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Mutation of a transcription factor, *TFCP2L3*, causes progressive autosomal dominant hearing loss, DFNA28

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We ascertained a large American family with an autosomal dominant form of progressive non-syndromic sensorineural hearing loss. After excluding linkage to known deafness loci, we performed a genome-wide scan and found linkage to marker GAAT1A4 on chromosome 8q22 (LOD = 5.12 at $\theta = 0$), and this locus was designated DFNA28. Sequencing of six candidate genes in the 1.4 cM linked region identified a frameshift mutation (1609–1610insC) resulting in a premature translation stop codon in exon 14 of the gene *TFCP2L3* (transcription factor cellular promoter 2-like 3). *TFCP2L3* is a member of a family of transcription factor genes whose archetype is *TFCP2*, a mammalian homolog of the *Drosophila* gene *grainyhead*. Northern blot analyses and *in situ* hybridization studies show that mouse *Tfcp2l3* is expressed in many epithelial tissues, including cells lining the cochlear duct, at embryonic day 18.5 and postnatal day 5.

INTRODUCTION

The incidence of hearing loss increases with age. Post-lingual progressive hearing loss affects ~50% of the elderly by the age of 80 (1), and can be caused by genetic or environmental factors such as exposure to ototoxic drugs, acoustic trauma, and the interaction of genes and the environment (2). The extent of the genetic contribution to post-lingual progressive deafness is unknown. Many families segregating post-lingual progressive deafness have been identified and used in mapping studies. The hearing loss phenotype in these families tends to be non-syndromic and segregates in an autosomal dominant fashion. To date, 41 loci for autosomal dominant non-syndromic hearing loss have been mapped and given the locus designations DFNA1, …, DFNA41 (Hereditary Hearing Loss Homepage, http://www.uia.ac.be/dnalab/hhh/). Of these, 16 genes have been identified (3–19).

The identified DFNA genes encode a wide variety of proteins, including gap junctions, ion channels, extracellular matrix components and two transcription factors. For several genes the function is unknown, and for nearly all of them the mechanisms of cochlear pathology are uncertain. There are few mouse models for progressive hearing loss (20), so our current understanding of progressive hearing loss relies heavily on the identification of genes through human mapping studies. Here we report a large North American family (LMG45) segregating non-syndromic autosomal dominant sensorineural and progressive hearing loss. We have identified a frameshift mutation (1609–1610insC) in a putative transcription factor, *TFCP2L3*, which is highly expressed in epithelial cells lining the cochlear duct.

RESULTS

The pedigree of LMG45 consists of five generations with 11 affected individuals (Fig. 1). Affected individuals show a mild to moderate bilateral sensorineural hearing loss across most frequencies that progresses to severe levels in the higher frequencies by the fifth decade. The age of subjective diagnosis of hearing loss varies, with the earliest case documented at 7 years of age (individual V-1). Figure 2A shows the pure-tone averages (PTAs) at 0.5, 1, 2 and 4 kHz for the better-hearing ear of LMG45 family members compared with 95th percentile normative population thresholds for males and females (21). Serial audiometry for individual V-1 are indicated in Figure 2A and B, demonstrating the progressive nature of the hearing loss phenotype. Also indicated in Figure 2A and B are serial

