Nonsyndromic Deafness DFNA1 Associated with Mutation of a Human Homolog of the *Drosophila* Gene *diaphanous*

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The gene responsible for autosomal dominant, fully penetrant, nonsyndromic sensorineural progressive hearing loss in a large Costa Rican kindred was previously localized to chromosome 5q31 and named *DFNA1*. Deafness in the family is associated with a protein-truncating mutation in a human homolog of the *Drosophila* gene *diaphanous*. The truncation is caused by a single nucleotide substitution in a splice donor, leading to a four–base pair insertion in messenger RNA and a frameshift. The diaphanous protein is a profilin ligand and target of Rho that regulates polymerization of actin, the major component of the cytoskeleton of hair cells of the inner ear.

DFNA1 defines the autosomal dominant, fully penetrant, sensorineural progressive hearing loss of kindred M of Costa Rica (OMIM 124900) (Fig. 1) (*1*, *2*). In this kindred, low-frequency deafness begins at about 10 years of age and progresses by age 30 to profound, bilateral deafness involving all frequencies. The ratio of affected to unaffected children of deaf parents is 1:1; males and females are equally likely to be affected. Deafness in kindred M is a sensorineural cochleosaccular dysplasia specific to the membranous structures of the inner ear. Speech development before onset, intelligence, life expectancy, and fertility are normal. Hearing loss in this kindred has been traced eight generations to a common ancestor, born in 1713 in Cartago, Costa Rica. As with other nonsyndromic forms of deafness, identification of the gene responsible is important for the understanding of human hearing because the wild-type product of the gene is likely to be critical to development and maintenance of hearing.

DFNA1 in kindred M was mapped to a region of 1 centimorgan on chromosome 5q31 by linkage analysis, then a complete 800-kb bacterial artificial chromosome (BAC) contig was constructed of the linked region (*3*). In order to identify all genes in the linked region, we sequenced BACs composing the contig after shotgun subcloning each into an M13 vector (*4*, *5*). We developed the computer program SeqHelp to organize sequences from the chromatograms, to call bases and align sequences using the computer programs PHRED and PHRAP, and to apply existing, publicly available, software to evaluate the novel genomic sequences (*6*). SeqHelp displayed putative coding regions, CpG islands, repeat sequences, and matches to known genes and expressed sequence tags (ESTs) from all databases in an interactive format for further analysis.

A previously unidentified human gene homologous to the *Drosophila* gene *diaphanous* (GenBank U11288) and to the mouse gene encoding p140mDia (GenBank U96963) was revealed by the genomic sequence of BACs 293C24, 45M22, and 249H5 (Fig. 2) (*7*). Given that the mouse and human predicted amino acid sequences are 97% identical for the regions identified from BACs, we estimated the sizes of gaps from the mouse sequence, constructed primers from the human coding sequence, and used these to amplify intervening exons from human cDNA and to carry out 5′RACE on polyadenylated [poly(A)$^+$] RNA from lymphoblastoid lines (*8*). A total of 3511 base pairs (bp) of coding sequence have been identified; about 250 bp remain to be determined. The human *diaphanous* gene comprises at least 18 exons with ~3800 bp of coding sequence and a 3′ untranslated region (UTR) of 918 or 1891 bp (*9*).

To screen the *DFNA1* gene for mutation in the M family, we designed primers to amplify exons and flanking splice junctions from genomic DNA of affected and unaffected members of the M family and from control individuals. Each product was screened for single-strand conformation polymorphisms (SSCPs). Variant bands were gel-purified, reamplified, and sequenced (*10*).

