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CHARACTERIZATION OF A DFNB1 DELETION ALLELE IN A MICHIGAN KINDRED OF GERMAN DESCENT

presented by

ELLEN SHIELDS WILCH

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PhD degree in Genetics

Karen Frideric
Major Professor's Signature

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CHARACTERIZATION OF A DFNB1 DELETION ALLELE IN A MICHIGAN KINDRED OF GERMAN DESCENT

By

Ellen Shields Wilch

A DISSERTATION

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ABSTRACT

CHARACTERIZATION OF A DFNB1 DELETION ALLELE IN A MICHIGAN KINDRED OF GERMAN DESCENT

By

Ellen Shields Wilch

A novel DFNB1 allele identified in a kindred segregating recessively-inherited hearing loss contains a 131.4 kb deletion, upstream of the transcriptional start sites of both *GJB2* and *GJB6*, the genes encoding the gap junction proteins connexin 26 and connexin 30, respectively. The four family members who are compound heterozygotes for this deletion and for the 35delG mutation of *GJB2* are all profoundly deaf, and have been since infancy. Heterozygous carriers of this deletion have normal hearing. We demonstrate that this allele segregates with a low-expression phenotype of *GJB2* and *GJB6* mRNA, assayed by allele-specific PCR of cDNA made from buccal cell RNA of four individuals (three assayed for *GJB2* expression, one assayed for *GJB6* expression). Considering the evidence of this allele together with that of other DFNB1 deletion alleles, it is likely that the pathogenicity of the DFNB1 deletion alleles is a result of loss of one or more distal *GJB2* enhancer elements. One or more critical *GJB2* regulatory elements is likely to exist within the genomic interval that is common to the three DFNB1 deletions that leave the *GJB2* sequence intact; this interval extends from about chr13:19,837,300-19,932,800 (Mar2006 assembly (NCBI36/hg18)). Assays of candidate enhancer elements identified by cross-species conservation have shown that some sequences within

the 131.4 kb deletion interval upregulate or downregulate transcription of a reporter gene in two human epithelial cell lines. Disruption of distal *GJB2* regulatory elements may underlie idiopathic hearing loss in persons with monoallelic mutation in the *GJB2* coding region. As mutations in *GJB2* are the most common cause of congenital hearing loss worldwide, understanding the *cis*-regulatory landscape of this gene is important in assessing pathogenicity of extragenic sequence variants in suspected DFNB1 alleles, and in interpreting the variation in expressivity of hearing loss in persons with biallelic coding region mutations of *GJB2*.

This dissertation is dedicated to the19th-century German immigrant settlers of Clinton and Ionia Counties, Michigan, and to their descendants.

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There are too many other people whose support and friendship I wish to acknowledge, but I will do my best to make a list:

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analyzed haplotype data, and maintained project data and other records. Dr. Jill Elfenbein coordinated the audiological testing and did much of the testing herself.

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- Love to Thom and the kids, Joe and Miriam.

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LIST OF ABBREVIATIONS

35delG	deletion of a single G nucleotide at c.35
array CGH	array comparative genome hybridization
CEPH	Centre d'Etude du Polymorphisme Humain
CNV	copy number variant
Cx26	Connexin 26
Cx30	Connexin 30
dB	decibel
DFNB1	Deafness locus, autosomal recessive, first identified
DFNB4	Deafness locus, autosomal recessive, fourth identified
DSB	double-strand break
GJB2	gap junction beta 2
GJB6	gap junction beta 6
K+	potassium
kb	kilobase
KCNJ10	potassium inwardly-rectifying channel, subfamily J, member 10
kDa	kilodalton
LINE	long interspersed nuclear element
MIM	Mendelian Inheritance in Man [sic]
mM	millimolar
mV	millivolt
Na+	sodium
nm	nanometer
nM	nanomolar

NHEJ	non-homologous end joining
OMIM	Online Mendelian Inheritance in Man [sic]
PCR	polymerase chain reaction
SLC26A4	solute carrier family 26, member 4
SNHL	sensorineural hearing loss
SNP	single nucleotide polymorphism
UTR	untranslated region

CHAPTER 1

THE MSU-DF5 KINDRED AND HEARING LOSS

THE MSU-DF5 KINDRED AND HEARING LOSS

The MSU-DF5 kindred is an expanded founder population in central Michigan, centered in Ionia and Clinton counties, and including the towns of Westphalia, Pewamo, and Fowler, among others. Beginning in the 1830s and continuing throughout the remainder of the 19th century, several hundred Catholic German immigrants homesteaded in this part of Michigan. Many, though not all, emigrated from the Eifel region of western Germany. A strong sense of community identity based on a shared German heritage and Catholic faith persists to this day. Marriage between the descendents of the original immigrants has been and remains common. Michigan State University faculty, most notably audiologist Dr. Jill Elfenbein and geneticists Dr. Rachel Fisher and Dr. Karen Friderici, began a long-term collaborative research project with this community in 1996, to investigate the etiology of inherited hearing loss within this population. To date, Dr. Fisher has gathered genealogical records from family and public resources that include 28,256 individuals. Ninety-eight percent of these individuals can be related in a single pedigree (Schutte DL, 2010). Among these, 223 community members have undergone audiological evaluations, given personal and family health histories, and donated DNA. Of the nineteen family members with congenital or prelingual, bilateral, nonsyndromic sensorineural hearing loss, eleven were found to be heterozygous for the 35delG mutation of *GJB2*, the gene encoding the gap junction protein connexin 26 (Cx26). Mutations in *GJB2* are the most common cause of inherited hearing loss. One person is homozygous for the L445W mutation of *SLC26A4*, the gene encoding the protein pendrin. Mutations in pendrin are also a common cause of inherited hearing loss. Three persons carry monoallelic coding region variants of *SLC26A4*; their diagnosis

remains under investigation. Four family members, all profoundly deaf, carry the 35delG mutation of *GJB2* on one chromosome, and share by descent on the other chromosome a novel deletion mutation that affects the regulation of *GJB2*. The identification and characterization of this mutation is the subject of this dissertation. The remainder of this chapter is a literature review, divided into three sections. The first is a description of the mammalian ear, with particular focus on the structures and mechanisms involved in the generation and maintenance of potassium gradients and the endocochlear potential, in which *GJB2* and its neighboring gene, *GJB6*, are highly implicated. The second section concerns the cell biology of the protein products of *GJB2* and *GJB6*, connexins 26 and 30, and the phenotypic consequences of mutations in those genes. The final section is a brief overview of emerging models of *cis*-regulation of gene transcription, with emphasis on the identification and function of distal regulatory regions.

THE CELL BIOLOGY OF HEARING

The mammalian hearing and vestibular sensory systems

The mammalian ear contains the sensory systems for hearing and for balance, gravity, and acceleration. There are many excellent reviews of the cell and molecular biology of the ear, and the reader is referred to a few of them for more detailed explanation (Dror & Avraham, 2009; Friedman & Griffith, 2003; Petit, 2006; Petit, Levilliers, & Hardelin, 2001; Zdebik, Wangemann, & Jentsch, 2009). The ear is conventionally divided into three regions: outer, middle, and inner (Figure 1-1). The outer ear comprises the pinna and the auditory canal. The geometry of these structures accounts for some degree of amplification of selective frequencies of sound waves

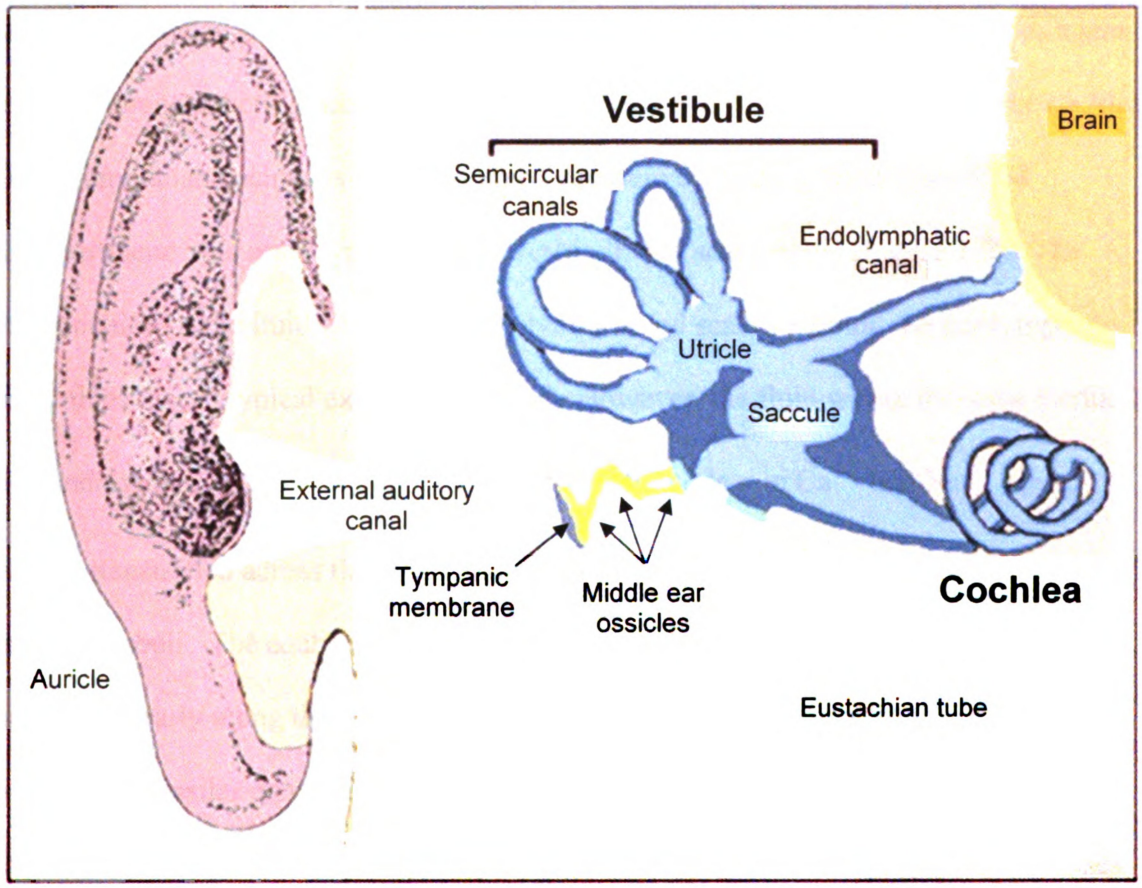

Figure 1-1. The human ear. The outer ear comprises the pinna (auricle) and external auditory canal; the middle ear is the air-filled compartment between the tympanic membrane and the fluid-filled vestibule and cochlea, which comprise the inner ear. Reproduced from (Petit, et al., 2001).

conducted through air to the middle ear. At the terminus of the auditory canal, sound waves vibrate the tympanic membrane, which is in physical contact with the malleus, one of the ossicles of the middle ear. Sound waves are further modified by the vibration along the ossicles, and are transmitted into the inner ear by vibration of the stapes against the membrane covering the oval window, an opening in the bony covering of the cochlea. The mammalian cochlea is a spiral-shaped structure containing three fluid-filled compartments: the scala tympani, scala vestibuli, and scala media (Figure 1-2). The composition of the fluid within the scala tympani and scala vestibuli, the perilymph, resembles that of typical extracellular fluid. However, the fluid within the scala media, the endolymph, is unusually high in K^+ (150 mM) and low in Ca^{2+} and Na^+. Sound waves transmitted across the oval window travel in perilymph along the length of the scala vestibuli. The cochlear hair cells and a variety of surrounding supporting cells are arrayed linearly along the length of the cochlea, comprising the organ of Corti, which rests on the basilar membrane. The shape and mechanical characteristics of the basilar membrane are such that the frequency of a sound wave generated by a pure tone causes vibration of only a specific region of the basilar membrane. The basilar membrane is tonotopically organized, with high frequency sound waves causing vibration at the base of the cochlea, and low frequency sound waves causing vibration at the cochlear apex. Displacement of the basilar membrane with respect to the tectorial membrane, a gel-like structure overlying the organ of Corti, causes deflection of the membrane-bound stereocilia that project from the apical surface of the hair cells. Opening of mechanically-gated channels on some stereocilia is the first in the cascade of events of

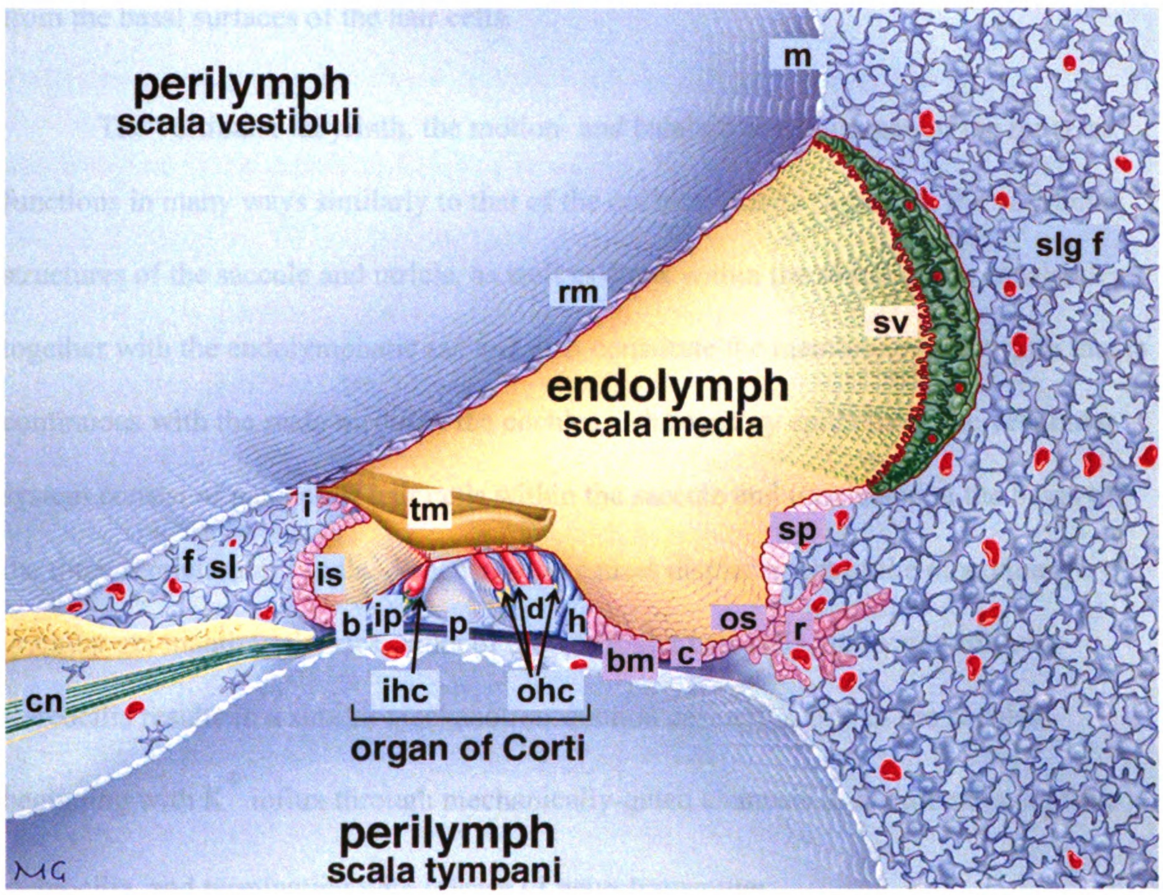

Figure 1-2. Cross-section through the cochlea. ihc, inner hair cell; ohc, outer hair cell; sv, stria vascularis; d, Deiter cell; p, pillar cell; h, Hensen cell; c, Claudius cell; r, root cells; i, interdental cell; is, inner sulcus cell; os, outer sulcus cell; sp, spiral prominence; tm, tectorial membrane; bm, basement membrane; r, Reissner's membrane. Reproduced from Cohen-Salmon et. al. (2002)

mechanotransduction, that depend on an initial, rapid influx of K^+ into hair cells via these channels, leading to depolarization of the cell with eventual release of neurotransmitter from the basal surfaces of the hair cells.

