REPORT

Expression of *GJB2* and *GJB6* Is Reduced in a Novel *DFNB1* Allele

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In a large kindred of German descent, we found a novel allele that segregates with deafness when present in *trans* with the 35delG allele of *GJB2*. Qualitative polymerase chain reaction–based allele-specific expression assays showed that expression of both *GJB2* and *GJB6* from the novel allele is dramatically reduced. This is the first evidence of a deafness-associated regulatory mutation of *GJB2* and of potential coregulation of *GJB2* and *GJB6*.

Mutations in *GJB2,* the gene encoding gap junction protein connexin 26 (Cx26), are the most common cause of prelingual-onset, recessively inherited, nonsyndromic, sensorineural hearing loss (SNHL) in humans. *GJB2* and *GJB6,* which encodes connexin 30 (Cx30), comprise the *DFNB1/A3* locus at 13q12 (fig. 1). In the vertebrate cochlea, Cx26 and Cx30 are coexpressed as heteromeric connexons in nonsensory cells of the organ of Corti as well as in subsets of cells of the spiral ligament and stria vascularis[1–3] and are implicated in the maintenance of cochlear K^+ gradients necessary for proper hair-cell function.[4,5]

More than 80 recessive mutations of *GJB2,* nearly all affecting proper translation of the Cx26 protein, and several dominant missense mutations are implicated in *DFNB1/A3* hearing loss. Others cause skin disease with accompanying SNHL, including Vohwinkel syndrome (MIM 124500), Bart-Pumphrey syndrome (MIM 149200), palmoplantar keratoderma, with deafness (PPK [MIM 148350]), and keratitis-ichthyosis-deafness (KID [MIM 148210]). Three dominant missense mutations of *GJB6* have been shown to cause the skin disorder Clouston syndrome (hidrotic ectodermal dysplasia [MIM 129500]).

In contrast to the abundance and diversity of *GJB2* hearing-loss mutations, only a single dominant mutation of *GJB6* causing nonsyndromic hearing loss had been published[6] before the identification of two large deletions—of 309 kb and 232 kb: del(*GJB6-D13S1830*) and del(*GJB6-D13S1854*), respectively—truncating the 5′ end of *GJB6* (fig. 1). These deletions segregate with hearing loss when present homozygously or heterozygously with each other or in *trans* with a recessive *GJB2* mutation.[7–11] Investigators have suggested, but have not demonstrated, that loss of appropriate regulation of *GJB2* from chromosomes bearing these deletions may underlie the hearing loss in these individuals,[7–11] which may be exacerbated by loss of one *GJB6* allele. Common et al.[12] showed immunohistochemical evidence that Cx26 expression is disrupted in certain skin-cell types in an individual bearing the larger deletion in *trans* with *GJB2* 35delG, suggesting disruption of a *GJB2 cis*-regulatory element located within this deleted interval. Mutation screening of *GJB2* in individuals whose hearing loss and family history are consistent with *GJB2* deafness reveals a significant number of subjects with only one identified mutation. Additional screening for *GJB6* deletions explains only some of these heterozygotes.

We report here evidence of a novel pathogenic *DFNB1* allele for which we demonstrate reduction in or loss of detectable expression of message from both *GJB2* and *GJB6*. Figure 2 shows a small portion of the pedigree of MSU-DF5, a large American kindred of mainly German descent. After obtaining written informed consent, we collected DNA and performed audiological testing on >200 family members. We screened all samples for *GJB2* 35delG and for *SLC26A4* L445W. Ten MSU-DF5 family members are hearing impaired due to homozygosity of 35delG (one, DF5-68, is shown in fig. 2); one deaf family member has received a diagnosis of Pendred syndrome (MIM 274600) and is homozygous for *SLC26A4* L445W (not shown in pedigree). Microsatellite and SNP genotyping across the *GJB2/GJB6* genomic region (fig. 1 and table 1) allowed us to define a number of haplotypes. Three distinct haplotypes bearing 35delG exist within the family, indicating more than one founder for 35delG in this community (two 35delG haplotypes appear in fig. 2). Notably, four deaf family members, DF5-20, -70, -122, and -194, are heterozygous for 35delG and share a common haplotype (indicated in fig. 2 by a black bar with a star) that is longer than 600 kb and shorter than 3.1 Mb on their non-35delG chromosome. These individuals are profoundly hearing impaired, in contrast to DF5-68, who is homozygous for 35delG and the father of DF5-70 (fig. 3). Of the 14 family members who carry the novel allele, all those with 35delG on their other chromosome (4 individuals) have hearing loss, whereas all those with any other allele (10 individuals) have normal hearing ($P < .001$, by Fisher's exact test). We also performed a linkage analysis (easyLINKAGE Plus