A guanine-to-thymine substitution in the splice donor of the penultimate exon of human *DFNA1* was observed in affected members of the M kindred (Fig. 3E). The guanine-to-thymine substitution at this site disrupts the canonical splice donor sequence

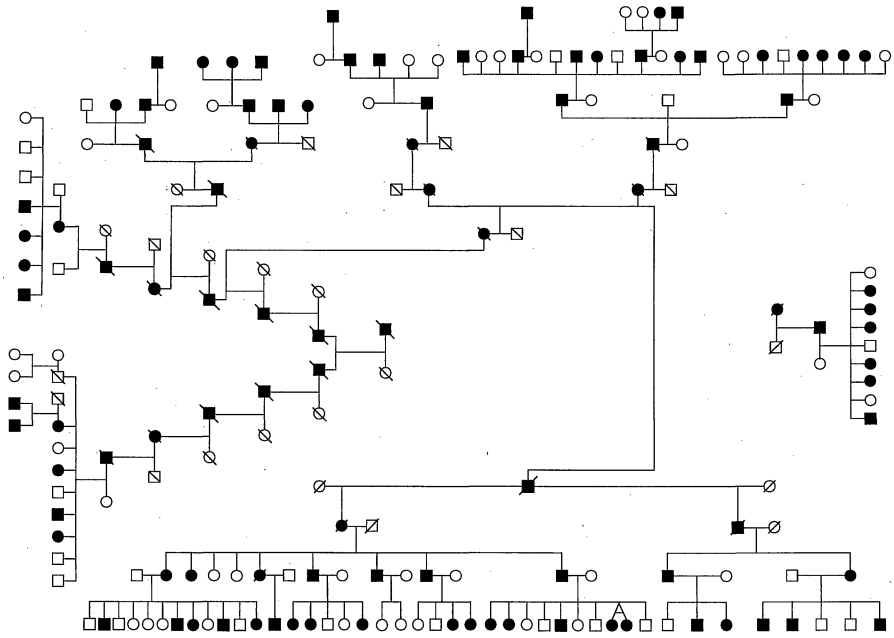

Fig. 1. The M kindred of Costa Rica. Hearing loss in this kindred is autosomal dominant, progressive and fully penetrant by age 30 and not associated with any other phenotype. Individuals with hearing loss are indicated by filled symbols and unaffected individuals by open symbols. All living individuals on the pedigree are included in the analysis. The pedigree is altered slightly, omitting young unaffected individuals, in order to protect privacy. The three-generation family drawn separately is related to the kindred, but the exact genealogy is unclear. All 78 affected individuals in the kindred share the *DFNA1* mutation, and all unaffected individuals over age 30 are wild type at the comparable site.

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AAGgtaagt (Fig. 3, A and B). To determine the consequences of this mutation at the level of RNA message, poly $(A)^+$ cDNA was prepared from lymphoblastoid cell lines of three affected members of the M kindred, from unaffected family members, and from unrelated, unaffected, controls. Insertion of TTAA was observed in cDNA of affected individuals (Fig. 3, C and D). The mechanism for the insertion was splicing at a cryptic site 4 bp 3′ of the wild-type site. The TTAA insertion leads to a frameshift, encoding 21 aberrant amino acids, followed by protein termination that truncates 32 amino acids (Fig. 4). All 78 affected members of the M kindred are heterozygous for the mutation. The site was wild type in 330 hearing, control individuals (660 chromosomes) of the following ancestries: 12 Costa Ricans unrelated to the M family, 94 Latin Americans from other countries, 32 Spanish, 154 Europeans (other than Spanish) and North Americans of European ancestry, and 38 African-Americans.

Expression of human *diaphanous* message in brain, heart, placenta, lung, kidney, pancreas, liver, and skeletal muscle was confirmed by Northern (RNA) hybridization (Fig. 3F). A single transcript of 4.7 kb was observed in all tissues, with highest expression in skeletal muscle. RNA from lymphoblastoid cell lines of affected and unaffected members of the M family similarly revealed a single transcript of 4.7 kb in all individuals, consistent with a 4-bp insertion in the mutant message. We confirmed expression of human *diaphanous* in the cochlea by reverse transcriptase–polymerase chain reaction (RT-PCR) of cochlear RNA using PCR primers that amplified the region of the gene that harbors the mutation in family M (*10, 11*). The sequence of the RT-PCR product from cochlear RNA was wild type. Hence, if alternate splice forms of the gene exist, normal cochlear transcripts include the region of the gene that is improperly spliced in affected members of kindred M.