The vestibular labyrinth, the motion- and balance-sensing organ of the inner ear, functions in many ways similarly to that of the cochlea. Endolymph fills the vestibular structures of the saccule and utricle, as well as ducts within the semicircular canals, that together with the endolymphatic sac and duct constitute the membranous labyrinth that is continuous with the scala media of the cochlea. The sensory epithelia of the vestibular system consist of patches of hair cells within the saccule and utricle and at the bases of the three semicircular canals. When motion causes displacement of the otoconium, a granular gel mantling the stereocilia of the vestibular hair cells, deflection of those stereocilia results in a similar mechanotransduction cascade to that of the cochlea, beginning with K^+ influx through mechanically-gated channels in the membrane of the stereocilia, and terminating with release of neurotransmitter.

There are two populations of hair cells in the mammalian cochlea. A single row of inner hair cells is separated by pillar cells from three rows of outer hair cells. The lateral wall membranes of outer hair cells are enriched in prestin, a motor protein responsible for shortening and stiffening of the outer hair cells in response to membrane depolarization. True hearing is accomplished by the inner hair cells, but the outer hair cells are responsible for amplification (about 50 dB) and frequency refinement, without which hearing is significantly compromised (reviewed in Dallos, 2008).

The stria vascularis

The stria vascularis (Figures 1-2 and 1-3) is a specialized tissue that forms part of the lateral wall of the scala media, and is integral to two processes critical for proper hearing, the generation of the endocochlear potential and the maintenance of high K^+ concentration in the endolymph (Kikuchi, Adams, Paul, & Kimura, 1994; Wangemann, 2002; Zdebik, et al., 2009). It is the most highly vascularized tissue in the human body, indicative of the metabolic energy required for its function. Two layers of epithelial cells form the stria vascularis. Strial marginal cells joined by tight junctions face the scala media. The basolateral membranes of marginal cells face the intrastrial space, which is also bound by strial intermediate cells. The membranes of both marginal and intermediate cells are highly convoluted and in close association with each other, each presenting a large surface area to the narrow (15 nm), highly vascularized intrastrial space. Intermediate cells are connected by gap junctions to an underlying layer of cells, the basal cells, which are also connected by gap junctions to fibrocytes of the spiral ligament. The integrity of the intrastrial space is maintained by the tight junctions of the marginal cells, the basal cells, and the endothelial cells of the capillaries.

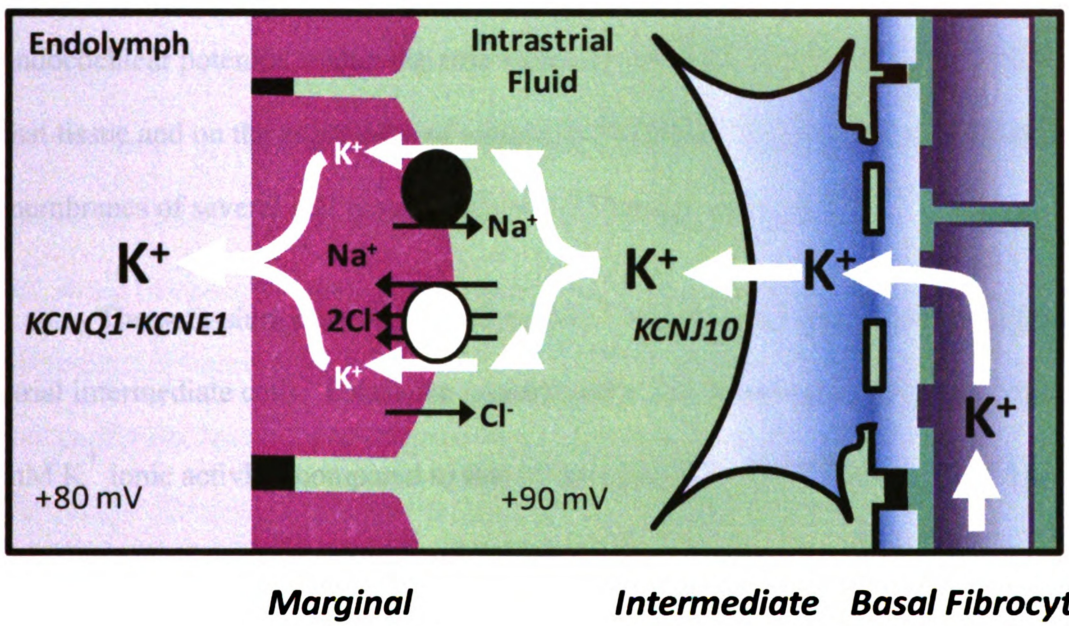

Figure 1-3. Schematic diagram of stria vascularis. Movement of potassium from cochlear lateral wall fibrocytes through the cells of the stria vascularis is responsible for the generation of the endocochlear potential. Reproduced from (Marcus, Wu, Wangemann, & Kofuji, 2002)

Generation of the endocochlear potential

The endocochlear potential is the large electrical potential of around +80 mV that exists in the endolymph of the scala media. It is this potential that drives the influx of K^+ into the mechanically-gated ion channels of the stereocilia at a rate and magnitude required for the appropriate downstream response of the hair cells. Generation of the endocochlear potential within the stria vascularis depends on the two-layer structure of that tissue and on the expression of various ion channels, pumps, and transporters on the membranes of several cell types within and adjacent to the stria.

The potassium channel KCNJ10 (Kir4.1) is expressed on the apical membrane of strial intermediate cells. Potassium concentration within the intrastrial space is low (5 mM K^+ ionic activity) compared to that of intermediate cells (60 mM), and diffusion of K^+ through these channels is estimated to be responsible for generation of most of the resting membrane potential of around +90 mV that exists across this membrane. Low potassium concentration is maintained in the intrastrial space by the action of two ion transport complexes that are highly expressed on the basolateral membrane of strial marginal cells, a Na^+,K^+ATPase (subunits coded for by *ATP1A1* and *ATP1B1* or *ATP1B2*) and a Na^+,K^+,$2Cl^-$cotransporter (NKCC1, coded for by *SLC12A2*). Potassium taken up by the marginal cells is then secreted via KCNE1/KCNQ1 channels expressed on the apical surface of the marginal cells that face the endolymph.

The gap junctional networks of the cochlea and supply of potassium to the endolymph

The rapid depolarization of hair cells that occurs in response to mechanical deflection of stereocilia depends on a rapid influx of K^+ into the hair cells, which requires a high concentration of potassium (150 mM) in the endolymph, as well as a high positive potential (+80 mV) in the endolymphatic compartment. Both of these physiological necessities are accomplished in coordination, via the movement of potassium and other ions between the various compartments of the cochlea. The basolaterally-expressed potassium channel KCNQ4 permits the rapid diffusion of potassium out of hair cells following a depolarization event, into perilymph surrounding the basolateral surfaces of hair cells and supporting cells of the Organ of Corti. It has been apparent since the 1980s that perilymph is the source of potassium to the endolymph (reviewed in (Hibino, Nin, Tsuzuki, & Kurachi, 2010)), but there is not yet universal consensus on how that is accomplished. (Zdebik, et al., 2009) discuss three models, all supported by experimental evidence and none mutually exclusive of the others, that are likely to encompass the reality (Figure 1-4). In the first model, K^+ may recirculate directly via the perilymph of the scala tympani, which underlies the basilar membrane. The second model suggests that K^+ may be taken up from the perilymph adjacent to hair cells by surrounding Deiter's cells and inner phalangeal cells, through KCC4 and KCC3 K^+Cl^- cotransporters. These channels may operate bi-directionally, allowing movement of potassium into these cells where the external potassium concentration is locally high (presumably in the narrow perilymphatic space adjacent to hair cells), and out of the cells where external

potassium concentration is low (in regions of the membrane facing the basilar membrane and scala tympani). Thus, these cells may serve as local buffers of K^+ only, rapidly sequestering K^+ away from hair cells and releasing it back into perilymph. The third model similarly posits K^+ uptake by Deiter's cells from the perilymph surrounding the hair cells, with subsequent gradient-driven cell-to-cell movement away from the organ of Corti via gap junctions.

Figure 1-4. Models of potassium movement in cortilymph. A, K^+ diffuses through open cortilymph/perilymph; B, K^+ moved into supporting cells is recycled via gap junctions; C, K^+ concentration is buffered by sequestration in supporting cells. Reproduced from (Zdebik, et al., 2009).

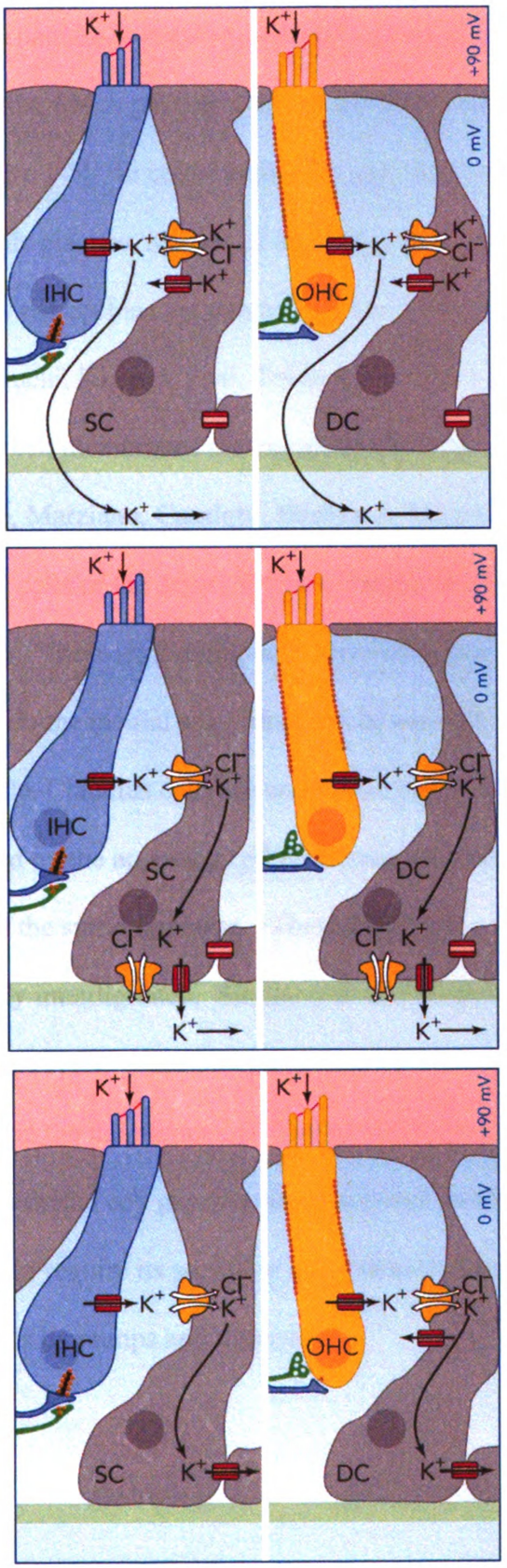

Figure 1-4

Gap junctions are channels formed by hexamers of connexin proteins in the membranes of adjacent cells, which provide a means for direct cell-to-cell transfer of ions and small molecules (Figure 1-5). In cells coupled by gap junctions, freeze-fracture electron microscopy reveals plaques, regions of densely packed gap junctional channels. Certain cell populations of the cochlea are extensively coupled by gap junctions (Kikuchi, et al., 1994; Kikuchi, Kimura, Paul, Takasaka, & Adams, 2000); some of the plaques observed in these cell membranes are exceptionally large, having over 100,000 individual channels(Forge, Marziano, Casalotti, Becker, & Jagger, 2003). Gap junctions couple all of the epithelial cells of the organ of Corti, with the exception of the inner and outer hair cells themselves. These supporting cells are coupled on both sides of the organ of Corti to adjacent cells; on the medial side, inner sulcus cells are coupled to interdental cells, and on the lateral side, Claudius cells are coupled to root cells. Notably, gap junctions are not expressed on the apposing cell membranes of root cells and the Type II and Type IV fibrocytes of the spiral ligament. These cell membranes are highly convoluted and extensively interdigitated. Similarly to the basolateral membrane of strial marginal cells, the Na+K+ATPase potassium pump and NKCC1 (SLC12A2) potassium transporter are expressed on the membranes of Type II and Type IV fibrocytes. Thus, transfer of K+ from the epithelial cell gap-junctional network to the fibrocytes of the spiral ligament is thought to require its secretion into extracellular perilymph and uptake into adjacent fibrocyte cells by pumps and transporters.