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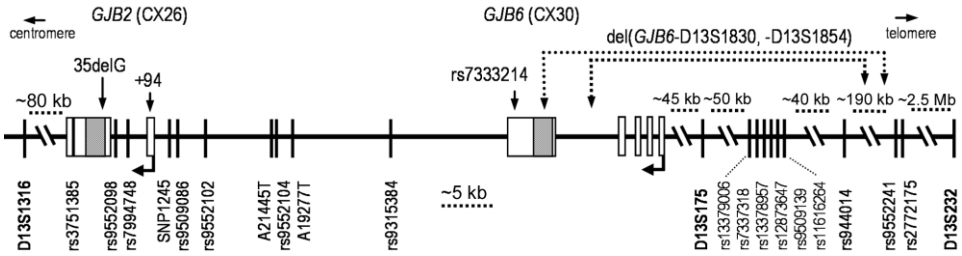

Figure 1. Map encompassing haplotyped region of 13q11-12, showing locations of *GJB2, GJB6,* genotyped microsatellite and SNP markers, and breakpoints of del(*GJB6-D13S1830*) and del(*GJB6-D13S1854*), all shown approximately to scale. The locations of the variants used for allele-specific expression assays—*GJB2* 35delG, *GJB2* +94, and *rs7333214*—are also indicated. Boxes indicate exons, and the hatched boxes indicate the coding regions of the two genes. Vertical lines show approximate locations of genotyped microsatellites (*in bold*) and SNPs. *D13S232* is the most proximal microsatellite showing recombination of the novel haplotype in affected individuals. The transcriptional start sites of *GJB2* and *GJB6* are indicated by right-angled arrows.

v.3.01RC1, SuperLink v1.4) at the *DFNB1* locus, using 28 individuals (shown in the boxed areas in fig. 2). We assigned the same mutant status to both the novel haplotype and the 35delG allele, and all other haplotypes were considered nonmutant. The resulting LOD score of 2.08 indicates that there is a good likelihood that the novel haplotype harbors a *DFNB1* mutation.

We sequenced the entire coding regions and 5′ and 3′ UTRs of both *GJB2* and *GJB6* in three of our affected probands, including splice sites, alternative exons and promoter regions, the entire single intron of *GJB2,* and several kilobases of sequence around each gene, and found no sequence variants unique to the novel haplotype. Heterozygosities found in sequencing also showed that *GJB2* and *GJB6* are intact on both chromosomes and that the affected individuals do not bear either del(*GJB6-D13S1830*) or del(*GJB6-D13S1854*). Southern blotting indicated no rearrangements or unusual methylation around *GJB2* (fig. 4).

We hypothesized that an unidentified *cis*-regulatory element of *GJB2* exists within the *DFNB1* locus and is disrupted on our novel pathogenic allele. Given the imprac-