The human diaphanous 1 protein, mouse p140mDia, and *Drosophila* diaphanous are homologs of *Saccharomyces cervisiae* protein Bni1p (*12*). The proteins are highly conserved overall (Fig. 5). The genes encoding these proteins are members of the formin gene family, which also includes the mouse limb deformity gene, *Drosophila* gene *cappuccino*, *Aspergillus nidulins* gene *sepA*, and *Schizosaccharomyces pombe* genes *fus1* and *cdc12* (*13*). These genes are involved in cytokinesis and the establishment of cell polarity. Rho-binding domains in the NH_2-terminal regions, polyproline stretches in the central region of each sequence, and formin-homology domains in the COOH-terminal region characterize many formins (*12*). Multiple mutants of mouse formin have been characterized (*13*). A truncated mouse formin allele ld^{In2} lacking the 42 COOH-terminal amino acids leads to mislocalization of the formin protein to the cytoplasm (*14*).

The biological role of human diaphanous 1 in hearing is likely to be the regulation of actin polymerization in hair cells. Actin polymerization involves proteins known to interact with diaphanous protein in *Drosophila* and mouse. The protein profilin binds actin monomers and is a regulator of actin polymerization (*15*). Mammalian and *Drosophila* diaphanous are effectors of Rho (*12*). Diaphanous acts in a Rho-dependent manner to recruit profilin to the membrane, where it promotes actin polymerization. As predicted by this model, transient expression of p140mDia induced homogeneous actin filament formation in COS cells (*7*). Rho-induced actin polymerization is conserved from yeast to mammals.

The *DFNA1* mutation in the M family is relatively subtle, in that it affects only the COOH-terminal 52 amino acids. Given that human *diaphanous* appears to be ubiquitously expressed, and that the only ob-

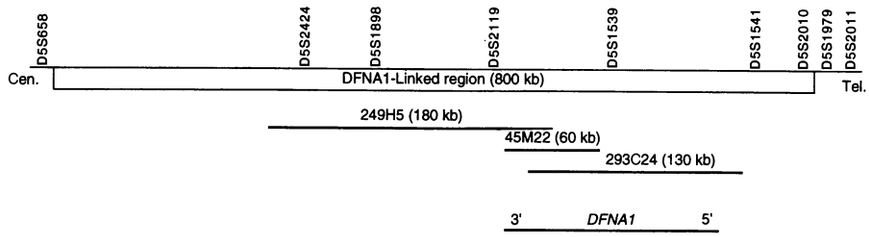

Fig. 2. *DFNA1* is a human homolog of *diaphanous* on the region of chromosome 5q31 linked to deafness in kindred M. Markers D5S658 and D5S1979 define the 1-cM linked region (*3*). BACs 249H5, 45M22, and 293C24 form a 300-kb portion of the 800-kb BAC contig spanning the region. The 5′ end of *DFNA1* is telomeric to the 3′ end of the gene. Cen., centromere; Tel., telomere.