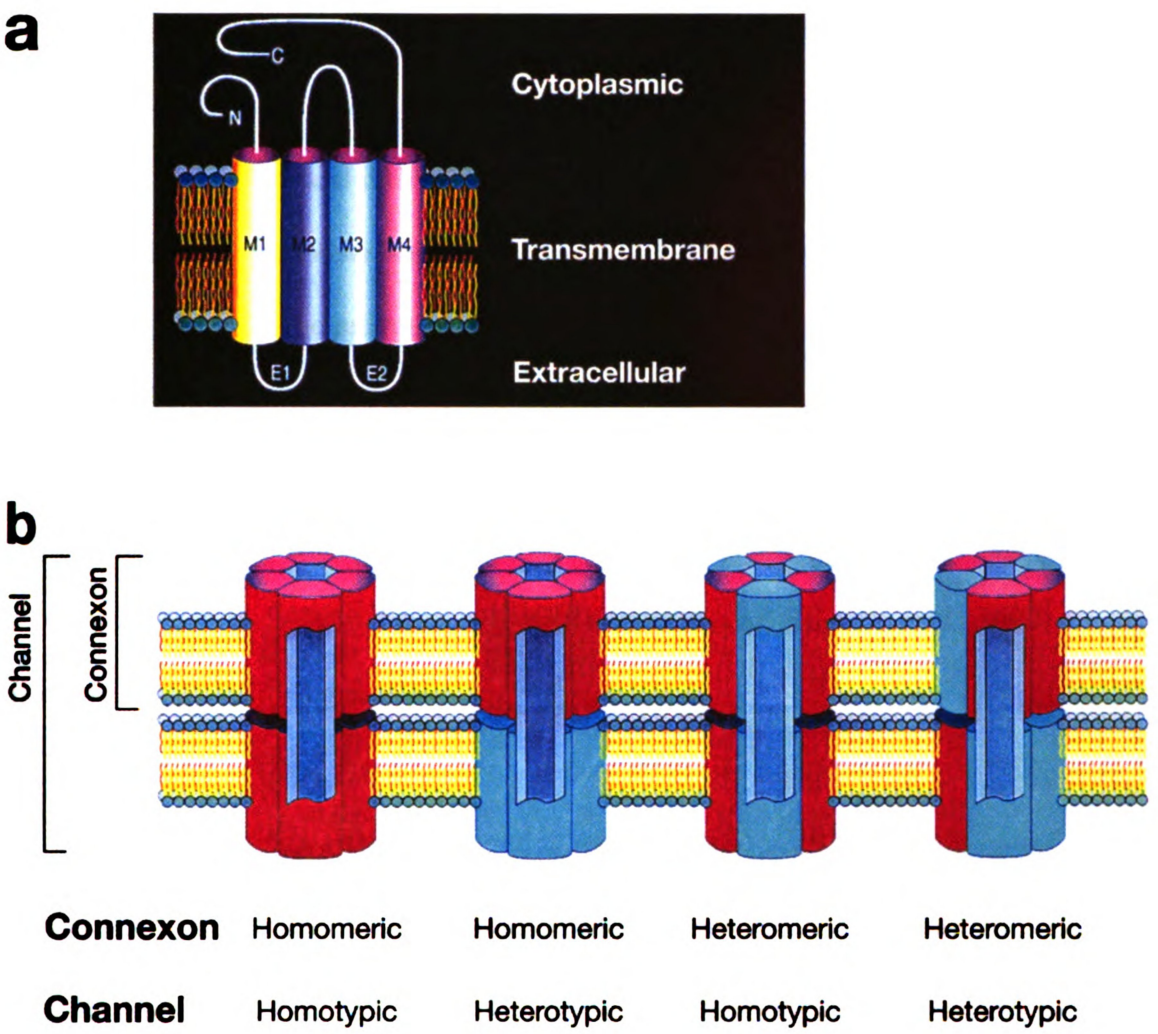

Figure 1-5. Schematic of apposing cell membranes coupled by gap junctions. Gap junction channels consisting of interacting hexameric connexons in apposing cell membranes provide channels for the selective transfer of ions and small molecules. Connexins are small, 4-pass, membrane-spanning proteins with cytoplasmic N- and C-terminal domains, one cytoplasmic loop, and two extracellular loops. Connexons and channels with different connexin composition have different selectivity and permeability. Reproduced from (Wei, Xu, & Lo, 2004).

All of the fibrocytes of the spiral ligament are coupled by gap junctions, and gap junctions are expressed between spiral ligament fibrocytes and the basal cells of the stria vascularis. Strial basal and intermediate cells are also extensively coupled by gap junctions. Thus, there exists between the organ of Corti and the stria vascularis a veritable highway for the recirculation of potassium from its site of disposal into perilymph from channels in the basolateral membrane of hair cells back to its site of secretion from strial marginal cells into endolymph (Figure 1-6). The model of primarily gap-junctional-mediated movement of K+ is supported by a mouse model of conditional knockout of Connexin 26 in the sensory epithelium ((Cohen-Salmon, et al., 2002), discussed in the following section).

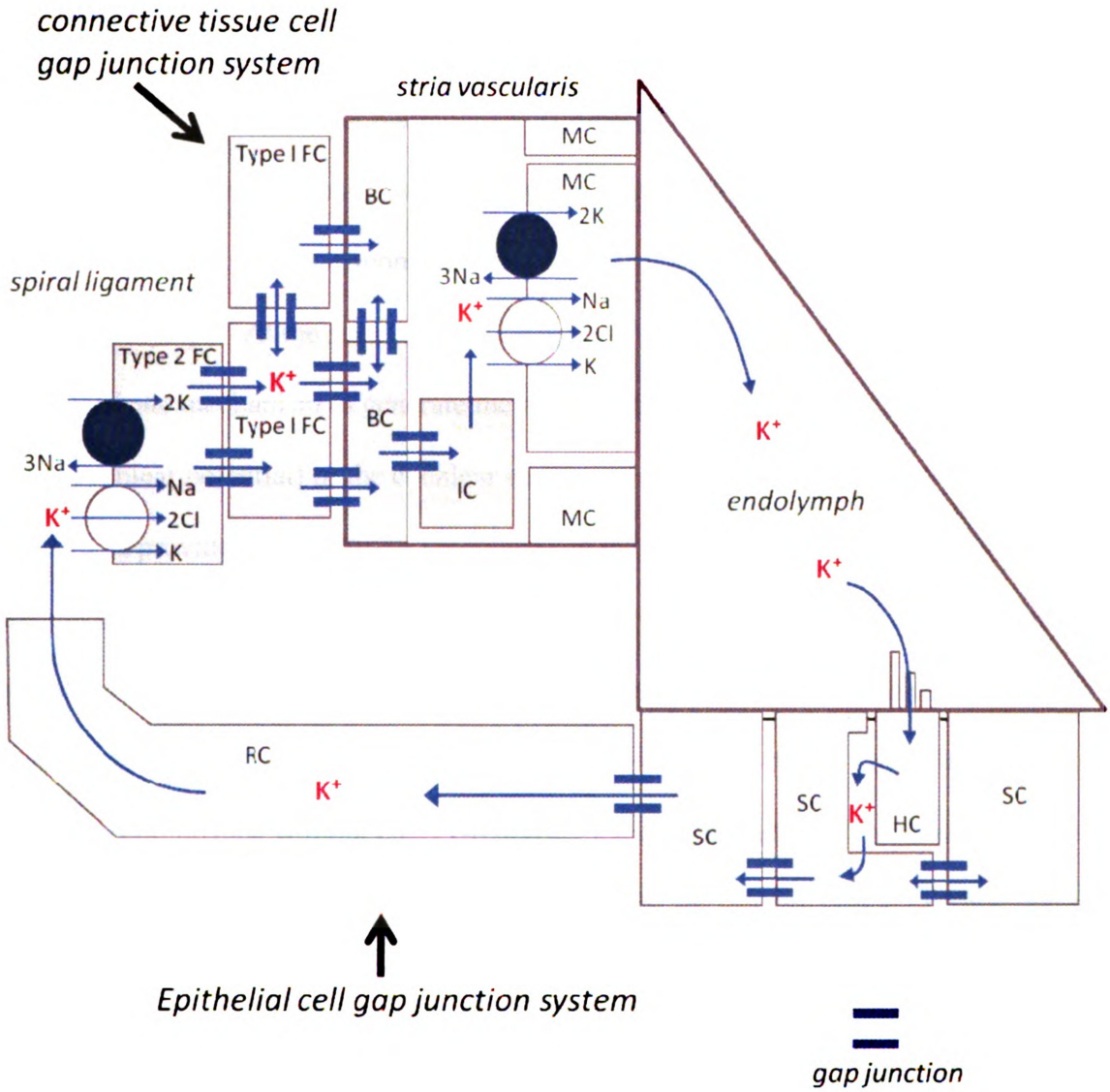

Figure 1-6. Schematic showing the circulation of potassium ions through the two gap junctional systems of the mammalian cochlea. FC, fibrocytes; MC, marginal cells, BC, basal cells; IC, intermediate cells; RC, root cells; SC, supporting cells, HC, hair cells. Reproduced from Zhao et al. (2006).

In summary, the intricate cell biology of the mammalian cochlea supports a hearing apparatus that functions over a broad frequency range with high sensitivity and selectivity. This sharpening and amplification is achieved by the function of the outer hair cells, a unique feature of mammalian cochlear anatomy. The hearing process is powered by the stria vascularis, a multi-layered tissue with a complex distribution of ion channels and transporters responsible for achieving the high concentration of potassium in the endolymph. At the same time, movement of potassium through the stria is coupled to an intricate mechanism to generate the required high electrochemical potential (endocochlear potential) of the cochlear endolymph. The metabolic energy required by the stria is provided by extensive vasculature in the intrastrial space. It is suspected that the physical separation of the stria from the organ of Corti is a requirement to minimize vibration from blood flow transmitted to the hair cells. Notably, the movement of potassium through the hair cells and within and through the sensory epithelial supporting cells is accomplished without energy-requiring membrane pumps. It must also be noted that the two cochlear gap-junctional networks, that of the sensory epithelium and that of the spiral ligament connective tissue and strial basal and intermediate cells, can be seen not only as providing routes of transport, but as establishing large reservoirs for the buffering of potassium concentration. A ready sink for potassium may be necessary surrounding the basolateral surfaces of hair cells, and a ready source of potassium, fixed as well with respect to electrochemical potential, has been established as a requirement for proper function of the stria.

CONNEXINS AND CONNEXIN MUTATIONS IN HEARING LOSS

The connexin gene and protein families

Connexins are proteins that hexamerize to form channels in the membranes of most non-circulating cells of vertebrates (Figure 1-5). These channels, called connexons or gap junction hemichannels, associate extracellularly with connexons of adjacent cells to form gap junctions, channels that directly connect the cytosolic spaces of adjacent cells, permitting the transfer of ions and small molecules. Hemichannels may be composed of more than one type of connexin; such connexons are referred to as heteromeric. In forming gap junctions between adjacent cells, homomeric or heteromeric connexons of one cell may associate with connexons on an adjacent cell that are of the same or different composition, thus forming homotypic or heterotypic gap junctions.

There are 21 connexin genes in the human connexin gene family, and 20 in the mouse. Connexins are four-pass membrane proteins, with intracellular N- and C-termini, one intracellular loop, and two extracellular loops. Connexin proteins are recognized by some degree of amino acid sequence conservation of some portions of the N-terminus, the membrane-spanning domains, and in the extracellular loops, in paralogs (connexins of the same species) as well as in orthologs (the same connexin across species). Both extracellular loops are characterized by conserved cysteines that make intramolecular disulfide bridges between the two loop domains. High sequence identity between paralogs within species allows for the assembly of heterotypic gap junctions.

Comparative genomics has shown that the large vertebrate connexin gene family is ancient, as there are orthologs to most connexins from mammals to bony fishes

(Cruciani & Mikalsen, 2006, 2007). Connexins are subdivided into four classes, α, β, γ, and δ, according to amino acid sequence and length. Most of the length variation occurs in the C-terminal domain, which is not highly conserved between orthologs. Connexin proteins are generally named according to their molecular weight, and the corresponding connexin genes are named according to class and order of discovery of the connexin protein or gene. Thus, connexin 26 (Cx26) is a member of the β class of connexins which are the shortest, ranging from 25-32 kDa. Human Cx26 is coded for by *GJB2*, the second designated gap-junction β-connexin gene. β-connexins have the shortest C-terminal sequences; α-connexins the longest.

Most connexin genes have a similar genomic structure, with one small 5' non-coding exon, a single intron, and one exon containing a small portion of the 5' UTR, the entire coding sequence, and the 3' UTR. However, some connexin genes have been found to have multiple 5' exons. Alternate promoter use and alternative splicing, associated with tissue-specific expression and/or variation in translation efficiency, has been documented for several connexins, among them Cx30 (Anderson, Zundel, & Werner, 2005), Cx32 (Neuhaus, Dahl, & Werner, 1995; Sohl, Eiberger, Jung, Kozak, & Willecke, 2001; Sohl, et al., 1996), Cx40 (Dupays, et al., 2003), Cx43 (Pfeifer, Anderson, Werner, & Oltra, 2004) and Cx45(Anderson, et al., 2005). Additionally, some connexin genes have more than one coding exon; among them, Cx31.3, Cx36, and Cx40.1.

Connexin genes are found on eight human chromosomes, both singly and in clusters. The genes coding for connexins 26, 30, and 46 (*GJB2*, *GJB6*, and *GJA3*) are

tandemly arranged within less than 100 kb at 13q12.11. Two other clusters of connexin genes occur on human chromosome 1.

Expression and function of human connexins

Connexins are expressed in the cell membranes of nearly all non-circulating cells of vertebrates. Most cell types express more than one connexin, and most connexins are expressed in more than one cell type; for instance, keratinocytes express at least six different connexins, and human Cx43 is expressed in at least 35 different tissues(Laird, 2006). Gap junction channels and hemichannels are known to permit the selective transfer of ions and molecules of less than about 1 kDa, including metabolites and signaling molecules such as cyclic adenosine monophosphate (cAMP) and inositol 1,4,5-trisphosphate (IP3) (Beltramello, Piazza, Bukauskas, Pozzan, & Mammano, 2005). Passage of ions and molecules through gap junction channels is not a passive process, dependent solely on channel pore size, but is in fact highly regulated. The gating status as well as the selective permeability of gap junction channels is determined by interactions between amino acid residues in the cytoplasmic domains of the connexon, within and between connexins. Changes in membrane voltage, transjunctional voltage, pH, Ca^{2+} concentration, and phosphorylation status are all known to affect the opening and closing of gap-junctional channels. A high-resolution (3.5 Å) structure of human Cx26 recently solved has yielded insights into the functionality of a number of the residues of the Cx26 protein (Maeda, et al., 2009). Orientation of the four transmembrane helices of each connexin creates of the hexameric connexon a broad, shallow bowl at the cytoplasmic entrance to the channel. Two of the four transmembrane

helices of each connexin extend significantly into the cytoplasm to create this broad surface. Hydrogen bonding between residues of the N-terminal helix and the first transmembrane helix of the adjacent connexin form a narrow-diameter ring of the six N-terminal helices of the connexon at the channel entrance. These hydrogen bonds may be disrupted by conformational changes in one or more of the connexins as a result of changes in the cytoplasmic chemical or electrical milieu, favoring interactions between residues of the N-terminal helices and consequent plugging of the channel. Certain features of the general structure of a Cx26 gap junction, as well as the gating model proposed by (Maeda, et al., 2009) are generalizable from Cx26 connexons to many others, including heteromeric connexons, as there is extensive conservation of many residues in the modeled domains, including nearly universal conservation across α and β connexins of the D2 and W3 residues of the N-terminal helix, the two residues most implicated in the gating function. (Maeda, et al., 2009) do not model the C-terminus or cytoplasmic loop of Cx26; these domains are highly variable across connexins. It is believed that the variability of these domains contributes to the specificity in permeability of connexons of differing composition. It is known that the C-terminus of many connexins (although not Cx26) is a target of phosphorylation, and that many kinases act on connexins, and that such interactions modulate gap junction function.