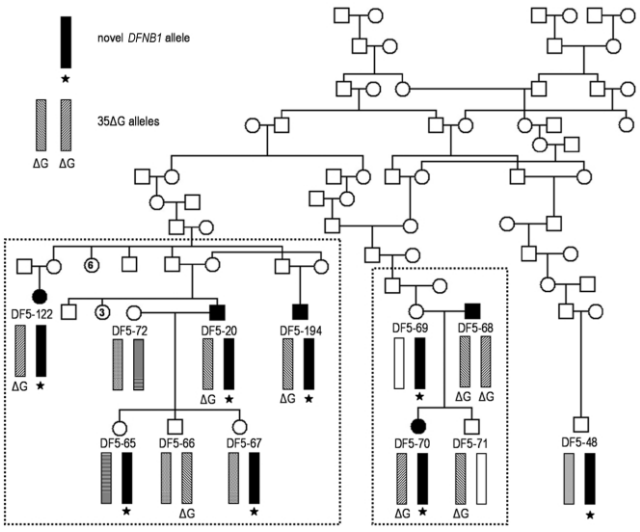

Figure 2. A small portion of the MSU-DF5 pedigree, with *DFNB1* haplotypes indicated for selected individuals. Five individuals with congenital SNHL are represented by blackened symbols. One of these, DF5-68, is hearing impaired because of homozygosity of *GJB2* 35delG (ΔG). The other four are heterozygous for *GJB2* 35delG and bear the same haplotype on their non-35delG chromosome (*black bar with star*). Individuals contained within the boxed areas were included in the linkage analysis.

Table 1. Selected Haplotype Assignments Based on Genotypes at Microsatellites and SNPs in a 620-kb Region of Chromosome 13 Including *GJB2* and *GJB6*

Marker	Location (bp)	Novel *DFNB1* Allele	A	B	C	D	E
D13S1316	19,580,089-442	6	6	6	5	5.5	6
rs3751385	19,660,956	C	T	T	C	C	C
35delG	19,661,685	+	ΔG	ΔG	+	+	+
rs9552098	19,662,895	C	T	T	C	C	C
rs7994748	19,664,130	T	C	C	T	C	T
GJB2 +94	19,664,944	C	C	C	A	C	C
SNP1245	19,665,308	T	C	C	T	C	T
rs9509086	19,665,957	C	A	ND	C	A	ND
rs9552102	19,668,529	T	A	ND	T	T	ND
A21445T	19,673,593	A	A	A	A	T	A
rs9552104	19,673,997	C	G	ND	C	C	C
A19277T	19,675,761	A	ND	A	ND	T	A
rs9315384	19,682,018	A	G	G	A	G	A
rs7333214	19,694,497	C	C	C	C	A	C
D13S175	19,746,380-734	4	3	3	2	4	2
rs13379006	19,795,790	T	C	C	T	C	C
rs7337318	19,795,911	C	G	G	C	G	G
rs13378957	19,795,932	A	C	C	A	C	C
rs12873647	19,796,279	T	C	C	T	C	C
rs9509139	19,796,316	A	G	G	A	G	G
rs11616264	19,796,330	A	T	T	A	T	T
rs944014	19,837,040	G	A	G	G	G	G
rs9552241	20,025,569	ND	ND	G	C	G	G
rs2772175	20,198,712	T	T	ND	A	A	A

NOTE.—The "A" and "B" columns indicate delG (ΔG) alleles carried by DF5-20 and DF5-70, respectively. The "C" column corresponds to the haplotype shown by a box with stippling, "D" to the haplotype shown by a box with horizontal lines, and "E" to the haplotype shown by a white box in figures 2, 5, and 6. Locations are based on UCSC Genome Browser, May 2004 assembly. + = wild type; ND = not determined.