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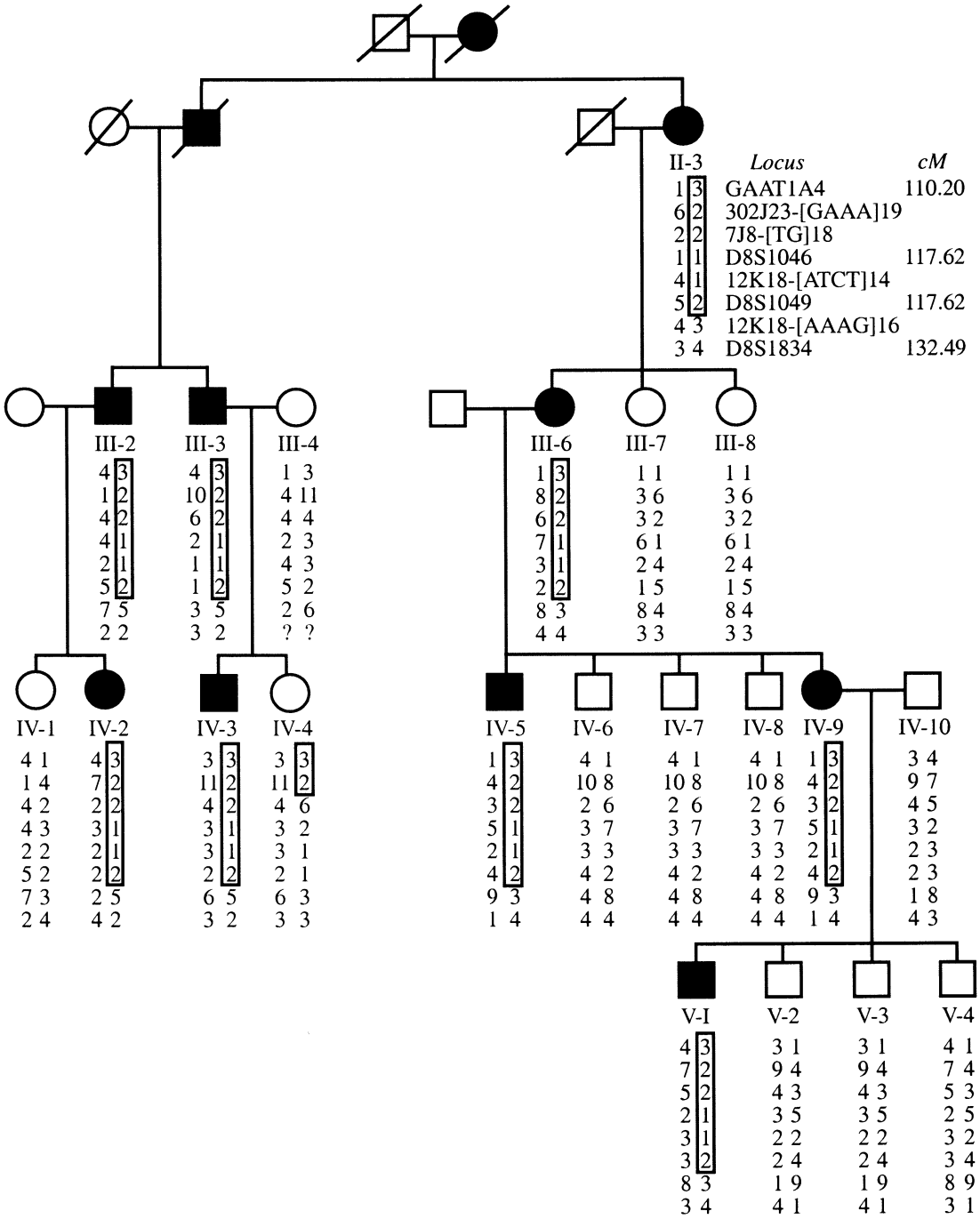

Figure 1. LMG45 pedigree showing the co-segregation of progressive hearing loss with a haplotype (boxed) of polymorphic markers on chromosome 8q22.

audiometry for individual IV-2, whose hearing loss is milder than that of the other affected individuals, but shows progression in all frequencies over a 3-year interval. PTAs for her better ear at both time points are greater than the 95th percentile for age-matched females.

Genotype analysis excluded linkage to the known DFNA loci, and a subsequent genome-wide scan detected linkage to marker GAAT1A4 (maximum LOD score of 5.21 at $\theta = 0$) at chromosome 8q22. Typing of additional polymorphic STRs defined an ~1.4 cM interval flanked by recombinant markers 302J23–$[GAAA]_{19}$ and 12K18–$[AAAG]_{16}$ (Fig. 3). All affected individuals have the same haplotype spanning ~7 cM on chromosome 8q22. An unaffected individual (IV-4) inherited a portion of this haplotype, defining a proximal linkage boundary and reducing the linked region to ~1.4 cM. Individual IV-4 is a female who was 25 years old at the time of audiological evaluation and showed no evidence of hearing loss.

The physical map of this region is represented by a contig of 15 overlapping BACs spanning 1.7 Mb of sequence, which include 26 predicted and 7 known genes according to the