Connexins, like other membrane proteins, are translated on rough endoplasmic reticulum, and oligomerized into connexons before delivery to the plasma membrane. Hemichannels diffuse within the plasma membrane to the periphery of gap junction plaques, sites of many closely packed gap junctions, where newly arriving hemichannels dock to hemichannels in adjacent cell membranes. Although it was believed that all

hemichannels are constitutively closed until successful docking with hemichannels on apposing cells, it is now known that hemichannels may function independently of gap junction formation. Connexins turn over rapidly, having half-lives of a few to several hours. Degradation of aging gap junctions occurs by invagination and budding off of unique double-membraned vesicles, called connexisomes, from the center of plaques(Laird, 2006). There is a growing body of evidence that connexin gap junction formation and function is tied to the formation and function of adherens junctions and tight junctions, that connexins interact with proteins of adherens junctions and tight junctions, and that connexins interact with cytoskeletal proteins and other cytoplasmic proteins (Dbouk, Mroue, El-Sabban, & Talhouk, 2009). Thus, regulation of gap junction function may occur, not solely through mechanisms that modulate the opening and closing of individual channels, but also by mechanisms that govern the rate of synthesis, assembly, and degradation of gap junctions.

The formation of gap junction plaques between and among sets of cells within tissues makes of those cells a functional, though nontraditional, syncitium. With respect to the ions and molecules that pass through particular configurations of gap junctions, diffusion can occur across large numbers of cells quickly and with little or no investment of metabolic energy. This has three main functional consequences for cells connected by gap junctions. Firstly, cells coupled by gap junctions may simply benefit from a long-distance, low-energy import and/or export mechanism. In the model of gap-junctional movement of K^+ away from the sensory epithelia of the cochlea, energy-requiring K^+ pumps can be positioned in the spiral ligament, minimizing 'noisy' vascularization near the organ of Corti. Secondly, cell-to-cell signals can be easily propagated and amplified.

(Beltramello, et al., 2005) provided evidence for gap-junction mediated diffusion of IP_3 in supporting cells of the organ of Corti, coupled to the propagation of intercellular Ca^{2+} waves. Gap junctions are known to be selectively permeable to other second messengers, including cAMP. In addition to coordinating physiological responses across populations of cells, gap junctional communication is important in development. Thirdly, extensive coupling of cells provides large sources and sinks. For example, the K^+ diffusion potential across the membrane of strial intermediate cells depends on an enormous reservoir of K^+, supplied by the extensive gap-junctional network connecting spiral ligament fibrocytes to the strial basal cells, which are coupled to the intermediate cells.

Distribution of connexins 26 and 30 in the mammalian ear

Connexins 26, 30, 31, 36, and 43 are known to be expressed in the mammalian ear. A number of studies have determined connexin expression by immunoprecipitation, freeze fracture, immunogold labeling, and immunohistochemistry, in cochlear preparations from mouse, guinea pig, human, and other mammals ((Forge, Becker, et al., 2003; Forge, Marziano, et al., 2003; Jagger, Nevill, & Forge, 2010; Liu, Bostrom, Kinnefors, & Rask-Andersen, 2009; Zhao & Yu, 2006). Connexins 26 and 30 are the most abundant connexins in the cochlea, with high levels of expression in all of the supporting cells of the sensory epithelium, and in several cell types comprising the lateral wall of the cochlea. Expression of Cx26 and Cx30 overlaps in several cell types, although there are regions of both gap-junctional networks where expression of one connexin predominates, or is expressed exclusively; co-labeling of both connexins in organ of Corti supporting cells suggests the existence of heteromeric and/or heterotypic

gap junctions (Liu, et al., 2009). Co-assembly of Cx26/Cx30 connexons or gap junctions was demonstrated in mouse cochlear preparations by co-immunoprecipitation (Ahmad, Chen, Sun, & Lin, 2003). Connexins 26 and 30 are known to make functional heteromeric gap junctions *in vitro* (Marziano, Casalotti, Portelli, Becker, & Forge, 2003; Yum, et al., 2007).

Mutations of GJB2 in hearing loss

Over 220 mutations of *GJB2* have been reported to cause recessively inherited nonsyndromic hearing loss (Hilgert, Smith, & Van Camp, 2009). The great majority of these are missense mutations. About one-quarter to one-third of the total are nonsense mutations or small deletions which cause a frameshift and premature stop codon. Many of these mutations result in translation of severely truncated, non-functional protein that is rapidly turned over. For instance, the 35delG mutation results in a premature stop codon after a single frameshift substitution in the 12^{th} codon (Denoyelle, et al., 1997). Cx26 protein is not detected in skin biopsies of persons homozygous for this mutation. One splice site mutation (IVS1+1G→A; (Denoyelle, et al., 1999)) and one promoter mutation (-44C→T; (Matos, et al., 2007)) have also been reported. Mutations associated with AR hearing loss (DFNB1A) are distributed across the protein, in every domain. Fewer than a dozen mutations of *GJB2* associated with AD hearing loss (DFNA3A) have been reported; these mutations also occur throughout the protein. Functional studies of a number of recessive and dominant mutations indicate a range of defects, including impaired trafficking to the plasma membrane, inability of hemichannels to form gap junctions with adjacent cells, and varying degrees of channel dysfunction of assembled gap junctions (variously summarized in (Bicego, et al., 2006; Mese, Londin, Mui, Brink,

& White, 2004; White, 2000). Differences between recessive and dominant mutations have been shown to be due to presence or absence of dominant-negative effects when mutant Cx26 constructs are expressed with wild-type Cx26 or Cx30, including mistrafficking of assembled connexons as well as channel dysfunction.

The DFNB1A phenotype is characterized by congenital, bilateral, sensorineural hearing loss that is not generally progressive, with a flat or sloping audiogram. There is considerable variation in the degree of hearing loss, which is very rarely nonpenetrant. The severity of hearing loss correlates with category of mutation; greater hearing loss is associated with a genotype of biallelic known or expected null or protein-truncating mutations, and milder hearing loss is associated with biallelic non-truncating (missense) mutations (Azaiez, et al., 2004; Cryns, et al., 2004; Snoeckx, et al., 2005). Of note, there is considerable variation in degree of hearing loss among affected persons with the same genotype, indicating genetic and/or environmental effects beyond that conferred by any particular combination of mutations. However, no modifier loci have been identified to date.

Although the vast majority of mutations of *GJB2* cause AR NSHL, several are responsible for dominantly inherited conditions and syndromes that include hearing loss along with skin disorders related to defects of keratinization. These mutations tend to occur in the N-terminal domain or in the first extracellular loop of the Cx26 protein. *GJB2* mutation may cause PPK (palmar-plantar keratoderma, hyperkeratosis of the palmar and plantar surfaces of the hands and feet), KID (keratitis-ichthyosis-deafness) syndrome (MIM 148210, vascularizing keratitis with progressive EKV

(erythrokeratoderma variabilis)), Vohwinkel syndrome (MIM 124500, mutilating keratoderma), or Bart-Pumphrey syndrome (MIM 149200, knuckle pads and leukonychia (white nails)).

Connexin 26 mouse models

Contrary to the human phenotype, where complete loss of Cx26 protein leads only to NSHL and no other apparent pathology, knockout of connexin 26 in mouse is embryonic lethal (Gabriel, et al., 1998). (Cohen-Salmon, et al., 2002) generated a transgenic, conditional mouse knockout of *Gjb2* in the sensory epithelium of the organ of Corti. Development of the cochlea was normal, and endocochlear potential (EP) in P12-P13 conditional knockout mice was comparable to that of wild-type mice. However, death of border cells and inner phalangeal cells, supporting cells in proximity to inner hair cells, was observed starting at P14. Cell death progressed to include other supporting cells, as well as the outer hair cells themselves. By 3 weeks of age, significant hearing loss was detected by ABR (auditory brainstem response). In adult mice, both EP and K^+ concentration of endolymph were reduced. The authors postulate that cell death results initially from failure of supporting cells around the inner hair cells to adequately transport K^+ out of the cortilymph (the perilymph in the extracellular spaces of the organ of Corti).

A mouse model expressing a human *GJB2* transgene bearing the R75W mutation associated with dominantly inherited hearing loss (Inoshita, et al., 2008; Kudo, et al., 2003; Minekawa, et al., 2009) confirms earlier evidence from expression of human R75W *GJB2* in Xenopus oocytes, that R75W connexins exert a dominant-negative effect

on wild-type Cx26 (Richard, et al., 1998). Defects in supporting cells in the organ of Corti in the R75W transgenic mouse were observed, and felt to underlie the dysmorphology of the organ of Corti and to explain the hearing loss phenotype, as the outer hair cells themselves maintained electromotility (Minekawa, et al., 2009). No vestibular phenotype is apparent in this mouse, even though Cx26 is expressed in vestibular supporting cells, and morphology and function of the stria vascularis appears normal (Kudo, et al., 2003).

GJB6: GJB2's mutation-poor, mysterious twin

Evidence for co-assembly of Cx26/Cx30 heteromeric connexons and gap junctions is now reasonably well established (see above). *GJB6* shares the DFNB1 locus with *GJB2* and is located about 30kb telomeric to *GJB2*. Connexins 26 and 30 show high amino acid sequence identity. Synteny at this locus is preserved in mammals, birds, and reptiles, but the duplication that gave rise to *GJB2* and *GJB6* in tandem is not observed in fish genomes (Cruciani & Mikalsen, 2006). While over 200 mutations of *GJB2* are now reported to cause nonsyndromic recessively inherited hearing loss (Hilgert, et al., 2009), no coding region mutations of *GJB6* at all are associated with recessive hearing loss, and only a single missense mutation is associated with dominant hearing loss. However, there are now four identified DFNB1 deletion mutations. Two are deletions in which *GJB6* is truncated, with abrogation of *GJB2* expression shown for one (F. J. del Castillo, et al., 2005; I. del Castillo, et al., 2002; Feldmann, et al., 2009; Lerer, et al., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002; Rodriguez-Paris & Schrijver, 2009). The third is a large (>920 kb) deletion in which the entire gene sequences of both *GJB2* and *GJB6* are missing (Feldmann, et al., 2009). The fourth is the

deletion identified in our lab, and that is the subject of this dissertation. This deletion segregates with reduced expression of both *GJB2* and *GJB6* (Wilch, et al., 2006) [Chapter 2 of this dissertation]; (Wilch, et al., 2010)[Chapter 3 of this dissertation). The *GJB6* coding region is closely scrutinized in individuals with suspected DFNB1 hearing loss; curiously, the only null alleles of *GJB6* that have been identified are those in which *GJB2* function is compromised as well. The Cx30 knockout mouse lacks the endocochlear potential and has significant hearing impairment that worsens in concert with degeneration of the sensory epithelium that begins at P18 (Teubner, et al., 2003). Although this mouse does not display an overt vestibular phenotype, loss of saccular hair cells has been documented (Qu, et al., 2007). Overexpression of Cx26 in this mouse rescues both hearing (Ahmad, et al., 2007) and saccular hair cells (Qu, et al., 2007).

I discuss *GJB6* and its relationship to *GJB2* further in Chapter 4 of this document. To summarize:

1. The pathogenicity of the three DFNB1 deletions so far identified that leave *GJB2* intact very likely results from loss of expression of *GJB2*.

2. The weight of evidence to date does not support a model of inheritance of monoallelic mutation of both *GJB2* and *GJB6* (digenic inheritance) as a cause of recessively inherited hearing loss.

3. One or more cis-regulatory elements controlling the expression of *GJB2* and *GJB6* exist somewhere within the genomic interval common to the three DFNB1 deletions discussed. This interval extends from about 150 kb to 250 kb upstream of the

transcriptional start site (TSS) of *GJB6* (~ 180 kb to 280 kb upstream of the TSS of *GJB2*).

4. Other mutations occurring in these yet-to-be identified *GJB2*-regulatory elements may explain the hearing loss in some persons with monoallelic mutation of *GJB2*. Sequence variation within these elements may underlie some fraction of the variable expressivity of hearing loss in persons with biallelic *GJB2* mutation.

5. We have begun to characterize sequence elements in this region that are capable of enhancing expression of a reporter gene in a cell culture assay system.

CIS-REGULATION OF TRANSCRIPTION AND CIS-REGULATORY MUTATIONS IN GENETIC DISEASE

Transcriptional enhancers and locus control regions

Investigations in the last twenty years have gone a long way toward building a rich conceptualization of the eukaryotic *cis*-regulatory landscape. Arrays of enhancer and silencer elements may be associated with transcriptional regulation of target genes, controlling transcription rates as well as temporal- and tissue-specificity of transcriptional activity. It is now known that such elements may exist at vast distances from the genes they regulate. These elements are thought to interact with basal and proximal promoters via looping of intervening DNA. Enhancer-bound transcription factors many interact directly with protein components of the basal transcriptional machinery, or with other proteins bound to the proximal promoter, to stabilize the pre-initiation complex and increase the rate at which the RNA Polymerase II complex is recruited to the transcription start site. Enhancer elements are also associated with the recruitment of

chromatin-modifying complexes. Some chromatin modifiers propagate covalent histone modifications along the chromatin, establishing transcriptionally-permissive regions of 'open' chromatin that spread in both directions from an enhancer element. Enhancers may provide more transient points of recruitment for other types of chromatin-remodeling proteins, such as SWI/SNF complexes, that function to 'nudge' nucleosomes with respect to their associated DNA, increasing accessibility of basal promoters to the transcriptional machinery. Enhancer elements may also up-regulate transcription by mediating the movement of target genes into 'transcription factories' or other nuclear subcompartments permissive to transcription.

A requirement for preventing promiscuous interactions between enhancers and non-target genes is fulfilled, at least in part, by insulator elements. One such element that is widespread across mammalian genomes and is relatively cell-type-invariant is defined by a number of binding sites for CTCF (CCCTC-binding factor). Insulators are thought to have two general activities. Enhancer-blocking is the selective prevention of association of a non-target gene with an enhancer in *cis*; this may involve CTCF/chromatin/protein interactions that define boundaries of active chromatin loops permitting interactions only between enhancers and genes in the same loop domain. Insulators are also found at the boundaries of active (euchromatic) and silenced (heterochromatic) chromatin domains, indicating a barrier function with respect to the spread of chromatin-modifying complexes.

Remote enhancers identified in human genetic disease

A number of genetic diseases can be caused by disruption of distal cis-regulatory elements. Among the most dramatic and best-characterized examples is preaxial polydactyly resulting from gain-of-function mutations of a conserved non-coding sequence element that drives expression of sonic hedgehog (*Shh*) in vertebrate limb development. Among humans, mice, and cats, twelve different single-nucleotide mutations have been identified within an 800 bp conserved region located 1Mb from the TSS of *Shh*. In transgenic mice, this sequence element is sufficient to drive expression of a reporter gene in a manner that capitulates the wild-type expression pattern of *Shh* in limb buds; replacement of the wild-type enhancer sequence with mutant constructs results in ectopic expression of *Shh* in a pattern that correlates to the polydactyly subphenotypes in humans and cats (Lettice, et al., 2003; Lettice, Hill, Devenney, & Hill, 2008; Lettice, et al., 2002).