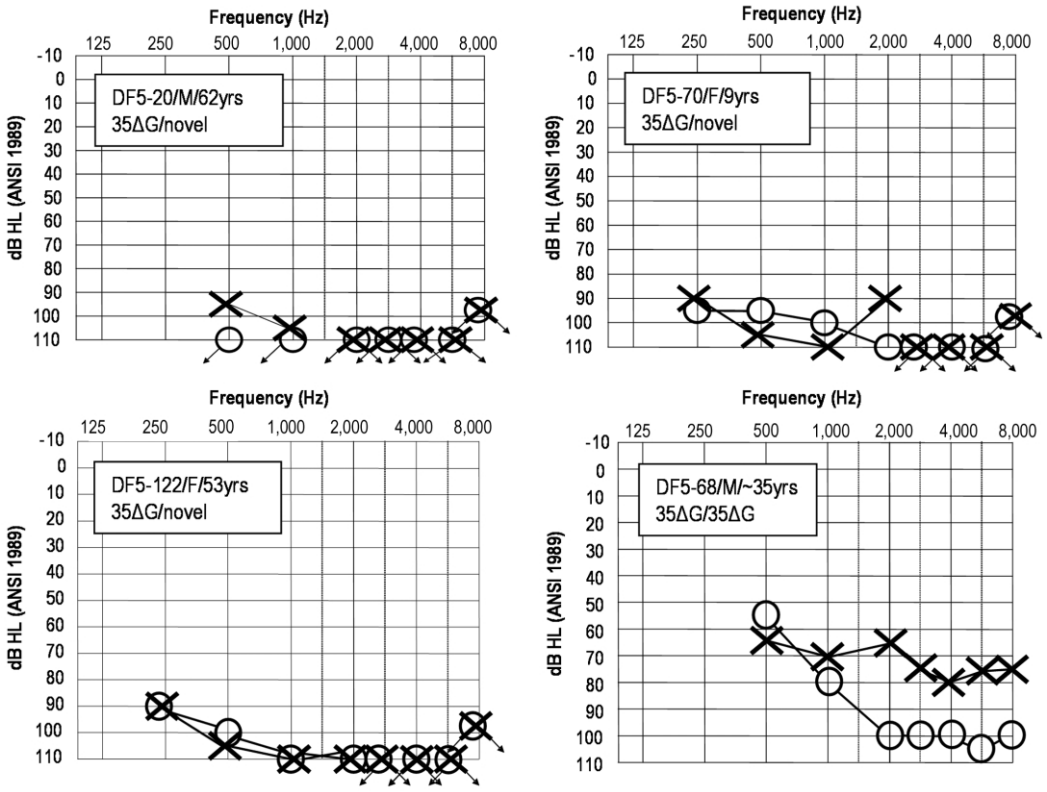

Figure 3. Audiograms of four MSU-DF5 family members (identified by ID/sex/age). dB HL = decibels hearing level; ANSI = American National Standards Institute. DF5-20, -70, and -122 are *GJB2* 35delG (35ΔG) heterozygotes bearing the novel pathogenic allele. Note that all three have profound SNHL at all tested frequencies. By contrast, DF5-68, homozygous for *GJB2* 35delG and the father of DF5-70, has significantly more residual hearing, particularly in his left ear. Although only pure-tone air-conduction thresholds are shown (circle = right ear; × = left ear; arrows = threshold beyond tested limit), bone conduction thresholds and immittance measures (not shown) indicate that the hearing loss in all four family members is sensorineural.

ticality of searching for candidate variants across the entire locus, we first sought to acquire evidence of loss of *GJB2* expression from this allele. We developed three allele-specific PCR assays to assess the relative abundance of transcript from each allele, using tissue both easily available and expected to express Cx26. To isolate RNA, buccal cells from several family members were collected on Cytosoft brushes and were immediately stored in 1 ml Trizol (Gibco/Life Technologies) on ice. Some samples were stored at −20°C for one to several days. RNA was isolated after the Trizol protocol, was resuspended in 20 μl H_2O, and was stored at −80°C. We followed a modified Superscript II (Invitrogen) reverse-transcription protocol for cDNA synthesis; 5–8 μl template RNA, 1 μl of 0.5 μg/μl oligo(dT) primer, 1 μl of 10-mM dNTP, and 2 μl H_2O were heated to 70°C for 10 min, were chilled on ice, and then were mixed and incubated at 42°C for 2 min after 4 μl of 5 × first-strand buffer and 2 μl of 0.1-M dithiothreitol was added. Addition of 1 μl Superscript II reverse transcriptase was followed by incubation at 42°C for 50 min and then incubation at 70°C for 15 min. A final incubation at 37°C for 20 min followed the addition of 1 μl RNaseH. A negative control to test for genomic DNA contamination was generated for each sample by omitting the Superscript II and replacing it with 1 μl H_2O. These negative controls were carried through the assays side by side with the corresponding sample. In no instance was product observed for these controls (data not shown).

The figure is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Figure 4. Southern blot of region around *GJB2,* in DF5-20 and an unrelated control. The legend is available in its entirety in the online edition of *The American Journal of Human Genetics*.