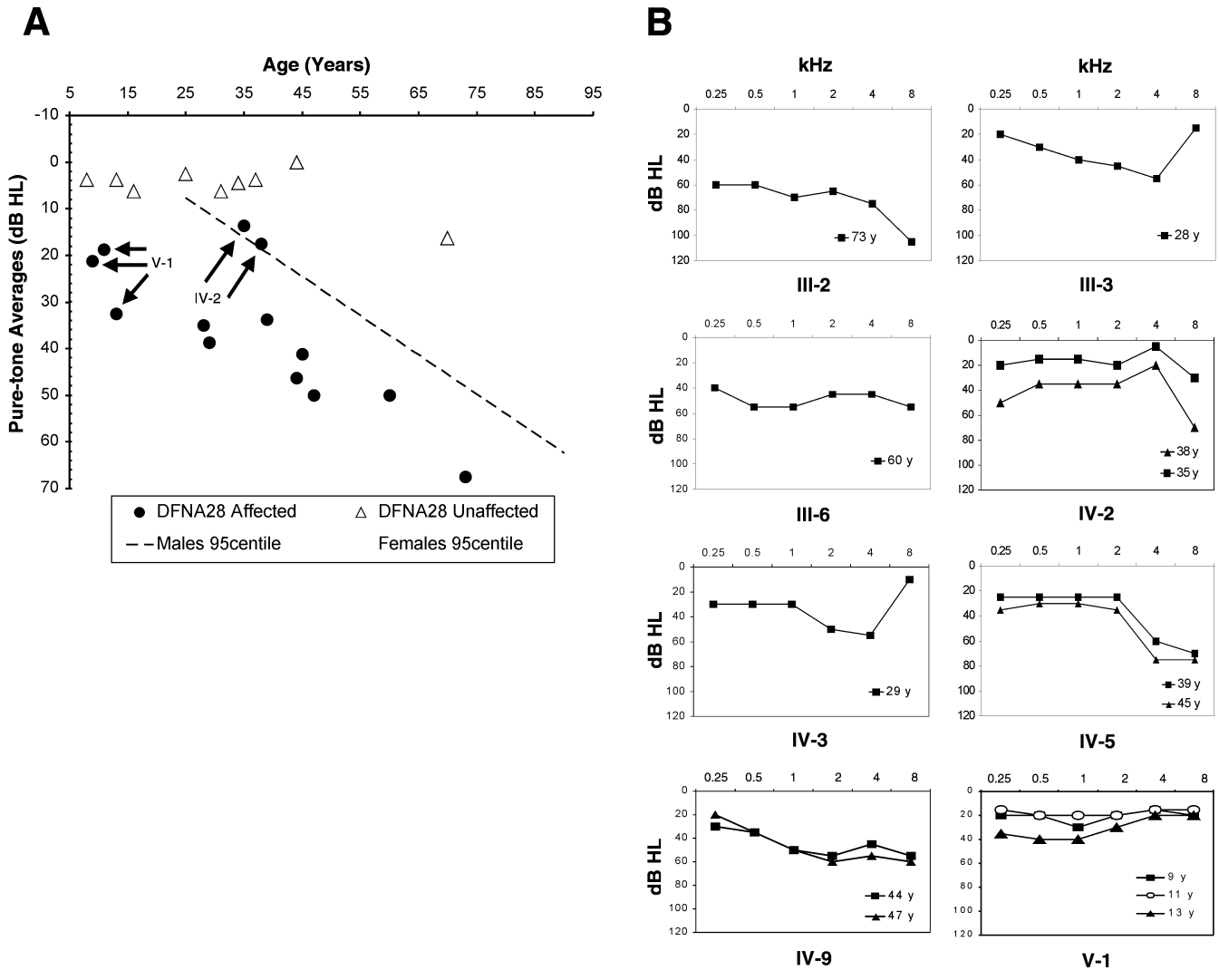

Figure 2. Hearing thresholds of LMG45 family members. (**A**) Pure-tone air-conduction averages (0.5, 1, 2 and 4 kHz) for the better-hearing ears of DFNA28 individuals. Affected individuals have hearing loss exceeding the 95th percentile for normal males and females. Population data are from the Baltimore Longitudinal Study (21). Individual IV-2 (arrow) has progressive hearing loss; her PTAs exceed the 95th percentile at 35 and 38 years of age, whereas individual V-1 (arrow) shows progressive hearing loss at an earlier age. (**B**) Hearing thresholds for the better ears of affected individuals in the LMG45 pedigree. Ages of individuals at the time of audiological examination are indicated in each panel.

annotation in the Human Genome Project draft assembly (UCSC browser, December 2001; http://genome.cse.ucsc.edu). On the basis of speculated roles in the development and maintenance of the cochlea, four candidate genes with known function were sequenced for mutation analysis: outer dense fibre of sperm tails (*ODF1*), progestin-induced protein (*DD5*), ribonucleotide reductase (*p53R2*) and neurocalcin δ (*NCALD*). Expressed sequence tags (ESTs) for all of these genes except for *ODF1* are represented in a fetal cochlea cDNA library (22). No mutations were detected in the coding regions of these genes or the intron/exon boundaries. Two remaining candidate genes of unknown function were sequenced: hematopoietic stem/progenitor cell 38 protein (*LOC51123*) (23) and *FLJ13782*. A 1 bp insertion in the 13th coding exon of *FLJ13782* was detected in all of the affected individuals of LMG45.

The GenBank *FLJ13782* mRNA (accession no. NM_024915) is 4793 nt with an open reading frame (ORF) predicted to encode a 625-amino-acid protein that has similarity to the transcription factor cellular promoter 2 (TFCP2) family of transcription factors. Following approval by the HUGO Nomenclature Committee, *FLJ13782* was designated *TFCP2L3*, and other members of the TFCP2-related family were officially renamed (Table 1). Human *TFCP2L3* cochlear cDNA (3761 nt) was generated using PCR primers homologous to exons predicted by alignment of genomic DNA to human and mouse ESTs. Comparison of the cochlear *TFCP2L3* cDNA with the GenBank mRNA sequence (accession no. NM_024915) confirms the same ORF encoding a 625-amino-acid protein. A start codon with an appropriate Kozak sequence begins at nucleotide 13 (24). Sequencing of all 16 exons of the *TFCP2L3* gene revealed a heterozygous insertion of a cytosine at 1610 in