Many human diseases are associated with cytogenetically-diagnosed translocations, deletions, duplications, and inversions. Some of these phenotypes have now been shown in some cases to be attributable to disruption of *cis*-regulatory elements whose target genes are located at a considerable distance from these regulatory sequences. For instance, aniridia (MIM 106210) is commonly caused by haploinsufficiency of *PAX6*; chromosomal rearrangements associated with aniridia have breakpoints that occur up to 125 kb away from *PAX6* itself. Analyses of this distant downstream region have now identified several sequence elements that have been shown in transgenic mouse studies to drive relevant, tissue-specific expression of a reporter (Kleinjan, et al., 2006; Kleinjan, et al., 2001).

Identification of remote cis-regulatory elements

Modularity of enhancer elements is an emerging theme, and is perhaps to be expected in genes with important developmental roles, with highly specific patterns of expression in a wide variety of cell types, and with constraints on timing of expression during development (Visel, et al., 2009). Highly investigated regulatory regions of developmentally important genes, in particular, are suggestive of cis-regulatory landscapes with certain general features. First, regulatory sequence may occupy a large span of genomic space, from several 10s of kilobases to more than a megabase. These large regions may appear as 'gene deserts' (Nobrega, Ovcharenko, Afzal, & Rubin, 2003); large spans of nongenic DNA are in fact associated with some developmentally important genes. Transposon exclusion zones may also mark extensive regions of cis-regulatory DNA. Second, cross-species conservation is, not surprisingly, associated with function in intergenic and intronic sequence (Pennacchio, et al., 2006; Prabhakar, et al., 2006; Woolfe & Elgar, 2008; Woolfe, et al., 2005). As discussed above, quantitative variation associated with genetic disease, related to ectopic expression or to loss of expression, has been clearly localized to highly conserved *cis*-regulatory sequence.

Thirty-five years ago, King and Wilson (King & Wilson, 1975)presciently suspected that regulatory rather than coding-sequence variation would be shown to be responsible for a great deal of phenotypic variation between closely related species. Because changes to amino acid sequence of a protein expressed in a variety of tissues may have negative pleiotropic effects, phenotypic variation resulting from mutation in a *cis*-regulatory region affecting timing or amount of expression in only a single tissue may be more likely to be tolerated than variation that is introduced by alteration of amino acid

sequence (Wray, 2007). In a meta-analysis of several surveys assessing evidence for positive selection in both protein-coding and noncoding sequence, Haygood et al. (Haygood, Babbitt, Fedrigo, & Wray, 2010) detected such a trend, finding less adaptive change in the coding regions of genes with patterns of broad expression, relative to those genes with expression limited to a single tissue. Thus, while conservation remains the most straightforward means by which to identify candidate enhancer elements, those studies which return the most striking results are perhaps cherry-picking. That is, key developmental regulators expressed early in development such as *SHH*, *PAX* genes, and *HOX* genes, are those most expected to have regulatory sequences intolerant of variation, and to preserve ancient, shared homology across many phyla. The likely story for many other genes whose diversity of expression across phyla may be responsible for phyletic diversity of phenotype characteristics may perhaps be less simple to predict. Functional studies such as that detailed in Chapter 4 of this dissertation are absolutely critical to understanding complicated patterns of *cis*-regulation.

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CHAPTER 2

Wilch E, Zhu M, Burkhart KB, Regier M, Elfenbein JL, Fisher RA, Friderici KH. (2006) Expression of GJB2 and GJB6 is reduced in a novel DFNB1 allele. ***American Journal of Human Genetics*** **79(1):174-9.**

ABSTRACT

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 show 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*.

TEXT

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 (Figure 2-1). 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 (Ahmad, Chen, Sun, & Lin, 2003; Forge, Becker, et al., 2003; Forge, Marziano, Casalotti, Becker, & Jagger, 2003), and are implicated in the maintenance of cochlear K^+ gradients necessary for proper hair-cell function (Kikuchi, Kimura, Paul, Takasaka, & Adams, 2000; Wangemann, 2002).

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 (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 (Grifa, et al., 1999) 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* (Figure 2-1). These deletions segregate with hearing loss when present either homozygously or heterozygously with each other or in *trans* with a recessive *GJB2* mutation (F. J. del Castillo, et al., 2005; I. Del Castillo, et al., 2003; I. del Castillo, et al., 2002; Lerer, et al., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002). 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 which may be exacerbated by loss of one *GJB6* allele as well. Common et al. (Common, et al., 2005) 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.

Figure 2-1. 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 variants used for allele-specific expression assays--*GJB2* 35delG, *GJB2* +94, and *rs7333214*, are also indicated. Boxes indicate exons, and the hatched boxes indicate 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.

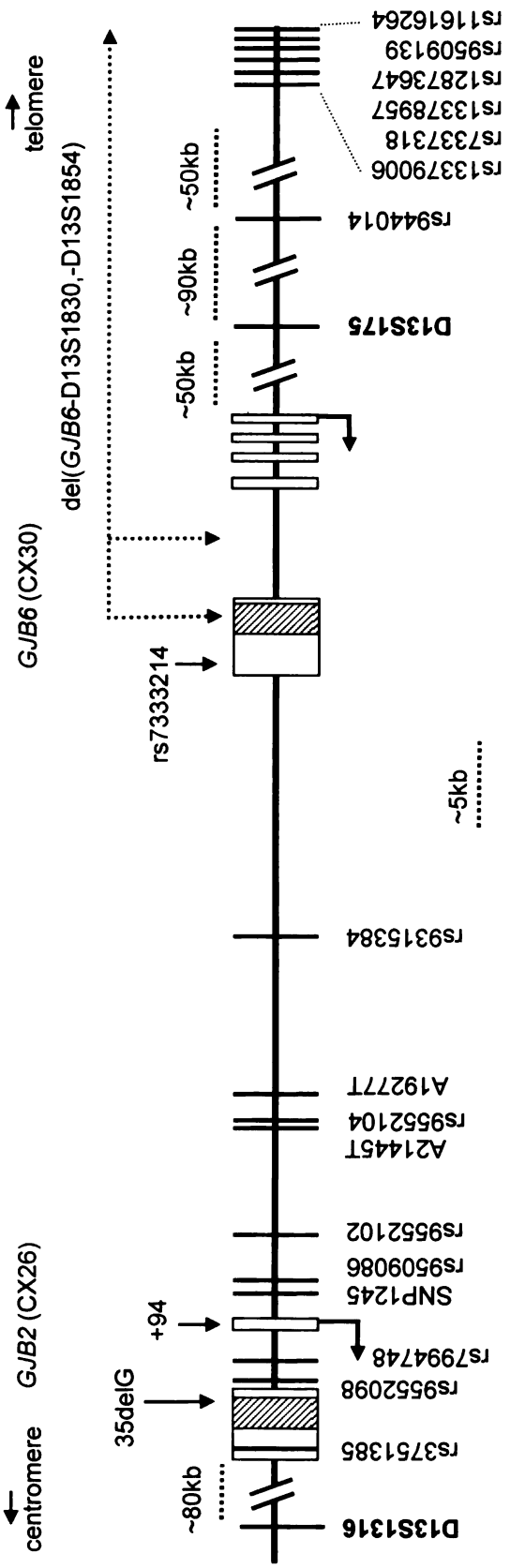

telomere
del(GJB6-D13S1830,-D13S1854)
GJB6 (CX30)
~50kb
~90kb
~50kb
rs11616264
rs9509139
rs12873647
rs13378957
rs7337318
rs13379006
rs944014
D13S175
rs7333214
~5kb
rs9315384
A19277T
rs9552104
A21445T
rs9552102
rs9509086
SNP1245
+94
rs7994748
rs9552098
35delG
rs37513865
~80kb
D13S1316
centromere
GJB2 (CX26)

Figure 2-1

We report here evidence for a novel pathogenic DFNB1 allele for which we demonstrate reduction in or loss of detectable expression of message from both *GJB2* and *GJB6*. Figure 2-2 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 Figure 2-2); 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 (Figure 2-1 and Table 2-1) 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 Figure 2-2). Notably, four deaf family members, DF5-20, -70, -122, and -194, are heterozygous for 35delG and share a common haplotype (indicated in Figure 2-2 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 is the father of DF5-70 (Figure 2-3). 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 v.3.01RC1, SuperLink v1.4) at the DFNB1 locus, using 28 individuals (shown in boxed areas in Figure 2-2). We assigned the same mutant status to both the novel haplotype and

Figure 2-2. 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.

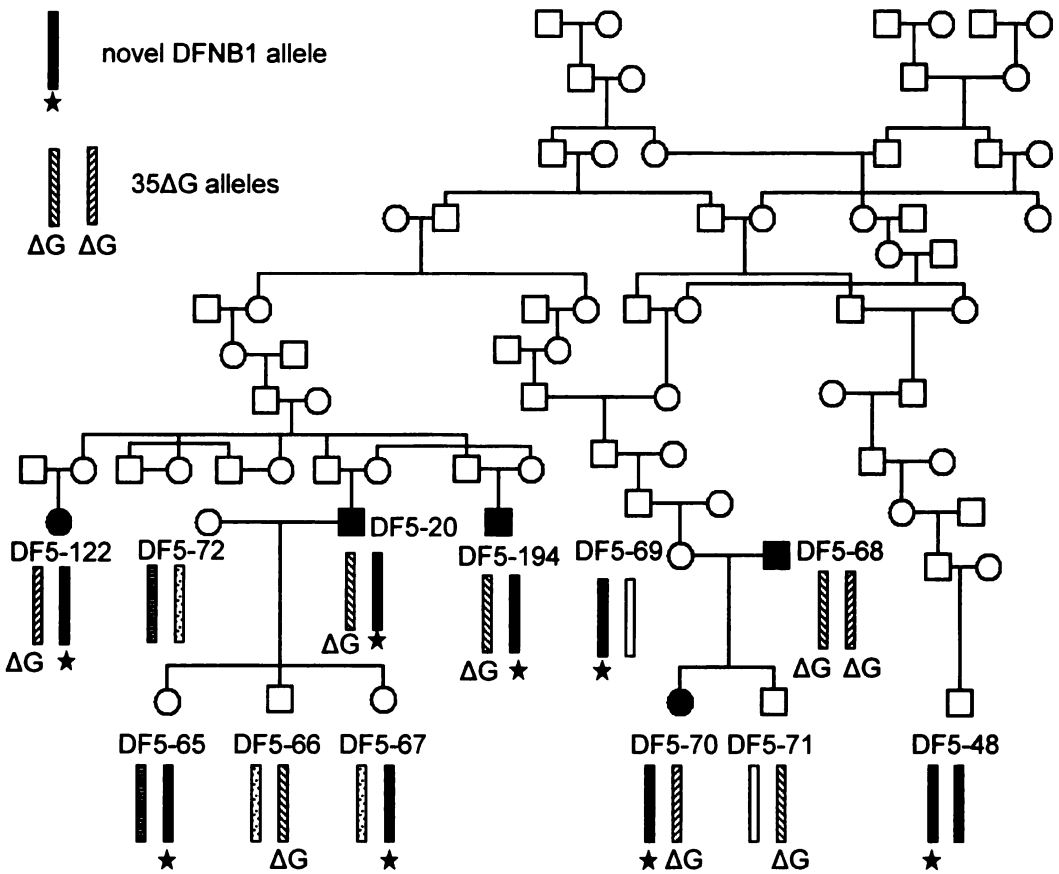

novel DFNB1 allele
35ΔG alleles
ΔG ΔG
DF5-122
DF5-72
DF5-20
DF5-194
DF5-69
DF5-68
DF5-65
DF5-66
DF5-67
DF5-70
DF5-71
DF5-48

Figure 2-2

Table 2-1. Selected haplotype assignments based on genotypes at microsatellites and SNPs within 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	19,695,038	A	A	A	A	T	A
rs9552104	19,695,038	C	G	ND	C	C	C
A19277T	19,695,038	A	ND	A	ND	T	A
rs9315384	19,695,038	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 2-3. Audiograms of four MSU-DF5 family members (identified by ID/sex/age). dB HL = decibels hearing level; ANSI = American Standards Institute. . DF5-20, -70, and -122 are 35delG (35ΔG) heterozygotes bearing the novel pathogenic allele. Note that all three 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; X = left ear, arrows = threshold beyond tested limit), bone conduction thresholds immittance measures (not shown) indicate that the hearing loss in all four family members is sensorineural.

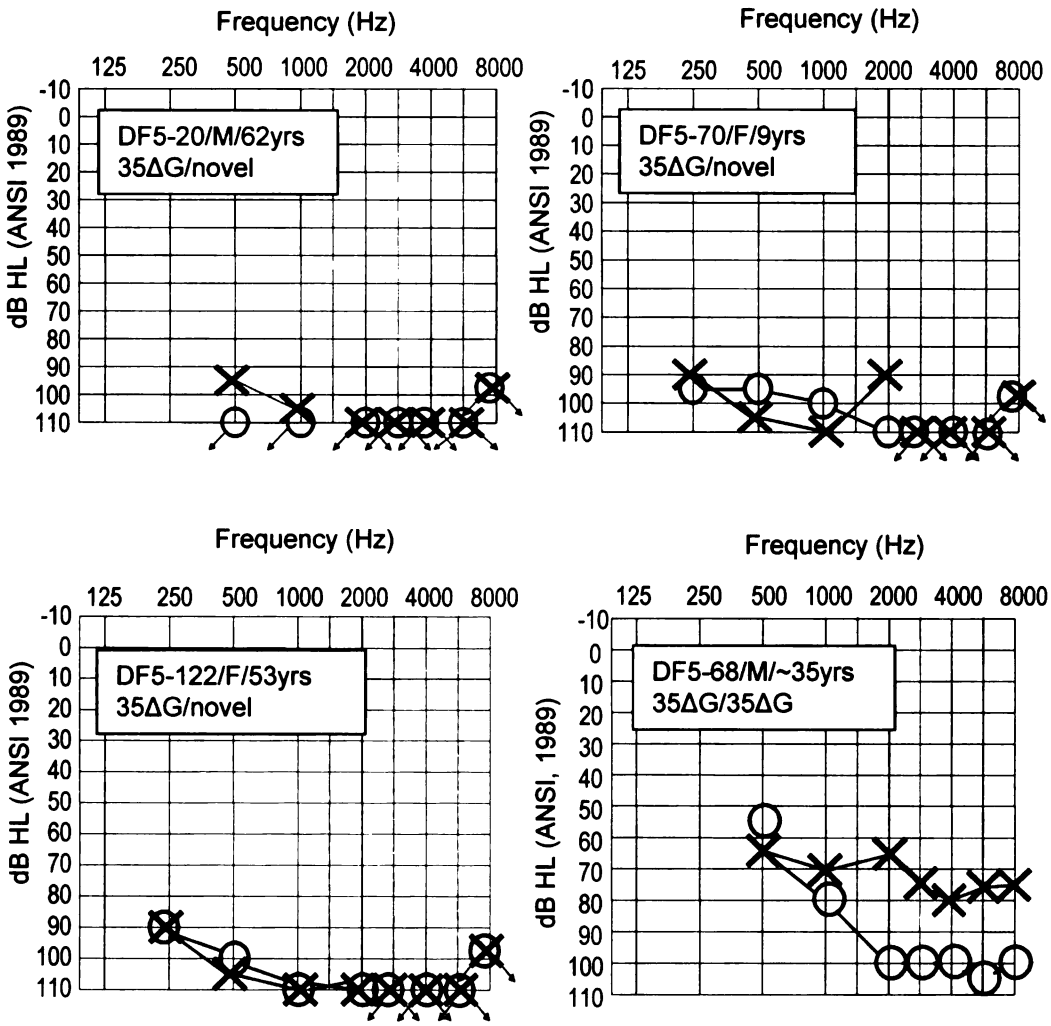

Frequency (Hz)
125 250 500 1000 2000 4000 8000
dB HL (ANSI 1989)
-10 0 10 20 30 40 50 60 70 80 90 100 110
DF5-20/M/62yrs
35ΔG/novel
Frequency (Hz)
125 250 500 1000 2000 4000 8000
dB HL (ANSI 1989)
-10 0 10 20 30 40 50 60 70 80 90 100 110
DF5-70/F/9yrs
35ΔG/novel
Frequency (Hz)
125 250 500 1000 2000 4000 8000
dB HL (ANSI 1989)
-10 0 10 20 30 40 50 60 70 80 90 100 110
DF5-122/F/53yrs
35ΔG/novel
Frequency (Hz)
125 250 500 1000 2000 4000 8000
dB HL (ANSI, 1989)
-10 0 10 20 30 40 50 60 70 80 90 100 110
DF5-68/M/~35yrs
35ΔG/35ΔG