We designed a cDNA-specific PCR assay to assess the relative abundance of 35delG and non-35delG product amplified from *GJB2* cDNA synthesized from 35delG heterozygotes (fig. 5*A*). A standard PCR-based assay for the identification of 35delG in genomic DNA[13] is based on the introduction of a restriction site for *Bst*NI by a 3′ mismatch primer (5′-GCTGGTGGAGTGTTTGTTCACACCCGC-3′)

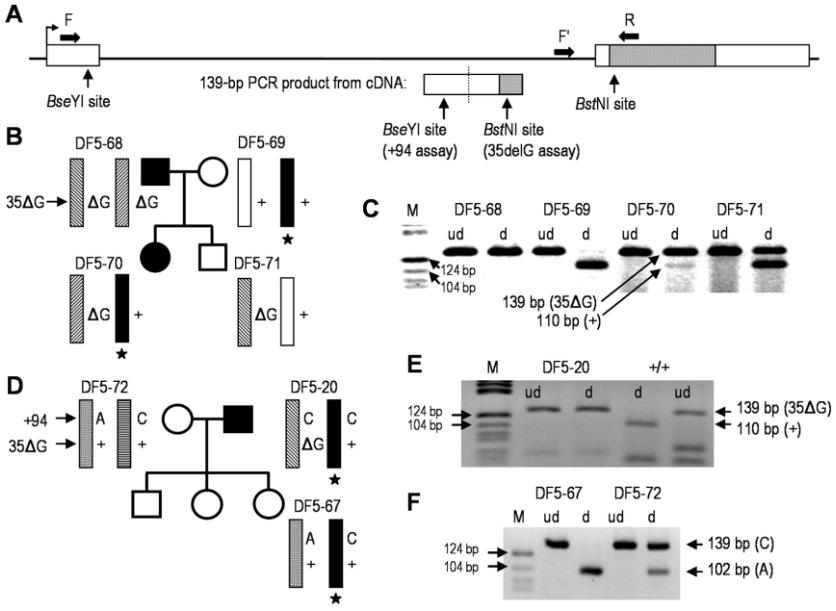

Figure 5. Allele-specific expression assays for *GJB2*. *A,* Schematic showing design of *Bst*NI (which assays for 35delG [35ΔG] genotype) and *Bse*YI (which assays for +94 genotype) digestion assay performed using 139-bp PCR product amplified from cDNA only. A forward primer (F) located in the first, noncoding exon of *GJB2* is separated on genomic DNA from the reverse mismatch primer (R), located in the coding region of *GJB2,* by a >3-kb intron (F′ = forward primer for genomic 35delG assay). The *Bst*NI site is introduced into product amplified from wild-type (non-35delG [indicated by "+"]) template. *B* and *D,* Pedigrees including DF5-70 and DF5-20, heterozygous for both *GJB2* 35delG and the novel pathogenic allele (*black bar with star*). Also indicated are the 35delG and +94 genotypes. *C* and *E,* Results of cDNA-specific assay for *GJB2* expression based on 35delG genotype. Products of 139 bp were amplified from all tested family members (ud = undigested, no enzyme added; d = addition of restriction enzyme). DF5-20, -70, and -71 (normal-hearing sibling of DF5-70) are all heterozygous for 35delG; however, after digestion with *Bst*NI, only DF5-71 clearly shows both 139-bp and 110-bp products, indicating representation of two different alleles at the cDNA level. DF5-20 and DF5-70, who both bear the novel pathogenic non-35delG allele, show either no or barely detectable 110-bp product, indicating underrepresentation of this allele in the pool of amplified product (M = Marker V [Roche]). *F,* Results of cDNA-specific assay for *GJB2* expression based on genotype at *GJB2* +94. Products of 139 bp (ud = undigested) were amplified from both tested family members, who are both heterozygous (A/C) at +94. Although the digested product (d) amplified from DF5-72 cDNA clearly indicates heterozygosity at the cDNA level, lack of any detectable 139-bp product in the digested DF5-67 sample indicates underrepresentation of the novel (C) allele among the amplified product.