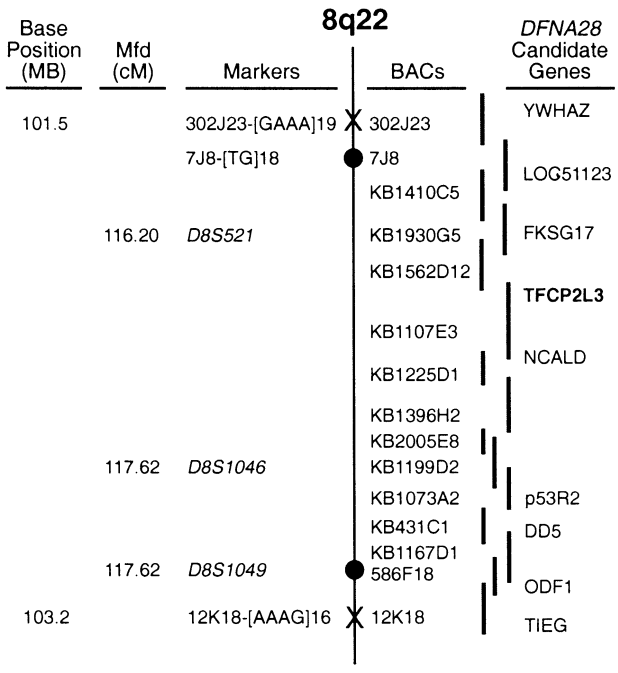

Figure 3. Genetic and physical map of the DFNA28 locus defined by proximal and distal breakpoints in family LMG45. The proximal breakpoint on BAC 302J23 and the distal breakpoint on BAC 12K18 are shown as meiotic cross-overs (X) on the chromosome. The linked proximal marker 7J8–$[TG]_{18}$ and distal marker D8S1049 are illustrated as black filled circles. Physical and genetic map coordinates are shown at the left. The physical distance is according to the December 2001 freeze of the Human Genome draft sequence (UCSC Genome Browser); the genetic positions (cM) are according to the Marshfield Medical Research Foundation map (http://research.marshfieldclinic.org/genetics/).

Table 1. Members of the TFCP2-related transcription factor family

Gene symbol[a]	Aliases		Refs
	Human	Mouse	
TFCP2	*LBP-1c*	*Tfcp2*	(55,56)
	LBP-1d	—	(56)
	LSF	—	(57)
	CP2	—	(58)
TFCP2A	*LBP-1a*	*Nf2d9*	(59,60)
	LBP-1b	—	(56)
	UBP-1	*Ubp-1*	(40)
TFCP2L1	*LBP-9*	*Crtr-1* (pending)	(59,61)
TFCP2L2	*LBP-32*	—	(59)
	MGR	—	(25)
TFCP2L3	*FLJ13782*	*Tfcp213*	This study
	BOM		(25)

[a]Approved by the HUGO Nomenclature Committee.

exon 13 (1609–1610insC), three nucleotides prior to the donor splice site (Fig. 4). This mutation occurs in all affected individuals of LMG45, and was not detected in the sequence analyses of unaffected LMG45 individuals or among 150 individuals from Caucasian and pan-ethnic human DNA panels (Coriell Cell Repositories).

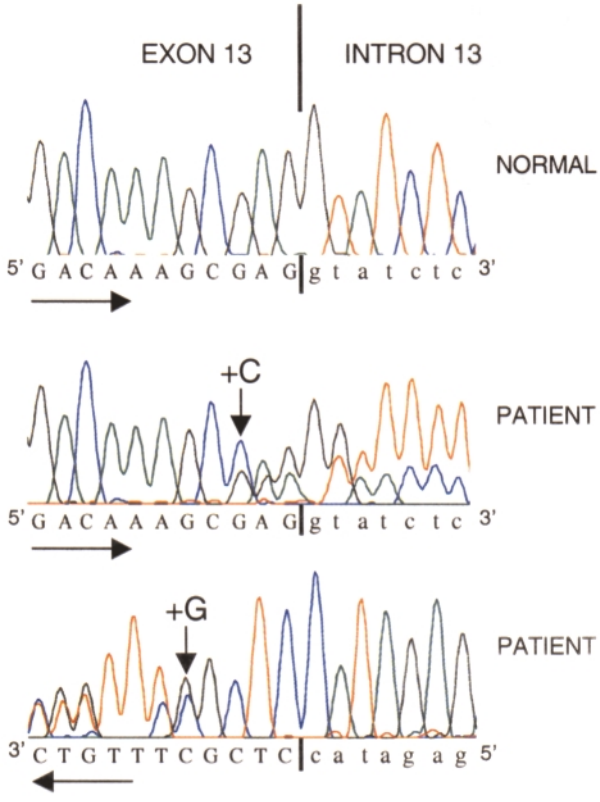

Figure 4. The nucleotide sequence of amplified genomic *TFCP2L3*, showing a heterozygous frameshift mutation in an affected individual (sense and antisense strand) compared with that of a normal individual (sense strand). The electropherogram shows the exon 13/intron 13 junction of *TFCP2L3*, with an insertion of a cytosine nucleotide at position 1962 (vertical arrow) in the sense strand. Heterozygosity for exon 13 *TFCP2L3* sequence, which was 1 bp out of register, was also observed when the antisense strand was sequenced. Horizontal arrows indicate the direction of DNA sequencing.

The mutation 1609–1610insC results in a shift in the reading frame, producing 10 novel codons and a premature stop codon in exon 14. If translated, this allele is predicted to encode a protein of 546 amino acids that contains the transactivation and DNA-binding domains, but lacks most of the dimerization domain at the C-terminus (Fig. 5) (25). However, the introduction of a premature translation termination codon in exon 13 may result in an unstable mRNA by marking the message for nonsense-mediated mRNA decay (26,27). No isoforms of *TFCP2L3* that skip exon 13 are predicted or described in the literature, nor were any recovered in our RT–PCR experiments on cochlear and prostate RNA.