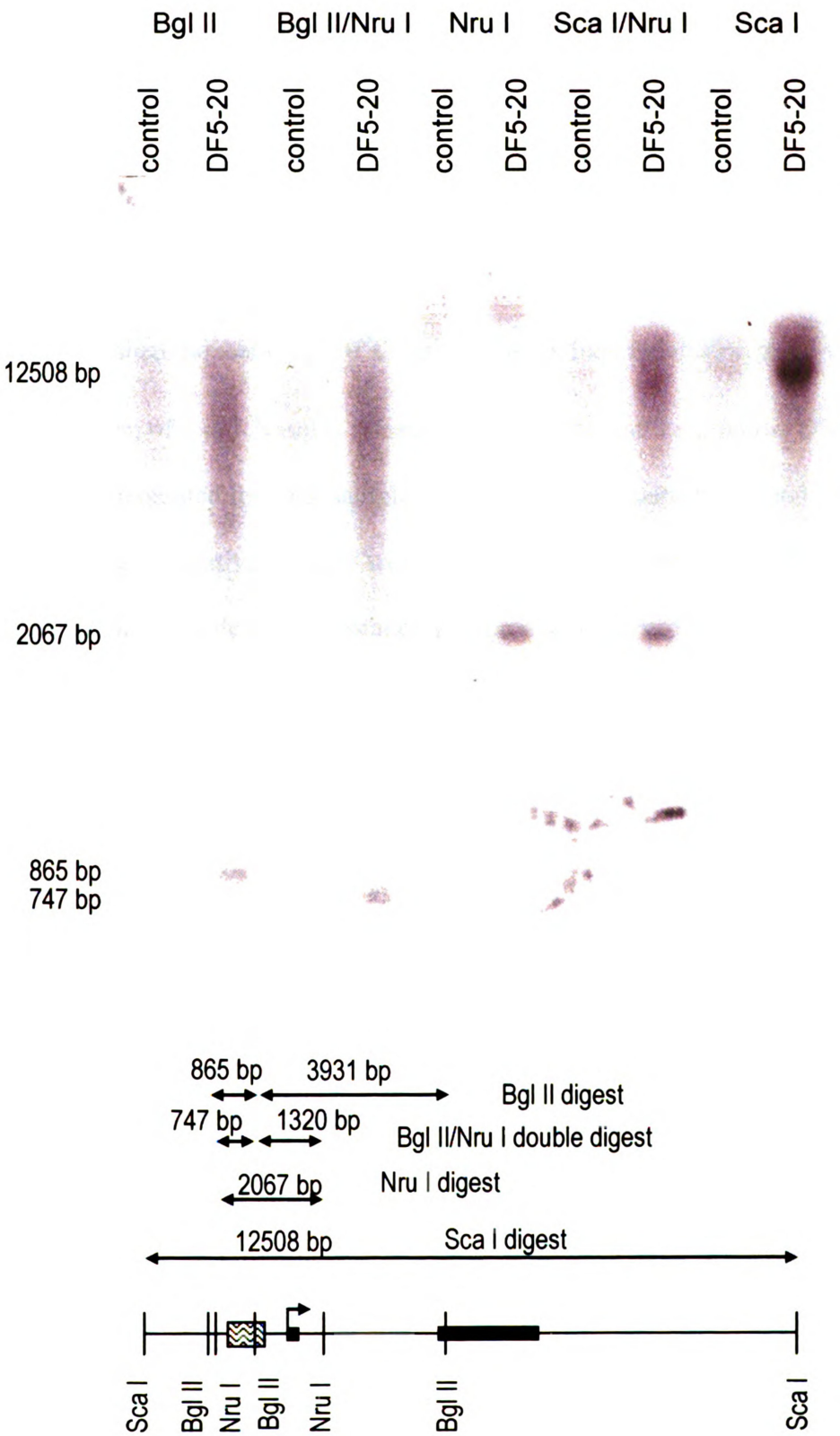
Figure 2-3

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* (Figure 2-4).

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 impracticality 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 1ml Trizol (Gibco/Life Technologies) on ice. Some samples were stored at –20°C for one to several days. RNA was isolated following the Trizol protocol, was resuspended in 20 µl H_2O, and was stored at –80°C. We followed a modified

Figure 2-4. Southern blot of region around *GJB2* (with transcriptional start site indicated by right-angled arrow, and exons by black boxes), for DF5-20 and an unrelated control. ^{32}P-labeled probe indicated by wavy-lined box. Detected probe is associated with expected digestion product sizes, and results are similar for both DF5-20 and the control. Since Nru I is a methylation-sensitive restriction enzyme, these results also preclude unusual methylation on either allele of DF5-20. Five μg aliquots of DNA from DF5-20 and an unrelated control were digested with 1 U of Bgl II, Nru I, or Sca I (all New England Biolabs) in single digests, or with 1 U of Bgl II or Sca II in combination with 1U of Nru I for double digests. Each digestion was incubated for 20 hours at 37°C in the appropriate concentration of NEB Buffer 3 (New England Biolabs) in 50 μl reactions. 45 μl of each digested sample (after addition of 5 μl loading dye to each tube) was run on a 0.7% agarose gel with Markers II and III (Roche), and transferred to a nylon membrane following a standard protocol. A 618 bp PCR product amplified from genomic sequence 500 bp- 1 kbp upstream of the transcriptional start site of *GJB2* was radioactively labeled with ~50 μCi [α-^{32}P]dATP following the protocol provided with the Invitrogen Random Primers DNA Labeling System, and purified by Chroma Spin column (BD Biosciences). The probe was hybridized to the membrane overnight at 65 °C following a standard protocol; the membrane was washed and exposed on a PhosphoImager overnight and then on X-ray film for 4 days (film image shown in figure).


Bgl II
Bgl II/Nru I
Nru I
Sca I/Nru I
Sca I
control
DF5-20
control
DF5-20
control
DF5-20
control
DF5-20
control
DF5-20
12508 bp
2067 bp
865 bp
747 bp
865 bp
3931 bp
Bgl II digest
747 bp
1320 bp
Bgl II/Nru I double digest
2067 bp
Nru I digest
12508 bp
Sca I digest
Sca I
Bgl II
Nru I
Bgl II
Nru I
Bgl II
Sca I

Superscript II (Invitrogen) reverse/transcription protocol for cDNA synthesis; 5-8 µl template RNA, 1µl 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 5X first-strand buffer and 2 µl of 0.1M dithiothreitol was added. Addition of 1 µl Superscript II reverse transcriptase was followed by incubation at 42°C for 50 min, then incubation at 70°C for 15 min. A final incubation at 37°C for 20 min followed 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 1µl 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).

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 (Figure 2-5A). A standard PCR-based assay for the identification of 35delG in genomic DNA (Wilcox, Osborn, & Dahl, 2000) is based on the introduction of a restriction site for *Bst*N1 by a 3' mismatch primer (5'-GCTGGTGGAGTGTTTGTTCACACCCGC-3') that overlaps the run of Gs between nt30 and nt35. The *Bst*N1 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

Figure 2-5. Allele-specific expression assays for *GJB2*. *A*, Schematic showing design of (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*N1 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 are also indicated. *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*N1, 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 product of DF5-67 indicates underrepresentation of the novel (C) allele among the amplified product.

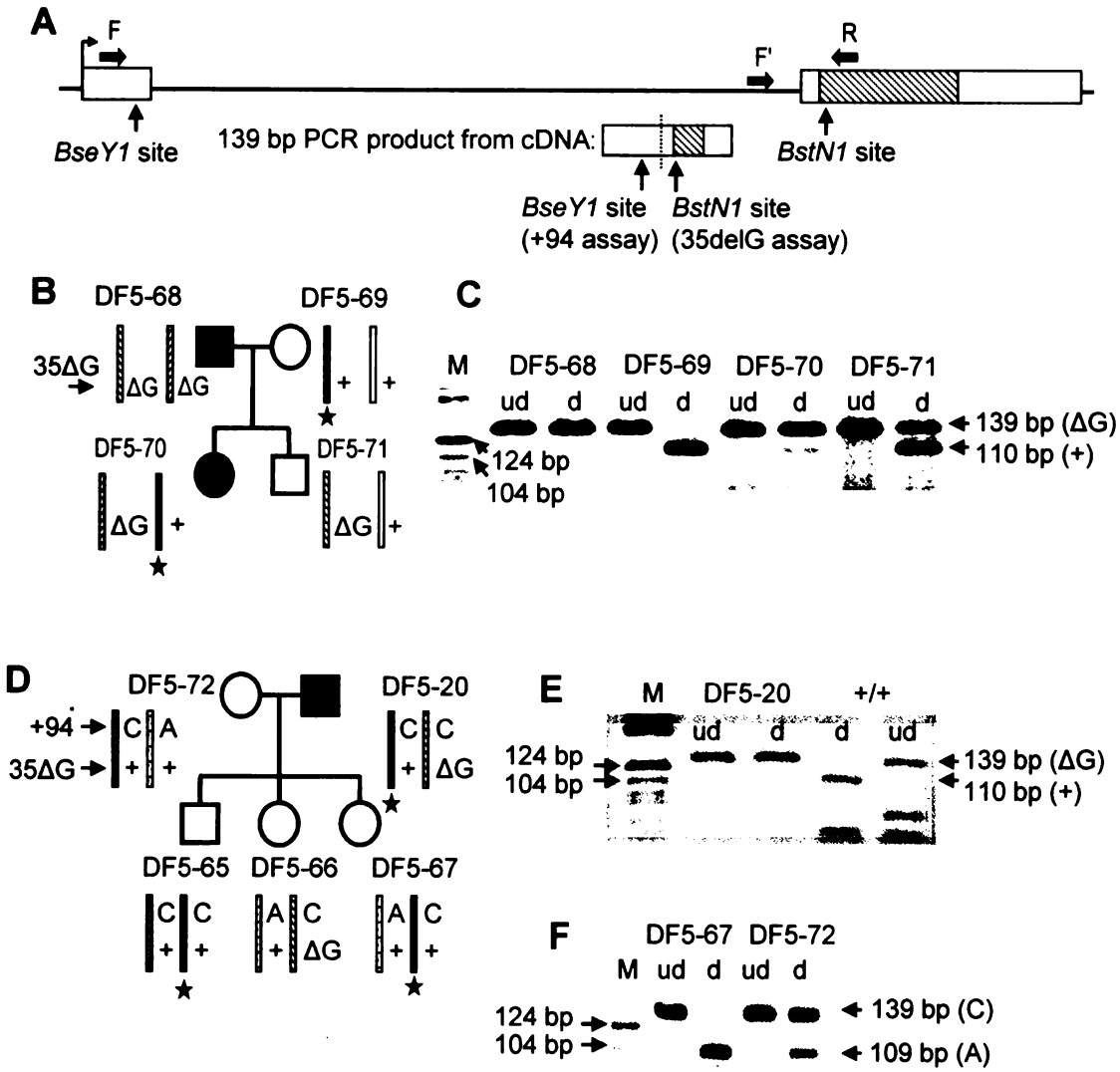

A
F
F'
R
BseY1 site
139 bp PCR product from cDNA:
BstN1 site
BseY1 site
(+94 assay)
BstN1 site
(35delG assay)
B
DF5-68
DF5-69
35ΔG
ΔG
ΔG
+
+
DF5-70
DF5-71
ΔG
+
ΔG
+
C
M
DF5-68
DF5-69
DF5-70
DF5-71
ud
d
ud
d
ud
d
ud
d
139 bp (ΔG)
110 bp (+)
124 bp
104 bp
D
DF5-72
DF5-20
+94
35ΔG
C
A
+
+
C
C
+
ΔG
DF5-65
DF5-66
DF5-67
C
C
+
+
A
C
+
ΔG
A
C
+
+
E
M
DF5-20
+/+
ud
d
d
ud
124 bp
104 bp
139 bp (ΔG)
110 bp (+)
F
DF5-67
DF5-72
M
ud
d
ud
d
124 bp
104 bp
139 bp (C)
109 bp (A)

Figure 2-5

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 PCR (1-5 µl template cDNA, 2 µl 10X Qiagen buffer, 4 µl Q solution (Qiagen), 0.16 µl 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 30s, 66°C for 30s, and 72°C for 30s, 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 >2h, 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 (Figure 2-5, B-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 (Figures 2-5A and 2-5D). 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*Y1 enzyme [New England Biolabs] for >2 h, 5 µl 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 (Figure 2-5F), indicating that no or very little of the PCR product had been amplified from template derived from the novel allele which 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 child of DF5-20, bears the novel haplotype and is heterozygous for *rs*7333214 in the 3'UTR of *GJB6*, allowing us to test this hypothesis (Figures 2-6A and 2-6B). Using a forward primer located in the fourth of the noncoding exons (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 10X Qiagen

Figure 2-6. Allele-specific expression assay for *GJB6*. *A,* Schematic showing design of *Hpy*CH4 IV digestion assay for allele-specific expression of *GJB6* mRNA 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 for *rs7333214*. *C,* Results of cDNA-specific assay for *GJB6* expression. Digestion of product amplified from DF5-72 yields 460-bp as well as 292-bp 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.

