptor of intron 17, 1777(-12)G->A, led to deletion of exon 18 and protein truncation at codon 668 in the myosin head domain (Fig. 3*b*). A mutation in the splice acceptor of intron 8, 732(-2)A->G, led to an unstable message, as revealed by the absence of message from this allele in persons who carried the mutation in their genomic DNA (Fig. 3 *b* and *c*). These three mutations fully explained the hearing loss of Family N, in that there was complete concordance of myosin IIIA genotypes and hearing loss. All homozygotes and compound heterozygotes are deaf. All simple heterozygotes are carriers with normal hearing.

Family N members had reported to us that the age of onset of hearing loss seemed to differ among relatives, even within the same sibship. With the identification of three different myosin IIIA mutations, this variability was explained by the correlation between genotypes and hearing thresholds measured by puretone audiometry. Between ages 25 and 50, hearing across all frequencies was significantly poorer among individuals homozygous for the nonsense mutation (III:2, III:6, IV:2, IV:5, and IV:7 of Fig. 2), than among individuals heterozygous for the nonsense mutation and either of the splice mutations (III:1, III:3, III:4, III:5, III:7, III:12, and IV:4 of Fig. 2). The value of the F test with 6 and 9° of freedom was 13.5, corresponding to $P = 0.0005$ (see also Fig. 6, which is published as supporting information on the PNAS web site). Hearing loss was equally severe in all affected individuals by the sixth decade.

Family N illustrates how in a single family, a phenotype may be caused by multiple, independent, individually rare alleles at the same locus. Genotypes of members of Family N and carrier frequencies among Iraqi Jews indicate that the nonsense mutation is likely to be the most ancient of the three mutations in the family. First, multiple recombination events have occurred in Family N between the nonsense mutation and other sites on chromosome 10p, leading to three different DFNB30-linked haplotypes with the nonsense allele (Fig. 2). In addition, among 172 Iraqi Jewish hearing controls, four (2.3%) carried the nonsense mutation; none carried either splice mutation. None of the three mutations were observed among 96 Ashkenazi Jewish hearing controls or among 96 Palestinian hearing controls. The existence of three different myosin IIIA mutations in the same kindred also suggests that other mutations in this gene may exist, although individual mutant alleles may be rare. We speculate that late-onset hearing loss in persons from other populations