Northern blot analysis of RNA from human spleen, thymus, prostate, testis, uterus, small intestine, colon and peripheral blood leukocytes showed expression of *TFCP2L3* mRNA in the human prostate (Fig. 6A). Two mRNA transcripts were detected in the prostate: a major 6.5 kb band and a minor 8.4 kb band. A larger transcript encompassing an additional 1616 nt of coding sequence, including sequence from exon 13, is predicted by NCBI genome annotation and may correspond to the 8.4 kb band observed on our northern blots. However, we did not recover this isoform in our RT–PCR experiments

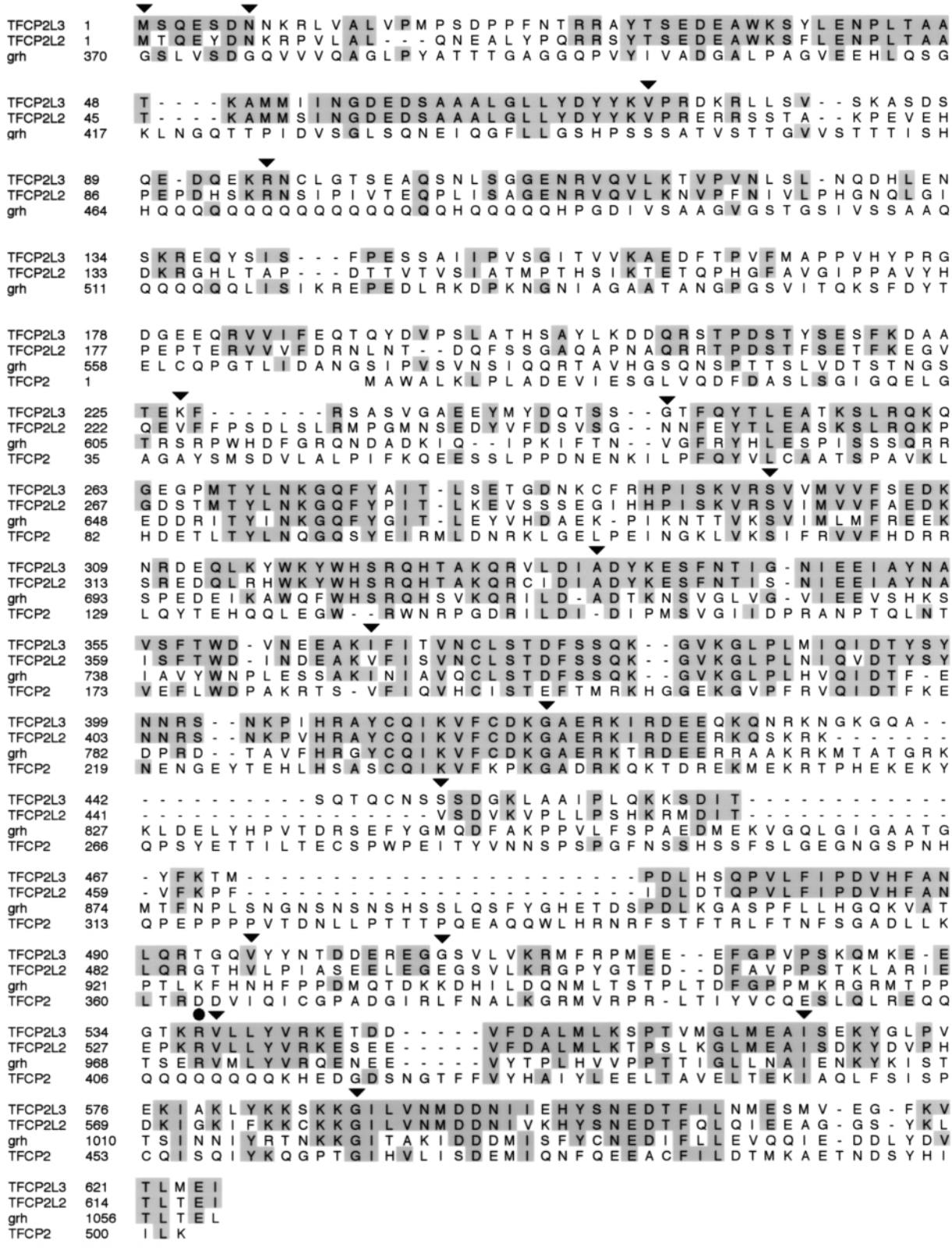

Figure 5. Multiple amino acid sequence alignment of TFCP2L3 compared with TFCP2, TFCP2L2 and *Drosophila* grh using ClustalW and SeqVu software. Dashes indicate sequence gaps. Amino acid identities with TFCP2L3 are shaded. Note that TFCP2L3 and TFCP2L2 share identities at the N-terminal and C-terminal ends, and all four proteins share homology in the middle region. The first three and last three exons of *Tfcp2l3* and *Tfcp2l2* have been experimentally determined to encode activation and dimerization domains, respectively (25). The region of homology in the middle comprises the DNA-binding domains that have been determined experimentally for each protein (25,62,63). The 5′ exon boundaries of TFCP2L3 are represented by black filled triangles. The frameshift mutation (black filled circle) of TFCP2L3 occurs in the codon for residue 537 and results in the substitution of proline (P) for arginine (R) followed by novel amino acid residues SALVREEGD and a stop codon.