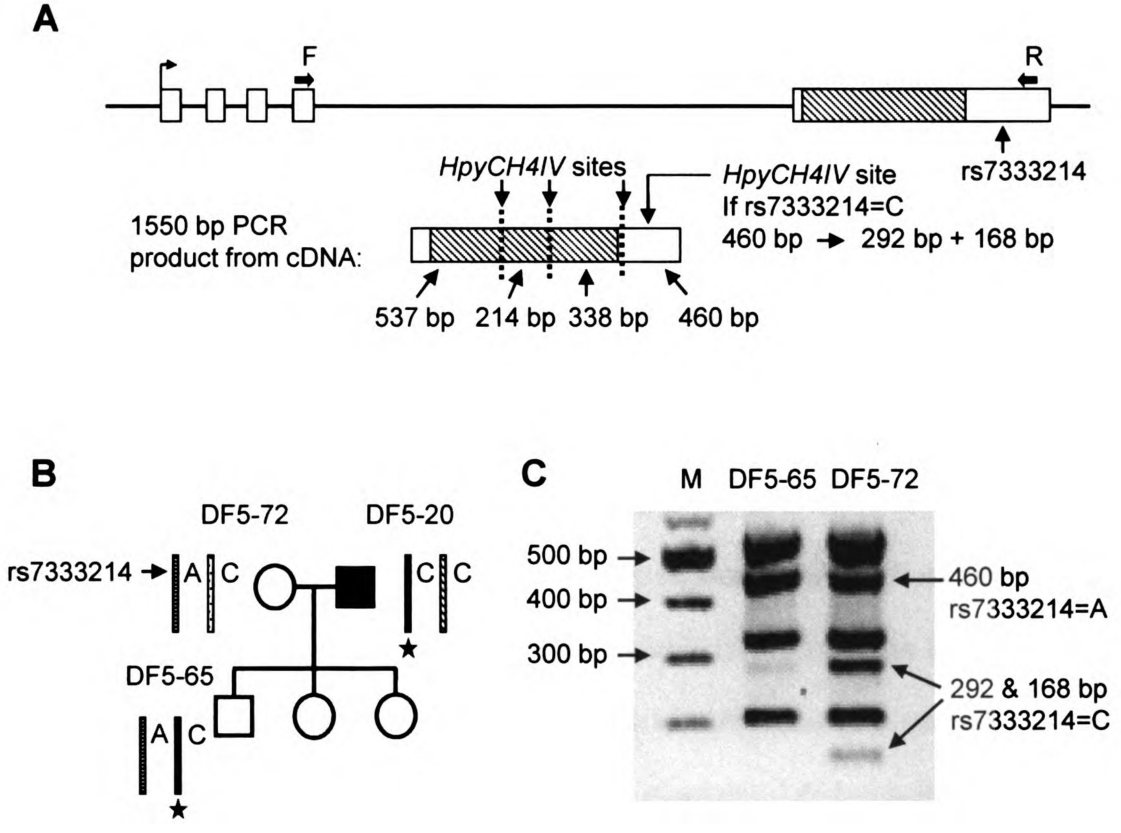

A
F
R
HpyCH4IV sites
HpyCH4IV site
If rs7333214=C
460 bp → 292 bp + 168 bp
rs7333214
1550 bp PCR
product from cDNA:
537 bp
214 bp
338 bp
460 bp
B
DF5-72
DF5-20
rs7333214
A
C
C
C
DF5-65
A
C
C
M
DF5-65
DF5-72
500 bp
400 bp
300 bp
460 bp
rs7333214=A
292 & 168 bp
rs7333214=C

Figure 2-6

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 30s, 57°C for 30s, and 72°C for 2 min, with a final extension at 72°C for 5 min. Digestion of this product for ~2h at 37°C with ~10 U *Hpy*CH4 IV (New England Biolabs) yields digestion products of 537, 460, 338, and 214 bp if an A (the rare variant) is present at *rs*7333214; the 460-bp product is digested to 292- and 168-bp products if a C is present at *rs*7333214 (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/30ml) in 0.5X 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 *rs*7333214 (Figure 2-6B) 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 (Figure 2-6C). 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 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 have 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 (Dean, 2006). 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 the hearing loss in the individuals in our kindred who are heterozygous for 35delG and this novel allele is similar to that of individuals who are del(GJB6-D13S1830)/35delG, who as a group have been shown to have more severe hearing loss than 35delG homozygotes (Snoeckx, et al., 2005). Beyond the identification of the basal promoters of both genes (Essenfelder, Larderet, Waksman, & Lamartine, 2005; Kiang, Jin, Tu, & Lin, 1997; Tu & Kiang, 1998), 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.

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WEB RESOURCES

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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CHAPTER 3

Wilch, E., Azaiez, H., Fisher, R. A., Elfenbein, J., Murgia, A., Birkenhager, R., et al. (2010). A novel DFNB1 deletion allele supports the existence of a distant cis-regulatory region that controls GJB2 and GJB6 expression. *Clin Genet, 78*(3), 267-274.

ABSTRACT

Eleven affected members of a large German-American family segregating recessively inherited, congenital, non-syndromic sensorineural hearing loss (SNHL) were found to be homozygous for the common 35delG mutation of *GJB2*, the gene encoding the gap junction protein Connexin 26. Surprisingly, four additional family members with bilateral profound SNHL carried only a single 35delG mutation. Previously, we demonstrated reduced expression of both *GJB2* and *GJB6* mRNA from the allele carried in *trans* with that bearing the 35delG mutation in these four persons. Using array comparative genome hybridization (array CGH), we have now identified on this allele a deletion of 131.4 kb whose proximal breakpoint lies more than 100 kb upstream of the transcriptional start sites of *GJB2* and *GJB6*. This deletion, del(chr13:19,837,344-19,968,698), segregates as a completely penetrant DFNB1 allele in this family. It is not present in 528 persons with SNHL and monoallelic mutation of *GJB2* or *GJB6*, nor have we identified any other candidate pathogenic copy number variation by arrayCGH in a subset of 10 such persons. Characterization of distant *GJB2/GJB6 cis*-regulatory regions evidenced by this allele may be required to find the 'missing' DFNB1 mutations that are believed to exist.

KEYWORDS

connexin 26, connexin 30, DFNB1, gene expression regulation, *GJB2*, *GJB6*, sensorineural hearing loss, sequence deletion

INTRODUCTION

Mutation in the Connexin 26 (Cx26) gap junction protein-encoding gene *GJB2* (MIM 121011) is the most commonly identified cause of congenital, recessively inherited, sensorineural nonsyndromic hearing loss (DFNB1A [MIM 220290]) (Hilgert, Smith, & Van Camp, 2009; Kenneson, Van Naarden Braun, & Boyle, 2002). In the inner ear, Cx26 and Cx30 (encoded by *GJB6* [MIM 604418], which lies 30 kb telomeric to *GJB2* on human chromosome 13) are expressed in non-sensory cells of the organ of Corti and in cells of the spiral ligament and stria vascularis. Cx26/Cx30 cochlear gap junctions have been implicated in maintenance of K^+ homeostasis in the inner ear (Kikuchi, Kimura, Paul, Takasaka, & Adams, 2000; Wangemann, 2002). However, more recent studies point to a complex suite of functions for gap-junctional networks throughout the cochlea involving participation of second messengers and including generation of the endocochlear potential (Beltramello, Piazza, Bukauskas, Pozzan, & Mammano, 2005; Teubner, et al., 2003; Zhao, Yu, & Fleming, 2005). This intricate set of roles depends on a varied distribution and composition of connexon hemichannels and gap junctions throughout the epithelial supporting cell and connective tissue gap-junctional networks of the cochlea (Ahmad, Chen, Sun, & Lin, 2003; Forge, Becker, et al., 2003; Forge, Marziano, Casalotti, Becker, & Jagger, 2003; Jagger & Forge, 2006; Zhao & Yu, 2006), therefore requiring tight regulation of expression of *GJB2* and *GJB6*.

Although more than 200 mutations of *GJB2*, two deletions involving *GJB6* (DFNB1B [MIM 612645]), and one deletion encompassing both *GJB2* and *GJB6*, constitute the reported set of DFNB1 mutations (F. J. del Castillo, et al., 2005; I. del Castillo, et al., 2002; Feldmann, et al., 2009; Hilgert, Smith, et al., 2009), population

screening commonly yields an excess of individuals with hearing loss who carry only a single identified *GJB2* mutation or DFNB1 deletion (Azaiez, et al., 2004). This finding strongly suggests that additional mutations that lie outside of the proximal promoter and transcribed regions of *GJB2* remain to be identified. Here we identify a novel 131.4-kb deletion, distant from the transcriptional start sites of both *GJB2* and *GJB6*, that segregates as a DFNB1 allele in the extended family in which it is found, and that also segregates with reduced expression of either *GJB2* or *GJB6* message in four family members assayed. This finding has relevance for the identification of distant *GJB2* and *GJB6 cis*-regulatory elements, as sequence variations within these elements may prove to constitute the bulk of 'missing' *GJB2* mutations in DFNB1 hearing loss.

MATERIALS AND METHODS

Human subjects

Participating MSU-DF5 subjects were ascertained through the Oyer Speech-Language-Hearing Clinic within the Department of Communicative Sciences and Disorders at Michigan State University. Audiologic examination of family members included otoscopy, tympanometry, and pure-tone and air- and bone-conduction thresholds. DNA was isolated from blood, saliva, or cheek swabs. Genealogical information was collated from family histories and from publicly available records. Additional subjects were ascertained from hearing loss referrals to the Molecular Otolaryngology Research Laboratories at the University of Iowa. Written informed consent for genetic and audiological testing was obtained from all participants and from the parents of minors. The Michigan State University Institutional Review Board and the University of Iowa Human Subjects Committee approved all procedures.

Array Comparative Genome Hybridization

To examine potential *cis*-regulatory regions for mutation, we looked first for copy number variants (CNVs) by array comparative genome hybridization (array CGH) using a finely tiled CGH microarray designed and manufactured by Nimblegen Systems. Included on the array were 384,000 overlapping oligomeric probes of 45-80 nucleotides in length from regions of non-repetitive sequence on human chromosome 13q11-12, spanning ~6.5 Mb from the most centromeric annotated sequence at chr13:18,000,000 to chr13:24,500,000 (Build 36.1). We provided Nimblegen with DNA from MSU-DF5-20, one of the four deaf family members with monoallelic mutation of *GJB2*. Nimblegen carried out the labeling and hybridizations on MSU-DF5-20 as well as on DNA from 10 additional unrelated *GJB2* heterozygotes with severe-to-profound non-syndromic sensorineural hearing loss (SNHL). Identification of the breakpoints of del(chr13:19,837,344-19,968,698) was done by bidirectional Sanger sequencing of the 628 bp polymerase chain reaction (PCR) product amplified with primers flanking the region of copy number loss (see the following section). Confirmation of regions of deletion and duplication in the additional *GJB2* heterozygotes and screening in 160 Centre d'Etude du Polymorphisme Humain (CEPH) controls was done by PCR with flanking primers; these products were not sequenced for breakpoint identification.

Deletion screening by PCR assay

To screen for del(chr13:19,837,344-19,968,698), we designed a two-product multiplex PCR assay. Two primers (5'-TGGGACCAGGTCTGTTGTT-3' and 5'-ATTGCGACTTGCTTTTCGTT-3') flank the deletion breakpoint and amplify a 628-bp

product from genomic DNA bearing the deletion; the second primer pair (5'-GCAGCCATCTCATGGTCTCT-3' and 5'-CCAACACAATTGGGTCACTCT-3') amplifies an 836-bp product from sequence within the deleted interval. In a 20-µl reaction containing 1.5 mM $MgCl_2$, 25 µM primer, 0.2 mM dNTP, and 0.5 U Taq polymerase, genomic DNA (10-40 ng) was denatured for 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. Products were resolved on a 1% agarose gel containing 0.3 µg/ml ethidium bromide. DNA from 220 MSU-DF5 family members, 160 CEPH controls, and 528 patients with hearing loss who are heterozygous for a known *GJB2/GJB6* mutation was assayed.

RESULTS

Identification of del(chr13:19,837,344-19,968,698) in an extended family segregating DFNB1 hearing loss

Figure 3-1 shows a small portion of the pedigree of MSU-DF5, an extended family descended from a founder community of 500 German-Catholic emigrant families who settled in Michigan in the 19th century. Eleven living family members with hearing loss are known to be homozygous for the common European founder mutation of *GJB2*, 35delG (only one of those individuals is included in Figure 3-1). Unexpectedly, four other family members with bilateral profound sensorineural hearing loss (SNHL) carry only a single 35delG mutation but share the same haplotype on their non-35delG chromosome. Previously, we documented allele-specific reduction of *GJB2* and *GJB6* expression from this allele in buccal cells from four heterozygous individuals (two deaf,

Figure 3-1. The MSU-DF5 subpedigree shown here includes the four profoundly deaf family members who are compound heterozygotes for del(chr13:19,837,344-19,968,698) (green shading) and for the 35delG mutation of *GJB2* (blue shading) which segregates within this family. Of the 220 family members screened, 27 unaffected persons are heterozygous for the deletion and are negative for 35delG; they are shown here, although a number of unaffected siblings who are negative for both mutations are not. The most recent common ancestors of the known carriers of del(chr13:19,837,344-19,968,698) were born in the early 18th century in northern Europe. We previously documented reduced expression of *GJB2* in three persons (red arrows) and *GJB6* in one person (yellow arrow) from the allele now known to bear del(chr13:19,837,344-19,968,698).