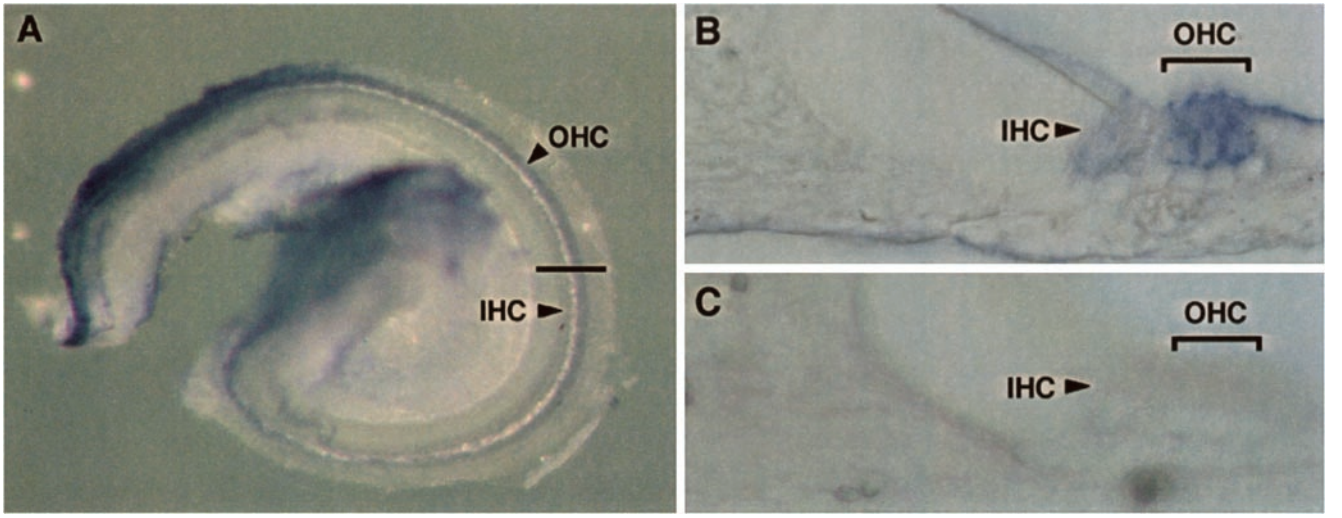

Fig. 5. Expression of myosin IIIA in mouse cochlear hair cells demonstrated by *in situ* hybridization. (*A*) Whole mount of mouse cochlea at P0 reveals specific labeling of hair cells by the *Myo3a* antisense probe (arrows). The line indicates the orientation of the cross sections. (*B*) Cross section of the cochlear duct showing specific labeling of the inner hair cells (IHC) and outer hair cells (OHC) with *Myo3a* antisense probe. (*C*) Cross section of the cochlear duct showing absence of labeling with the *Myo3a* sense probe.

may be caused by other, as-yet-unknown mutations in myosin IIIA with more attenuated phenotypes.

To evaluate myosin IIIA expression in the inner ear, we determined the mouse myosin IIIA gene sequence and genomic structure. When human myosin IIIA was first identified, its expression in retinal epithelium and pancreas was demonstrated (8). We used the mouse myosin IIIA sequence to test whether the gene is expressed in cochlea. RT-PCR amplification of RNA from mouse inner ear, using primers in the myosin IIIA tail domain, yielded two products, one corresponding to predicted myosin IIIA exons 30–35 and the other an in-frame deletion lacking exon 34 (Fig. 4). Expression appeared strong from P0 onward. Because the *Drosophila* homolog of myosin IIIA binds to the PDZ scaffolding protein INAD, we also evaluated expression in cochlea of mouse *InaD* (GenBank accession no. AF326527). *InaD* is expressed in cochlea at all tested ages (Fig. 4). Whole-mount RNA *in situ* hybridization on cochlear ducts revealed myosin IIIA expression to be restricted to the neurosensory epithelium, specifically to inner and outer hair cells (Fig. 5).

Discussion

Inherited hearing loss is genetically heterogeneous, caused by mutations in genes controlling molecular motors, hair-cell structure, neuronal innervation, signal transduction, and a variety of other processes (1, 10). Identification of myosin IIIA as DFNB30 adds a fifth myosin to those already associated with human hearing loss (see http://dnalab-www.uia.ac.be/dnalab/hhh/). Recessive hearing loss with congenital or very young onset can be caused by mutation of myosin VIIA or XV, and dominant hearing loss that progresses with age can be caused by mutation of myosin VIIA, VI, or myosin heavy chain 9 (*MYH9*). The hearing loss of Family N is unique in that it is recessively inherited yet progressive. It remains to be seen whether other, as-yet-unidentified mutations in myosin IIIA lead to dominant progressive hearing loss.

Initially, it was surprising that affected relatives of Family N retain normal vision, because myosin IIIA is expressed in both retina (8) and cochlea (Figs. 4 and 5), and mutations in *Drosophila NINAC* lead to retinal degeneration (12–15). However, in human retina there may be functional redundancy of class III myosins. In particular, myosin IIIB, which shares 65% identity with myosin IIIA in the kinase, head, and neck domains, has been identified in human retina (GenBank AF369908). Also, it is possible that as-yet-undetected myosin IIIA mutations in other families may lead to loss of both vision and hearing. By analogy, some mutations in myosin VIIA lead to nonsyndromic deafness, whereas other mutations in the same gene lead to both hearing loss and retinitis pigmentosa, or Usher 1B (reviewed in ref. 1).

The demonstration that a class III myosin is required for normal human hearing adds both information and complexity to the puzzle of sensory system evolution. The kinase, head, and neck domains of class III myosins are highly conserved across species (8, 9). Based on the conservation of these domains between NINAC and myosin IIIA, both the kinase and actin-binding functions are likely to be conserved as well (16). In contrast, NINAC and myosin IIIA tail domains share no detectable sequence similarity. The NINAC tail domain interacts with the PDZ scaffolding protein INAD (11, 13, 17), whose mammalian homolog is expressed in cochlea (Fig. 4). The ligand(s) of the myosin IIIA tail domain are not yet known, so it remains a mystery whether myosin IIIA forms a signaling complex with a PDZ scaffolding protein. The identification of the myosin IIIA tail domain ligand will enable the test of the functional equivalence of the NINAC-INAD signaling complex in the *Drosophila* eye and myosin IIIA and its ligand(s) in the mammalian ear (18, 19).

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