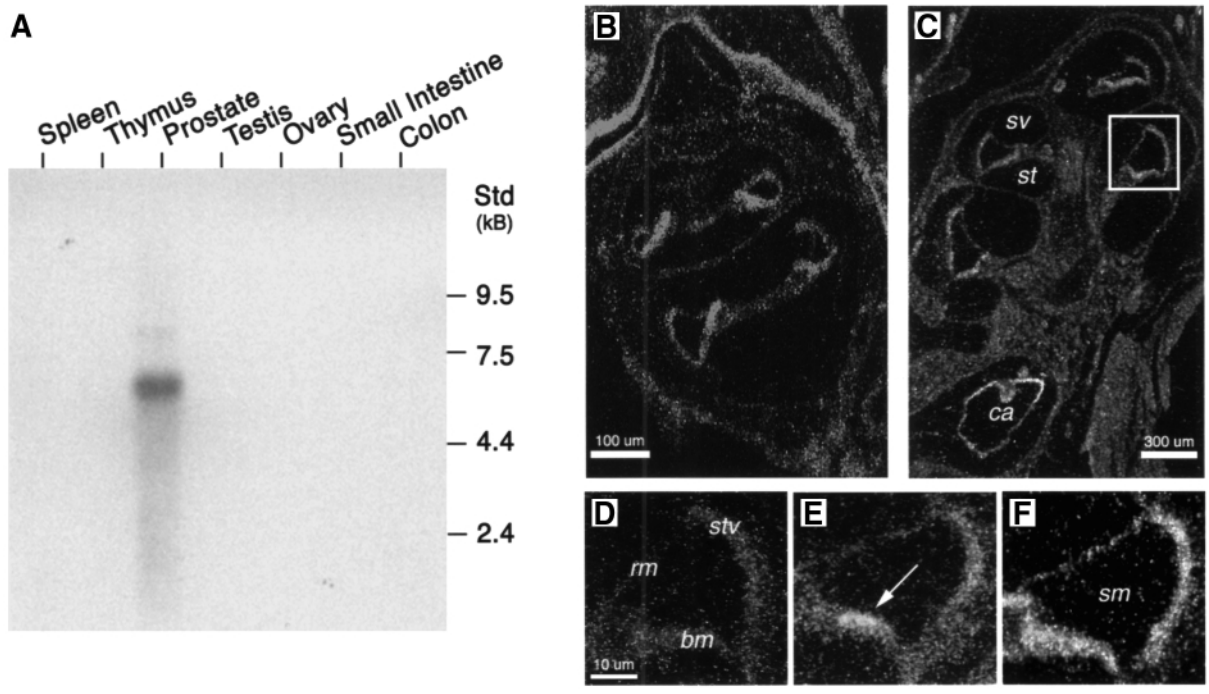

Figure 6. *TFCP2L3* mRNA expression in the human and mouse. (**A**) Human multiple-tissue northern blot (Clontech) was probed with an α-^{32}P-labeled 495 bp fragment of *TFCP2L3* mRNA spanning the 3′-UTR, revealing 6.5 and 8.4 kb mRNA transcripts after a 6 h exposure to autoradiograph film. Following a 7-day exposure, a 6.5 kb band was observed in the colon, small intestine and thymus (data not shown). Positions of bands from a 9.5–2.4 kb RNA ladder are indicated along the right margin. (**B**) *In situ* hybridization of a *Tfcp2l3* antisense RNA probe. Expression is observed in the apical and basal turns in the mouse cochlea at E18.5. (**C**) At age P5, expression is observed in the crista ampullaris (ca) and in the cochlear duct (boxed). (**D**) Background signal in the P5 cochlea duct with sense *Tfcp2l3* RNA probe. (**E**) For comparison, myosin XV is expressed in the inner and outer hair cells (arrow) in an adjacent section. (**F**) Expression of *Tfcp2l3* antisense RNA probe in the P5 cochlear duct is mostly in the epithelial tissues lining the scala media. st, scala tympani; sv, scala vestibuli; sm, scala media; bm, basilar membrane; rm, Reissner's membrane; stv, stria vascularis.

on cochlear and prostate RNA. Following a 7-day exposure, a band of ~6.5 kb was detected in the colon, small intestine and thymus. Analysis of a human multiple-tissue northern dot blot (Clontech 7776-1) confirmed that *TFCP2L3* was highly expressed in the prostate, as well as the placenta, and also showed lower-level expression in a variety of epithelial tissues such as thymus, kidney, lung, salivary gland, mammary gland and digestive tract (results not shown). This is consistent with the data reported by Wilanowski *et al.* (25), who demonstrated *TFCP2L3/Tfcp2l3* expression in a variety of human and mouse epithelial tissues, with high levels in the mouse embryo at embryonic day (E) 14.5 and E15.5 and weaker levels in adult epithelial tissues.