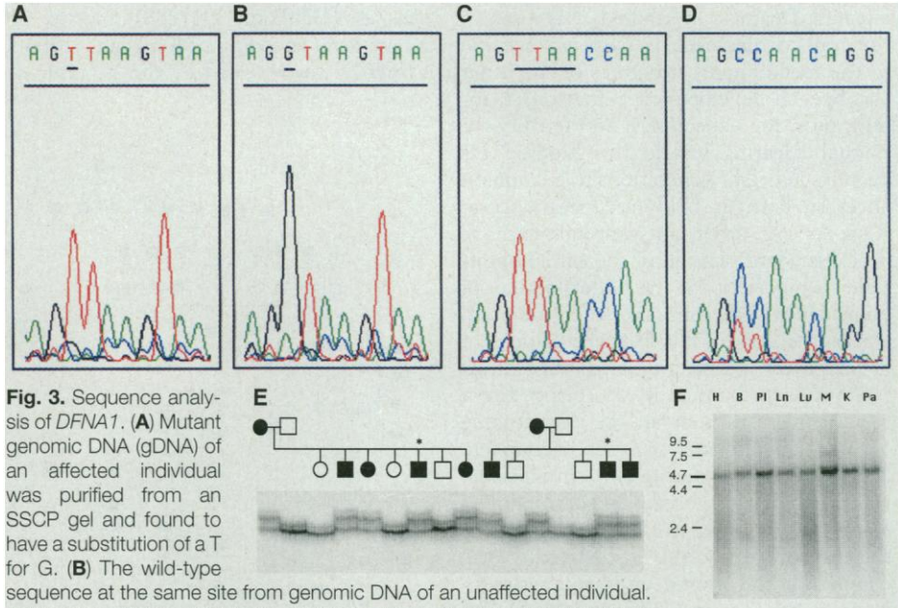

Fig. 3. Sequence analysis of *DFNA1*. (**A**) Mutant genomic DNA (gDNA) of an affected individual was purified from an SSCP gel and found to have a substitution of a T for G. (**B**) The wild-type sequence at the same site from genomic DNA of an unaffected individual. (**C**) Mutant cDNA purified from an SSCP gel indicates insertion of TCAA in the cDNA sequence. The insertion is generated by splicing at the GT immediately 3′ to the genomic mutation. Affected individuals have both the misspliced form and the wild-type allele. (**D**) Wild-type cDNA sequence at the splice junction. (**E**) Heterozygosity for the genomic G→T mutation, revealed by SSCP analysis, cosegregates with deafness in all branches of the M family. Individuals noted with an asterisk in these two branches defined the proximal and distal boundaries of the 1-cM linked region on chromosome 5q31. (**F**) Human *diaphanous* appears to be broadly expressed as shown by hybridization of IMAGE clone 926002 to a Northern blot containing human poly(A)$^+$ RNA from heart (H), brain (B), placenta (Pl), lung (Ln), liver (Lv), skeletal muscle (M), kidney (K), and pancreas (Pa). A single major transcript of 4.7 kb was observed in poly(A)$^+$ RNA from all tissues tested except skeletal muscle, which exhibited low-level expression of an additional transcript of ~6 kb. After hybridization, this Northern blot was washed at final stringency at 60°C in 0.1× saline sodium citrate and 0.1% SDS for 15 min. It is possible that the larger transcript observed in skeletal muscle represents a tissue-specific isoform of human diaphanous.

served phenotype in the M family is hearing loss, it seems likely that the hair cells of the cochlea are particularly sensitive to proper maintenance of the actin cytoskeleton. Hence, this mutation may represent a partial loss of function of the human diaphanous 1. One process in the inner ear that might be uniquely sensitive to disruption of actin polymerization is the amplification of sound reception by the inner hair cells, which is due to the concerted action of outer hair cells and pillar cells. The relay of kinetic energy from outer hair cells to inner hair cells relies critically on the presence of a rigid structure of actin fibers. Additional structural support in hair cells is provided by the cuticular plate, a dense network of actin fibers at the apical ends of hair cells into which stereocilia are anchored. Organization of the actin fiber network of the cuticular plate is not well understood but is probably dynamic. The *DFNA1* mutation may impair maintenance of organization of the actin fibers of the cuticular plate.

Hair cell stereocilia may be an alternate or additional site affected by the aberrant protein. The structural support providing rigidity to the stereocilia comprises largely cross-linked actin filaments packed in a paracrystaline array (*16*). Upon acoustic overstimulation, the paracrystaline array becomes disordered (*17*). Reordering of the array is controlled by an as yet unidentified protein or proteins. It is possible that normal human diaphanous 1 is involved in this process. In the M family, mutant human diaphanous 1 might compete with the wild-type protein to repair damage from normal exposure to sound. The generation of a mouse model with the *DFNA1* mutation in p140mDia, followed by testing the effects of acoustic exposures on these mice, will provide an experimental test of this hypothesis.

A second human homolog of *Drosophila* gene *diaphanous* was revealed during the cloning of *DFNA1*. This second human diaphanous gene maps to chromosome Xq22 (*18*). Nonsyndromic X-linked deafness, *DFN2*, also maps to Xq22 (*19*), suggesting the second human *diaphanous* gene as a candidate gene for *DFN2* hearing loss.