that overlaps the run of Gs between nt 30 and nt 35. The *Bst*NI restriction site is present in PCR product amplified from wild-type (non-35delG) alleles only and is absent in PCR product amplified from 35delG alleles. We paired a 5′ primer located in exon 1 (5′-CGCAGAGACCCCAACGCCGAGA-3′) with the 3′ mismatch primer to generate a 139-bp PCR product from cDNA template only, which, when incubated with *Bst*NI, will yield digestion products of 110 bp and 29 bp only if the 35delG mutation is not present. For each individual we assayed, we amplified from cDNA template and from the corresponding negative control. A 20-μl volume PCR (1–5 μl template cDNA, 2 μl 10 × Qiagen buffer, 4 μl Q solution (Qiagen), 0.16 μl of 25-mM dNTP (Invitrogen), 1 μl of each 20-μM primer, 5 U Invitrogen *Taq* DNA polymerase, and remainder H_2O) was denatured for 3 min at 94°C, followed by 40 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Of each PCR, 10 μl was digested with ~10 U *Bst*NI (New England Biolabs) for >2 h, and 3 μl of each reaction was run on a 3.5% NuSieve 3:1 agarose gel containing 0.3 μg/ml ethidium bromide.

The results (fig. 5*B*–5*E*) for the two deaf 35delG heterozygotes that we assayed, DF5-20 and DF5-70, indicate that, although they are heterozygous for 35delG at the genomic DNA level, the barely detectable or absent 110-bp product indicates underrepresentation of *GJB2* transcript from the novel (non-35delG) allele. Results from the parents and sibling of DF5-70 are consistent with their genotypes and provide appropriate controls. Unbiased amplification and digestion of both 35delG and non-35delG alleles is shown by the result for DF5-71, the sibling of DF5-70, who is also heterozygous for 35delG but carries a normal non-35delG chromosome. Sequencing of genomic DNA across the 139-bp target sequence (not shown) confirmed that there are no differences between these two individuals that would give rise to a bias in either amplification or digestion.

To demonstrate that loss of expression of this allele is

not unique to individuals carrying the 35delG mutation on their other chromosome 13, we developed a second assay to determine allele-specific *GJB2* expression in a normal-hearing adult offspring of DF5-20 bearing the novel haplotype (fig. 5*A* and 5*D*). DF5-67 carries a previously unreported exon 1 SNP at position +94. *Bse*YI digestion of the 139-bp product amplified with the same primers as described above will yield 102-bp and 27-bp products only if an A is present at position +94 (10 μl of each PCR product was digested with 5 U *Bse*YI enzyme [New England Biolabs] for >2 h, 5 μl of 1% SDS was added, and electrophoresis was performed as above). Although both DF5-67 and -72 are heterozygous for +94 at the genomic level, all of the product amplified from DF5-67 cDNA was digested (fig. 5*F*), indicating that no or very little of the PCR product had been amplified from template derived from the novel allele that bears the common variant, C. Again, sequencing of genomic DNA across the target sequence shows no differences between DF5-67 and DF5-72 that would yield biased amplification or digestion of the resulting amplimers.

We hypothesized that loss of expression of *GJB2* might be accompanied by loss of expression of *GJB6,* since these two genes lie within 30 kb of each other and their products are coexpressed in the cochlea. DF5-65, a normal-hearing adult offspring of DF5-20, bears the novel haplotype and is heterozygous for *rs7333214* in the 3′ UTR of *GJB6,* allowing us to test this hypothesis (fig. 6*A* and 6*B*). Using a forward primer located in the fourth noncoding exon (5′-CACCATTGGCTTCTAGGCAC-3′) and a reverse primer located close to the 3′ end of the 3′ UTR (5′-CCACACTGTTCCGTCTACAT-3′), we amplified a 1,550-bp product from cDNA template only, in a 20-μl PCR under the following conditions: 2 μl of 10× Qiagen buffer, 4 μl Q solution (Qiagen), 0.16 μl of 25-mM dNTP (Invitrogen), 1 μl of each 20-μM primer, 5 U *Taq* DNA polymerase (Invitrogen), and remainder H_2O were denatured for 5 min at 94°C, followed by 50 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. Digestion of this product for ~2 h at 37°C with ~10 U *Hpy*CH4IV (New England Biolabs) yields digestion products of 537, 460, 338, and 214 bp if an A (the rare variant) is present at *rs7333214*; the 460-bp product is digested to 292- and 168-bp products if a C is present at *rs7333214* (the common variant, carried, in this case, on the novel allele). Two microliters of each reaction was run on a 1.5% agarose gel (10 μg ethidium bromide/30 ml) in 0.5× Tris-borate-EDTA buffer. Comparison of the result for DF5-65 with that for a control (DF5-72, his mother) who is also heterozygous for *rs7333214* (fig. 6*B*) and whose DNA is identical across the target sequence, shows that the 292- and 168-bp products, derived from amplification of the novel allele, are significantly underrepresented in DF5-65 (fig. 6*C*). This is consistent with the interpretation that expression of *GJB6* as well as that of *GJB2* is diminished from the novel allele. Additionally, since the cDNA specificity of each assay depends on the amplification of PCR product