We demonstrated cochlear expression of *TFCP2L3/Tfcp2l3* by RT–PCR of the complete ORF with the adjacent 2 kb of 3′ untranslated region (3′-UTR) from both human and mouse temporal bone RNA (data not shown). *In situ* hybridization studies showed that *Tfcp2l3* was expressed in the mouse otocyst at E11.5, and was prominent in epithelial derivatives of the otic placode in the vestibule and cochlear duct at E18.5 (Fig. 6B). At postnatal day (P) 5, these epithelial cells of the cochlear duct, which surround the endolymph-containing scala media, continued to express low levels of *Tfcp2l3*, while little or no expression was seen in the mesenchyme-derived cells lining the scala tympani and scala vestibuli (Fig. 6C and F).

DISCUSSION

The development and maintenance of the mammalian cochlea requires the synchronized action of many transcription factors (28–30). Syndromic hearing loss is caused by mutations of genes encoding the transcription factors *PAX3* (Waardenburg syndrome types I, III and craniofacial–deafness–hand syndrome) (31–33), *MITF* (Waardenburg syndrome type II) (34) and *SOX10* (Waardenburg syndrome type IV) (35). The additional clinical features exhibited in patients with mutations in these transcription factor genes presumably reflect the phenotypic effects of the genes that they regulate. It is also interesting to note that hearing loss, when present, is usually congenital in these patients. Dominant mutations in two other transcription factor genes, *EYA4* (DFNA10) (36) and *POU4F3* (DFNA15) (14), are associated with non-syndromic hearing loss. In patients with mutations in these genes, the hearing loss is late in onset and progressive.

We have identified a mutation in a third transcription factor gene, *TFCP2L3*, as being responsible for non-syndromic autosomal dominant hearing loss (DFNA28). The predicted translation product of *TFCP2L3* has sequence similarity to a group of proteins comprising the transcription factor cellular promoter 2 (TFCP2) family (Table 1). The TFCP2 family of transcription factors share a novel DNA-binding domain that is distinct from previously described DNA-binding domains such

as the zinc finger, helix–turn–helix or basic leucine zipper (37). TFCP2L3 and TFCP2 share 51% similarity and 34% identity in the amino acid sequence of the DNA-binding domain, and show 42% similarity and 27% identity overall (Fig. 5).

Many *in vivo* and *in vitro* target genes of TFCP2-like transcription factors have been identified. TFCP2 binds to many promoters, including those of human immunodeficiency virus type 1 (38–40), simian virus 40 (41) and mouse thymidylate synthase (42). *TFCP2* has been proposed as a genetic susceptibility factor in Alzheimer's disease (43).

The regulatory targets of *Drosophila grh* include *dopa decarboxylase* and the homeotic genes *engrailed, fushi tarazu* and *Ultrabithorax* (44–47). *TFCP2L2* is the mammalian gene closest in sequence similarity to *grh*, and gel shift assays demonstrates that TFCP2L2 can bind several constructs containing sequences known to be targets of *grh* (25). Moreover, TFCP2L2 binds to the human *engrailed-1 homolog* promoter and activates transcription.

TFCP2L3 and TFCP2L2 share 83% similarity (72% identity) in the DNA-binding region and 68% overall similarity (55% identity). Recently, Wilanowski *et al.* (25) suggested that TFCP2L3 and TFCP2L2 can form homo- and heterodimers through a conserved domain at their C termini. Thus, the genes regulated by TFCP2L3 may show considerable overlap with those regulated by TFCP2L2.

Given that there is no obvious clinical feature, other than hearing loss, distinguishing the carriers of the 1609–1610insC mutation of *TFCP2L3*, it is perhaps surprising that *TFCP2L3* should be so widely expressed. Northern dot blot analysis showed *Tfcp2l3* expression in a variety of epithelial tissues, such as prostate, thymus, kidney, mammary gland, pancreas and digestive tract. *In situ* hybridization showed that *Tfcp2l3* mRNA is expressed in the mouse cochlea most abundantly during embryonic development, and less so during early postnatal stages of development. In the cochlea, *Tfcp2l3* message appears to be confined to the epithelial cells of placodal origin, which line the endolymph-containing scala media. Its role in differentiation and maintenance of these cells is unknown, but the fact that hearing is initially normal in individuals carrying the 1609–1610insC mutation suggests that the mechanism that results in hearing loss has more to do with a role in epithelial cell maintenance. The wide variation in the onset of hearing loss, and in the shape of the audiometric curves in affected individuals, also argue for a considerable role for other factors, either environmental or genetic, in determining the events leading to hearing loss. The possibility that there is some functional redundancy between TFCP2L3 and TFCP2L2 may also account for the lack of any obvious phenotype in the epithelial tissues of individuals with the 1609–1610insC mutation of *TFCP2L3*. Functional redundancy between *Tfcp2* and *Tfcp2a (Nf2d9)* was the likely reason for lack of phenotype in mice nullizygous for *Tfcp2* (48)

There are no known mutations in the mouse homolog, *Tfcp2l3*. A mouse model for *DFNA28* may help to elucidate the pathological events that result in hearing loss, and possibly aid in the identification of *Tfcp2l3* target genes both in the auditory system and in other tissues. It would also be interesting to know, given the broad epithelial expression pattern of *Tfcp2l3*, whether other clinical manifestations of DFNA28-associated deafness would be revealed in a mouse mutant.