In the past 50 years, the incidence of maternal viral disease during pregnancy has declined dramatically in most countries, with a consequent decrease in childhood deafness. Most remaining cases of deafness at birth, in childhood, or among young

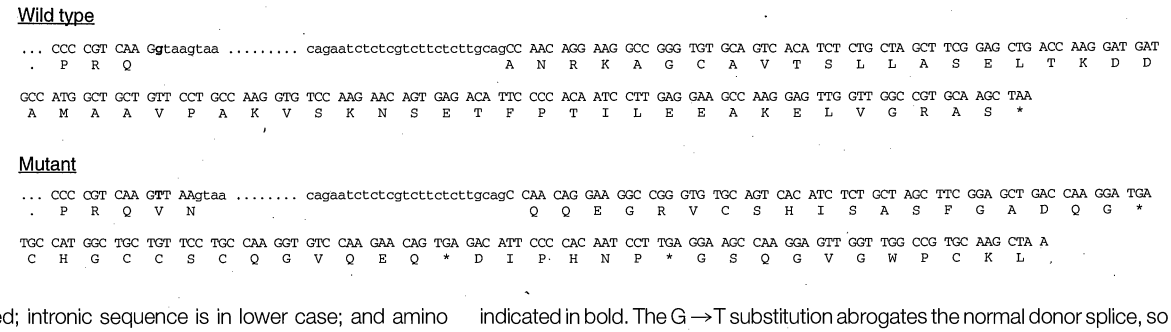

Fig. 4. Mutation in human *diaphanous* associated with deafness in kindred M. The wild-type *HDIA1* sequence of the splice junctions of the penultimate and ultimate exons and coding sequence of the ultimate exon are shown at the top of the figure. Sequence present in the RNA message is capitalized; intronic sequence is in lower case; and amino acid sequence is indicated. A guanine residue (g) at the donor splice junction is the site of the *DFNA1* mutation. The *DFNA1* mutant sequence of the same regions is shown at the bottom of the figure. The mutant thymine (T) is indicated in bold. The G→T substitution abrogates the normal donor splice, so splicing occurs instead at the Ag four nucleotides 3′ of the normal site. Consequently, TTAA is inserted in the mutant message, causing a frameshift and premature stops, as indicated.

Fig. 5. Wild-type amino acid sequence of human diaphanous 1 protein, inferred from human genomic DNA and cDNA sequence, compared with mouse p140mDia. Human sequence is above the mouse sequence; numbering is based on the mouse homolog; amino acids that differ between human and mouse are underlined. For human sequence, splice junctions inferred from genomic sequence are indicated by vertical bars above the last amino acid in each exon. Dots indicate regions for which human sequence is still in progress. The polyproline region and FH2 region of formin homology are indicated in bold. The *DFNA1* mutation in human diaphanous 1 lacks the 52 COOH-terminal amino acids, which are double underlined.

adults, are genetically influenced (*20*). In the past 5 years, at least 40 chromosomal locales for inherited, nonsyndromic human deafness have been mapped by linkage in families, and thus far five of the responsible genes have been identified: myosin 7A, the POU domain gene POU3F4, connexin 26, mitochondrial 12S rRNA, and mitochondrial $tRNA^{Ser(UCN)}$ (*21*). The association of autosomal dominant, nonsyndromic deafness with the human *diaphanous* gene adds a new and complementary piece to this puzzle. The M kindred provides a rare mutation that may reveal universal biology and augment our understanding of hearing.