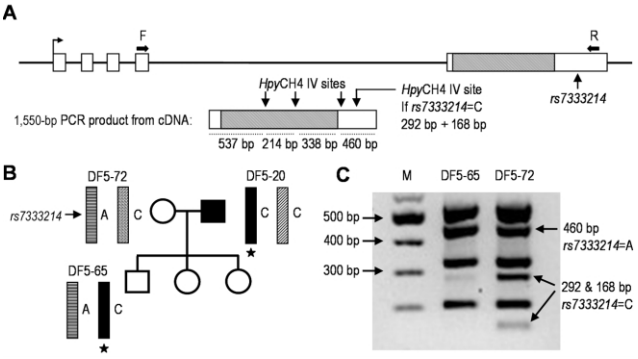

Figure 6. Allele-specific expression assay for *GJB6*. *A,* Schematic of design of *Hpy*CH4IV digestion assay for allele-specific expression of *GJB6* mRNA performed using 1,550-bp PCR product amplified from cDNA only. A forward primer (F) located in exon 5 of *GJB6* is separated on genomic DNA from the reverse primer (R), located in the *GJB6* 3′ UTR, by a >6-kb intron in addition to ~1,500 bp of the final exon. *B,* Pedigree showing *rs7333214* genotypes of assayed subjects. DF5-65 bears the novel pathogenic allele (*black bar with star*) and is heterozygous at *rs7333214*. *C,* Results of cDNA-specific assay for *GJB6* expression. Digestion of product amplified from DF5-72 yields 460-bp as well as 292- and 168-bp products, indicating that both *GJB6* alleles are expressed. Digestion of product amplified from DF5-65 yields a robust 460-bp band, but the band at 292 bp is only barely visible. This indicates underrepresentation of the novel allele among the amplified product.

from only cDNA from which a long intron has been properly spliced out, failure to accumulate PCR product might also be expected from an allele that is mutant for proper splicing. It is highly unlikely that *GJB2* and *GJB6* on this chromosome both contain unidentified splice mutations.

This study provides evidence that the MSU-DF5 family is segregating a novel *DFNB1* allele that is characterized by significant reduction in expression of both *GJB2* and *GJB6*. This loss of expression is heritable, *cis*-acting, and not due to a simple parent-of-origin epigenetic modification. We sought and found no coding-region mutations, splice-site mutations, or any other sequence variant unique to the novel allele within or close to *GJB2* or *GJB6,* suggesting that loss of function of an as-yet-unidentified *cis*-regulatory element(s) is responsible for our observations. It is possible that a locus-control region (LCR) regulates the coexpression of *GJB2* and *GJB6*. An LCR was initially described in the β-globin locus, where it is absolutely required for expression of any of the genes in this cluster. Other examples include the growth hormone and T_H2 cytokine loci.[14] Such an element(s) may be located within the common interval deleted on chromosomes bearing del(*GJB6-D13S1830*) or del(*GJB6-D13S1854*). The severity of hearing loss in the individuals in our kindred who are heterozygous for 35delG and this novel allele is similar to that in individuals who are del(*GJB6-D13S1830*)/35delG, who as a group have been shown to have more severe hearing loss than 35delG homozy-

gotes.[15] Beyond the identification of the basal promoters of both genes,[16–19] nothing is known of *cis*-acting elements that regulate *GJB2* and *GJB6*. The common observation of an excess of deaf individuals bearing only a single identified *GJB2* mutation strongly suggests that *GJB2* regulatory mutations remain to be identified.

Acknowledgments

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Web Resource

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for Vohwinkel syndrome, Bart-Pumphrey syndrome, PPK, KID, hidrotic ectodermal dysplasia, and Pendred syndrome)

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