MATERIALS AND METHODS

Clinical data

Written informed consent was obtained from all of the LMG45 family members who participated in this study approved by the NINDS IRB Protocol 97-DC-0180 at the National Institutes of Health. Pure-tone audiometry was performed at 0.25, 0.5, 1, 2, 4, 6 and 8 kHz for air conduction and at 0.5, 1, 2 and 4 kHz for bone conduction on 21 family members. Pure-tone averages (PTA) were calculated for decibels of hearing loss at 0.5, 1, 2 and 4 kHz. Individuals were considered affected if they showed bilateral PTAs greater than the 95th percentile when compared to with age- and sex-dependent control audiometric curve (21).

Linkage analysis

Genomic DNA was extracted either from whole blood using a Puregene kit (Gentra Systems) or from buccal swabs by a standard protocol (49). Twenty-one family members, including nine affected patients, were genotyped using the Marshfield screening panel (version 9a; Marshfield Medical Research Foundation). Additional simple tandem repeats (STRs) in the DFNA28 region were identified by inspection of publicly available BAC sequence data. Fluorescent dye-labeled STR amplicons were analyzed on a PE/Applied Biosystems 377 DNA sequencer using Genescan software (Applied Biosystems). LOD scores were calculated using the FASTLINK version of the LINKAGE software package (50). Conservative estimates of gene frequency (1/10 000), phenocopy rate (1/1000) and penetrance (95%) were used in the calculation of LOD scores.

DNA sequencing analysis

Six candidate genes in the DFNA28 interval were evaluated by direct sequencing of PCR products generated from genomic DNA of affected and unaffected LMG45 individuals. Primers were designed to amplify each coding exon with adjacent intron/exon boundaries. DNA sequencing results were analyzed using the PHRED/PHRAP/CONSED software suite (51,52). 150 DNA samples from a Human Diversity Panel (Coriell Cell Repositories HD01-09 and HD100CAU) were also amplified and sequenced to determine the population frequencies of candidate mutations.

cDNA analysis

Based upon the sequence of mouse and human ESTs and predicted *TFCP2L3* exons from human genomic sequence (BAC KB1107E3), several primers were designed to amplify *TFCP2L3* cochlear cDNA. Human *TFCP2L3* cDNA was amplified from human fetal-cochlea cDNA that was prepared using mRNA kindly provided by Dr C.C. Morton (Harvard University). A 3761 nt fragment containing the ORF and part of the 3′-UTR of human *TFCP2L3* was amplified using Advantage2 polymerase (Clontech) with a forward primer (5′-GATCAAACATGTCACAAGAGTCG), and a reverse primer (5′-CTGGATCCACGATGCAAAC).

Northern blot analysis

A 495 nt cDNA fragment corresponding to part of the human 3′-UTR of *TFCP2L3* mRNA (GenBank accession no. AK023844, nucleotides 3273–3767), was used to probe a northern blot. Following reverse transcription of normal human lymphoblast cell line RNA with oligo-dT primers (Clontech), *TFCP2L3* cDNA was amplified by PCR using forward (5′-CTGGGAGAGAGAAGCTGTGG) and reverse (5′-CTGG-AGCCACGATGCAAAC) primers and then cloned into pGEM-T Easy (Promega). The DNA probe was labeled with [α-^{32}P]dCTP using Redi-Prime Random primers (Amersham Pharmacia). A multiple human tissue northern blot (Clontech, 7776-1) was prehybridized with Ultrahybridization solution (Clontech) at 42°C for 30 min, hybridized for 16 h at 42°C with denatured α-^{32}P probe (1.5×10^7 c.p.m), and washed in 1× SSC and then in 0.1% SDS at 42°C. The blot was exposed to BioMaxMR film (Kodak) for 6 h and developed.

In situ hybridization

Oligo-dT-primed cDNA was prepared from total RNA obtained from mouse C57BL/6J temporal bones dissected at P1. A 389 nt fragment spanning part of the mouse 3′-UTR *TFCP2L3* mRNA (GenBank accession no. BC004782, nucleotides 664–1052), was generated by PCR using forward (5′-GGTATACTCCTTCCTGGGAG) and reverse (5′-GCCCAACCACTCAAGAAATG) primers and cloned into pGEM-T Easy (Promega). Probes were prepared by *in vitro* transcription of linearized plasmid using T7 polymerase (Stratagene) for the generation of sense and antisense RNA probes in the presence of [α-^{35}S]UTP. E18.5 P5 mouse heads were hemi-dissected and brain tissue was removed prior to overnight fixation in 4% paraformaldehyde in 1× phosphate-buffered saline. Tissue was embedded in paraffin, and serial cross-sections (10 µm) were collected onto 2% gelatin-treated microscope slides for *in situ* hybridization as previously described (53,54). Hybridization was performed at 52°C for 16 h using 54 000 c.p.m/µl [α-^{35}S]UTP-labeled RNA.

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