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2. This project has been approved by the Committee on Human Subjects in Research of the Ministry of Health of Costa Rica, and by the Human Subjects Division of the Institutional Review Board of the University of Washington. The criterion for deafness in the family is a hearing threshold greater than 50 dB at 250 Hz and 500 Hz. Of the participants, 78 are deaf and 69 are older than 30 years with normal hearing. All deaf relatives are included in the analysis, as are all hearing relatives older than age 30 years and all persons marrying into the family. No relatives younger than age 30 with normal hearing are included in the analysis or on the pedigree of Fig. 1. Cell lines were established from lymphocytes of 147 informative relatives with techniques established in our laboratory (*1*).
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5. Sequencing of BACs was performed as follows: 30 μg of BAC DNA was sonicated to 50 to 5000 bp, then treated with mung bean exonuclease. Blunt-ended fragments were electrophoresed on agarose gels, and DNA in the 1.5- to 3-kb range was excised from the gel for DNA isolation with a Qiaex gel extraction kit (Qiagen). Recovered fragments were ligated into Sma I–digested, phosphatase-treated M13mp18 vector. Ligations were electroporated into *Escherichia coli* strain DH12S. Transformations were plated in LB top agarose with DH12S lawn cells, X-Gal, and isopropyl-β-D-thiogalactopyranoside (IPTG) onto LB plates and incubated overnight at 37°C. The following day, clear plaques were picked and inoculated into 1 ml of LB with DH12S host cells in 96-well 2-ml plates. Phage cultures were incubated for 24 hours at 37°C, with shaking at 250 rpm. We prepared single-stranded M13 DNA by standard methods using polyethylene glycol (PEG) precipitation of phage particles and NaI solution to remove proteins. A detailed copy of the DNA preparation method can be found on the Internet at http://chroma.mbt.washington.edu/~kwseq/preps/amy_NaI_prep.html. This preparation method yielded 1 to 2 μg of M13 DNA for sequencing. The resulting DNA pellets were diluted in 30 μl of water, and 6 μl was used in 10-μl sequencing reactions with dichloroRhodamine Dye Terminator Chemistry from ABI. The remaining DNA was stored at −80°C for future use. Sequencing reactions were precipitated with 100 μl of 70% EtOH and 5 mM $MgCl_2$ at room temperature for 15 min. Precipitated reactions were pelleted by centrifugation for 15 min at 3500 rpm in a Beckman SH-3000 rotor with 96-well plate adapters. Supernatants were removed by centrifugation of the inverted plate at 500 rpm for 1 min, then pellets were dried at 37°C for 5 min. Pellets were resuspended in 3 μl of formamide loading dye, denatured at 95°C for 2 min, then placed on ice. A 1-μl sample of sequencing reaction was loaded onto a 36-cm Longranger gel (FMC) and electrophoresed on an ABI377 automated sequencer. ABI377 collection software version 1.1 was used to support 48-well combs and 9-hour data collection in the 2× collection mode. The chromatograms generated by ABI Sequence Analysis software version 3.0 were transferred to a UNIX-based Sun workstation for contig assembly and BLAST analysis. The computer program PHRED (P. Green and B. Ewing, 1996. Available at http://bozeman.mbt.washington.edu/phrap.docs/phred.html) was used to assign bases to the electropherograms. After eliminating vector sequences, the program PHRAP (*ibid.* Available at http://bozeman.mbt.washington.edu/phrap.docs/phrap.html) was used to analyze the sequences, identify overlapping individual sequences, and assemble them into contigs. To achieve about sixfold coverage of a region, we sequenced an average of 600 M13 subclones per BAC.
6. The SeqHelp program incorporates several sequence analysis programs and creates output in HTML files for browsing with any World Wide Web browser. The core programs used by SeqHelp are PHRED to read the ABI sequence files and assign bases, PHRAP to generate contigs of overlapping sequences, Repeat Masker (A. Smit, Univ. of Washington, 1996) to identify and mask common repetitive elements before database searching, and BLAST [S. Altschul, W. Gish, W. Miller, E. Myers, D. J. Lipman, *Mol. Biol.* **215**, 403 (1990)] comparison of project-specific sequences to the NR and dbEST databases at the National Center for Biotechnology Information. A full description of this program and its uses is currently in review (M. K. Lee *et al.*, in preparation). An example of the SeqHelp output for analysis of the *BRCA1* genomic region is available at http://polaris.mbt.washington.edu.
7. D. H. Castrillon and S. A. Wasserman, *Development* **120**, 3367 (1994); N. Watanabe *et al.*, *EMBO J.* **16**, 3044 (1997).
8. We purified poly $(A)^+$ RNA from lymphoblastoid cell lines using oligo-dT cellulose [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)]. The 5′ cDNA sequence was obtained with the 5′RACE (rapid amplification of cDNA ends) System, version 2.0 (Gibco-BRL). We performed 5′RACE on 1 μg of poly $(A)^+$ lymphoblast RNA according to the manufacturer's specifications. First strand cDNA synthesis was primed with the human *diaphanous*–specific primer H2a (5′-AGTCATCCATCTCCATGCGAATG-3′). After cDNA synthesis and homopolymeric 3′ tailing with terminal deoxynucleotidyl transferase (Tdt), first strand cDNA was amplified with the human *diaphanous*–specific primer H2b (5′-ATGCGAATGTCATCCAGCCGTC-3′), a nested primer that anneals 3′ to H2a. 5′RACE products of ~1 kb were gel-purified and TA cloned into the pGEM-T vector (Invitrogen) according to the manufacture's directions. The 5′RACE clones were amplified with M13-40F and M13-40R. PCR products of 5′RACE clones were purified. Templates were sequenced with M13-40 R primers and the gene-specific primers H6f (5′-TTGCGGGATATGCCTCTG-3′) and H7a (5′-GGTTGTTGTTGAGAGACACAC-3′). Sequencing was done with dichloroRhodamine Dye Terminators (ABI) and an ABI 377 sequencer as described in (*2*).
9. IMAGE clones 51234, 52194, 124697, 261240, 262633, 612749, and 926002 are cDNA clones of portions of human *diaphanous* [G. Lennon, C. Auffray, M. Polymeropoulos, M. B. Soares, *Genomics* **33**, 151 (1996)]. The ESTs for all clones are confined to the most 3′ exon of human *diaphanous*.
10. PCR primers used to amplify the variant sequence that includes the involved splice donor region are Dia9F (5′-TGTGGGAGAGGGGAAATCAAG-3′) and Dia9R (5′-TTGCTCTTTAGCCGCAGACTGG-3′). The 278-bp product was labeled by incorporation of a [^{32}P]deoxycytidine triphosphate during PCR, diluted 1:10 in formamide loading buffer, denatured at 95°C for 2 min, then placed on ice for 10 min. The samples (8 μl) were loaded onto an MDE (FMC Biochem) gel and electrophoresed at 6 W for 18 hours at room temperature to resolve SSCPs. Gels were dried and exposed to x-ray film for 18 hours. Variant bands on SSCP gels were individually excised from dried gels, eluted with water, and used as a template for reamplification with the Dia9F and Dia9R primers. PCR products were purified by centrifugation through 300 μl of Sephacryl-300 resin then sequenced with the Dia 9F and Di9R primers. Sequencing was done with dichloroRhodamine Dye Terminators (ABI) and an ABI 377 sequencer as described in (*2*). PCR amplification for cDNA analysis of the variant region was done with primers Dia8-10F (5′-CGGCGGAAGACAGAAGAAAAG-3′) and Dia8-10R (5′-TAGCAGAGATGTGACTGCACACCC-3′), which are designed to amplify a 234-bp product that spans the second to last exon and ends in the last exon of human *diaphanous*. PCR products were labeled and analyzed by SSCP as describe above. Variant bands were sequenced with the Dia8-10F and Dia8-10R primers.
11. Total cochlear RNA was extracted by using the guanidine isothiocyanate method [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]. Total cochlear RNA was reverse transcribed with Superscript MMLV RTase (Gibco BRL) according to manufacturer's instructions. A fraction of the resulting cDNA was used as template in a gene-specific PCR reaction with the Dia8-10F and Dia8-10R primers (*10*). PCR products were resolved on an agarose gel and visualized with ethidium bromide staining. A control reaction with no reverse transcriptase was negative as was a control reaction with no RNA template.
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18. The X-linked human *diaphanous* is represented by several IMAGE clones including 626664, a 3.1-kb cDNA clone from a HeLa cDNA library. When searched against the GenBank database, a portion of this clone was identical to genomic DNA from PAC 117P19, sequenced and mapped by the Sanger Center to Xq21.3. The Drosophila Related Expressed Sequences Homepage available at http://www.tigem.it/LOCAL/drosophila/dros.html [S. Banfi, G. Borsani, A. Bulfome, A. Ballbio, *Hum. Mol. Genet.* **6**, 1745 (1997)] indicates that a human homolog of *Drosophila diaphanous* maps to human chromosome Xq22.
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