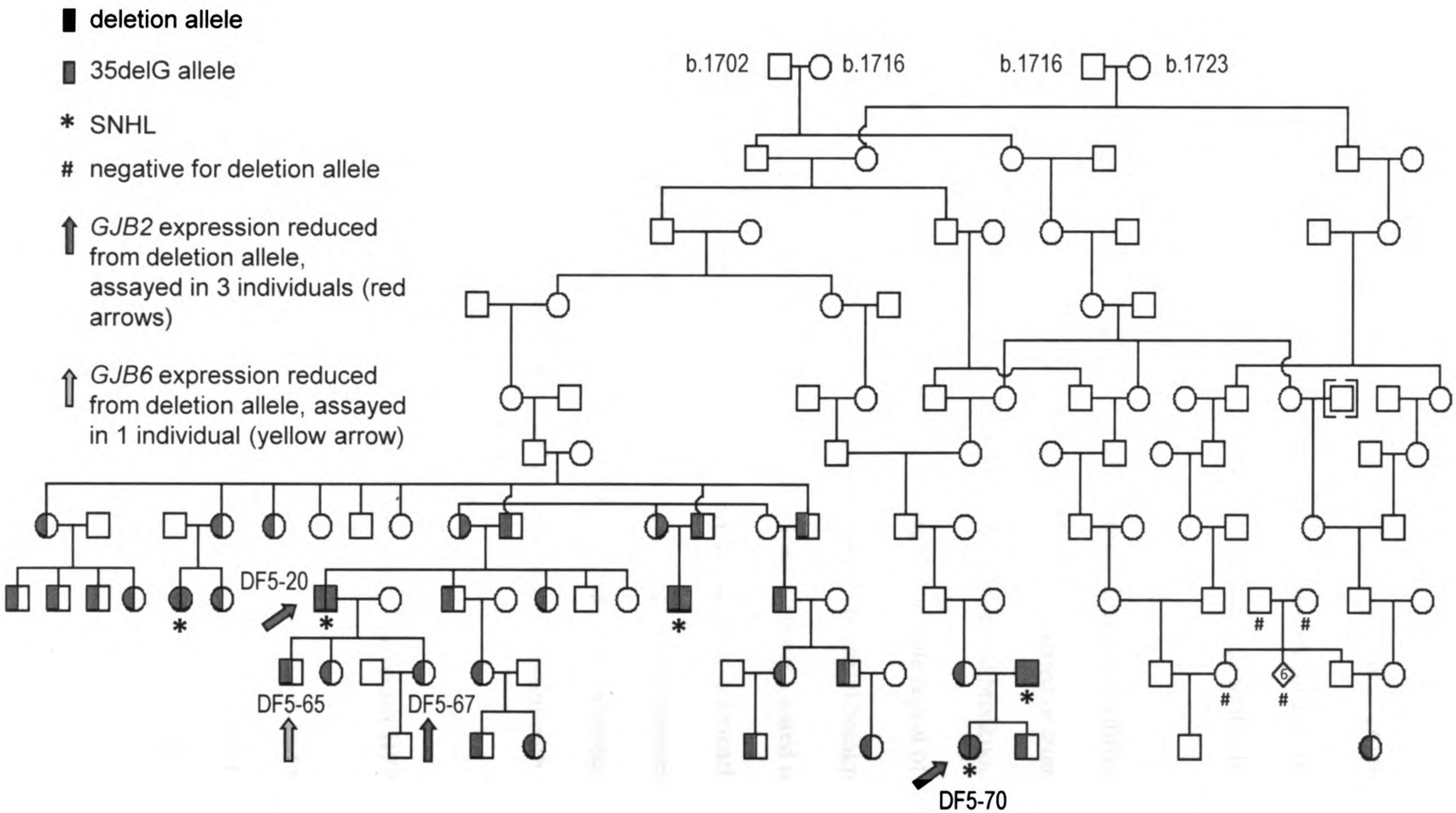
deletion allele
35delG allele
* SNHL
# negative for deletion allele
GJB2 expression reduced from deletion allele, assayed in 3 individuals (red arrows)
GJB6 expression reduced from deletion allele, assayed in 1 individual (yellow arrow)
b.1702
b.1716
b.1716
b.1723
DF5-20
DF5-65
DF5-67
DF5-70

Figure 3-1

with 35delG in *trans*; two hearing, with normal *GJB2* and *GJB6* alleles in *trans*) (WILCH *et al.* 2006). Sequencing of the coding regions, 5' and 3' UTRs, proximal promoter regions, and splice sites of both *GJB2* and *GJB6*, as well as several kilobases of sequence upstream of *GJB2* failed to identify any other sequence variant unique to this low-expression allele.

A single copy region identified by array CGH (Figure 3-2A) was confirmed by PCR and sequencing (Figure 3-2B) to be a deletion of 131.4 kb that is carried *in trans* with the 35delG mutation in the four deaf 35delG heterozygotes. The proximal breakpoint of this deletion is in intergenic sequence at chr13:19,837,344 within a simple repeat of $(TG)_{21}$, leaving 9 TG dinucleotides intact on the deleted chromosome. The distal breakpoint occurs at chr13:19,968,698 within a long interspersed nuclear element located in the second intron of *CRYL1*. The sequence flanking the breakpoints does not clearly indicate a mechanism by which the deletion was generated. No homology exists between the sequences including and immediately adjacent to the breakpoints. The existence of only a single base pair of microhomology at the breakpoint junction is consistent with non-homologous end joining and double-strand break repair.

Del(chr13:19,837,344-19,968,698) segregates as a completely penetrant DFNB1 allele and is >300 years old

Of the 220 MSU-DF5 family members tested, 27 are heterozygous and none are homozygous for the deletion. The deletion is present on the chromosomes from which reduced expression of *GJB2* and *GJB6* message was documented (in MSU-DF5-20, -65, -67, and -70), and in all four deaf 35delG heterozygotes, indicating that the deletion-

Figure 3-2A. Characterization of novel DFNB1 deletion allele del(chr13:19,837,344-19,968,698). Custom array comparative genome hybridization (array CGH) across a 6.5 Mb region of human chromosome 13 indicates a region of single copy number loss in MSU-DF5-20.

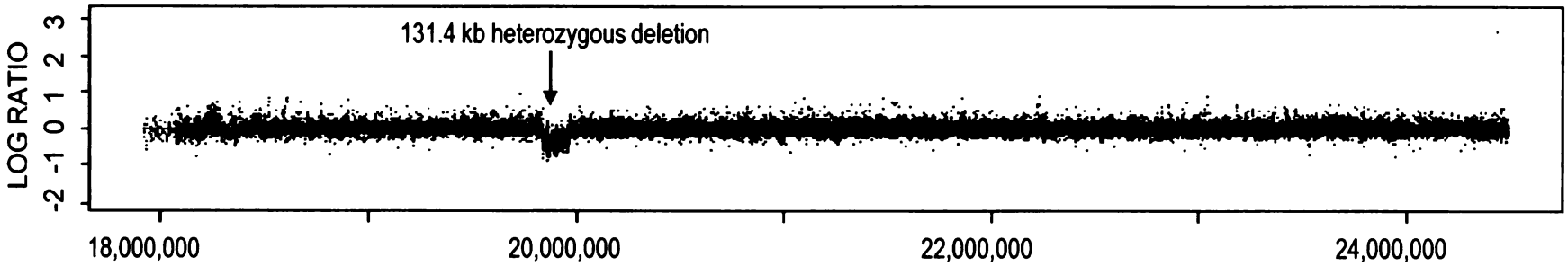
131.4 kb heterozygous deletion
LOG RATIO
-2 -1 0 1 2 3
18,000,000
20,000,000
22,000,000
24,000,000

Figure 3-2A

Figure 3-2B. Del(chr13:19,837,344-19,968,698) breakpoint junctional sequence amplified from MSU-DF5-20 DNA and aligned with human genome reference sequence (Build 36.1) shows a single basepair of microhomology at the breakpoint.

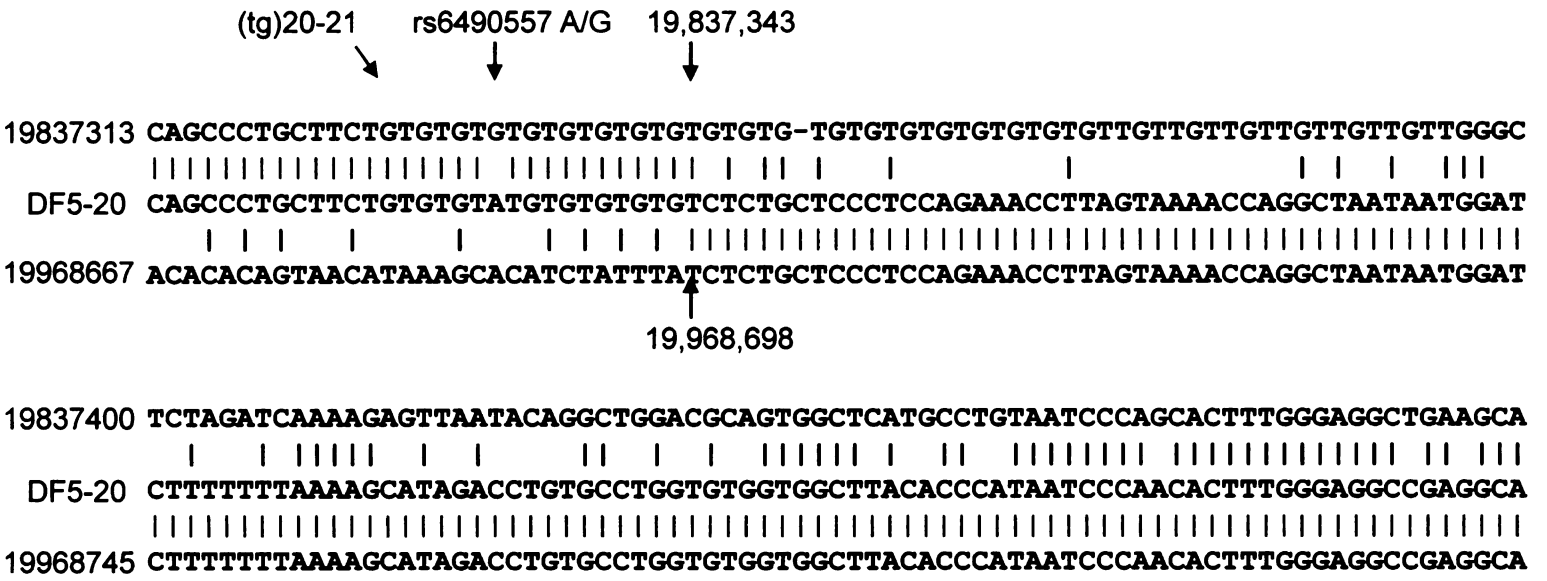
(tg)20-21
rs6490557 A/G
19,837,343
19837313 CAGCCCTGCTTCTGTGTGTGTGTGTGTGTGTGTG-TGTGTGTGTGTGTGTTGTTGTTGTTGTTGTTGTTGGGC
DF5-20 CAGCCCTGCTTCTGTGTGTATGTGTGTGTCTCTGCTCCCTCCAGAAACCTTAGTAAAACCAGGCTAATAATGGAT
19968667 ACACACAGTAACATAAAGCACATCTATTTATCTCTGCTCCCTCCAGAAACCTTAGTAAAACCAGGCTAATAATGGAT
19,968,698
19837400 TCTAGATCAAAAGAGTTAATACAGGCTGGACGCAGTGGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCTGAAGCA
DF5-20 CTTTTTTTAAAAGCATAGACCTGTGCCTGGTGTGGTGGCTTACACCCATAATCCCAACACTTTGGGAGGCCGAGGCA
19968745 CTTTTTTTAAAAGCATAGACCTGTGCCTGGTGTGGTGGCTTACACCCATAATCCCAACACTTTGGGAGGCCGAGGCA

Figure 3-2B

bearing chromosome segregates in this pedigree as a completely penetrant recessive DFNB1 allele (Figure 3-1). This deletion was not identified in 160 CEPH controls.

Under the assumption of identity-by-descent, and by choosing the least number of meioses to relate carriers, the deletion chromosome can be traced back to four individuals born in Germany between 1702 and 1723, all equally likely to have contributed this allele to the MSU-DF5 pedigree. Although the deletion allele is thus at least 300 years old and may exist outside of this pedigree, it has not been identified among 528 persons with hearing loss, who are heterozygous for a single *GJB2/GJB6* mutation, from the United States, Brazil, Iran, and several European countries (Table 3-1).

Assessment of 35delG-heterozygotes with SNHL for incidence of pathogenic copy number variation across the DFNB1 locus

To determine if deletions may constitute a frequent mechanism for DFNB1 deafness, we performed array CGH on DNA from 10 unrelated 35delG-heterozygous patients with severe-to-profound SNHL. We identified copy number variants (CNVs) across the region (Table 3-2), including 5 deletions ranging in size from 130 bp to 450 bp, and 3 duplications between 65 bp and 960 bp in size. Three regions were associated with either duplication or deletion. CNVs were confirmed by PCR with flanking primers, although the breakpoints were not precisely identified. All of these CNVs were present in 160 CEPH controls at frequencies higher than 20%.

DISCUSSION

Of the nearly 100 loci mapped for human inherited non-syndromic hearing loss since the early 1990s, 46 genes have been identified with sufficient evidence to establish

Laboratory (Investigators)	N	Reported ethnicity or nationality
USA/Iowa (Smith/Azaiez)	55	
USA/Virginia (Pandya)	132	96 caucasian, 4 black, 25 hispanic, 7 other
Spain (del Castillo)	50	
Brazil (Sartorato/da Silva Costa)	40	
France (Le Marechal)	41	
Netherlands (Kremer/Hoefsloot)	27	
Germany/Freiburg (Birkenhäger)	62	
Germany/Koln (Bolz/Kubisch)	10	
Germany/Mainz (Haaf/Schneider)	31	23 German, 5 Turkish, 2 Moroccan, 1 Spanish
Italy (Murgia)	22	
Belgium (Van Camp/Wuyts)	58	40 Belgian, 18 Iranian
Total	**528**	

Table 3-1. Number of affected individuals with monoallelic *GJB2/GJB6* mutation screened for del(chr13:19,837,344-19,968,698).

Position*	Variation	Length* (bp)
Chr13:19686728-19686792	dup	65
Chr13:19691120-19691940	dup	820
Chr13:19722020-19722070	del/dup	50
Chr13:19749870-19750200	del	340
Chr13:19768000-19768130	del	130
Chr13:19818780-19819200	del	420
Chr13:19835210-19835420	del/dup	200
Chr13:19863060-19864020	del/dup	960
Chr13:19934100-19934550	del	450
Chr13:19969740-19970700	dup	960
Chr13:19974330-19974480	del	150

CNV, copy number variant; del, deletion; dup, duplication.
* Length and position are approximate.

Table 3-2. List of CNVs in the DFNB1 region.

causation. Mutations in two of these genes, *GJB2* and *SLC26A4*, cause a significant proportion of hearing loss globally, with loss-of-function mutations of *GJB2* estimated to be responsible for more than half of all recessively inherited SNHL in developed countries (Hilgert, Smith, et al., 2009). This knowledge has changed the medical evaluation of families segregating presumed autosomal recessive SNHL and has made accurate genetic counseling, recurrence chance estimation, phenotype-genotype correlations and prognosis for progression of hearing loss possible when two mutant alleles of *GJB2* are identified. It is widely accepted that unidentified mutations of *GJB2* exist that are *cis*-regulatory in nature. Deletion alleles that abrogate expression of *GJB2* without disrupting the gene sequence are important for identifying candidate *cis*-regulatory regions for mutation analysis.

The deletion we have described, del(chr13:19,837,344-19,968,698), represents the fourth DFNB1 deletion allele, and the third in which *GJB2* is left intact. Del(*GJB6*-D13S1830) (I. del Castillo, et al., 2002; Lerer, et al., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002) and del(*GJB6*-D13S1854) (F. J. del Castillo, et al., 2005), deletions of 309 kb and 232 kb, respectively, truncate *GJB6* and extend telomerically (Figure 3-2C). Lerer et al. (Lerer, et al., 2001) showed *GJB2* expression from both alleles in lymphocytes from a patient heterozygous for del(*GJB6*-D13S1830) and on this basis suggested that a digenic interaction between *GJB2* and *GJB6* may be responsible for the hearing loss in these individuals. Our allele-specific expression assay results on del(chr13:19,837,344-19,968,698), which suggest otherwise, were consistently replicated using stratified epithelial cell-derived cDNA from buccal swabs, whereas assays of lymphocyte-derived cDNA from the same individuals gave inconsistent results

Figure 3-2C. Del(chr13:19,837,344-19,968,698) overlaps with DFNB1 deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) over a 95.4 kb interval upstream of the transcriptional start sites (indicated by right-angled arrows) of *GJB2* and *GJB6*. *CRYL1* is also shown.

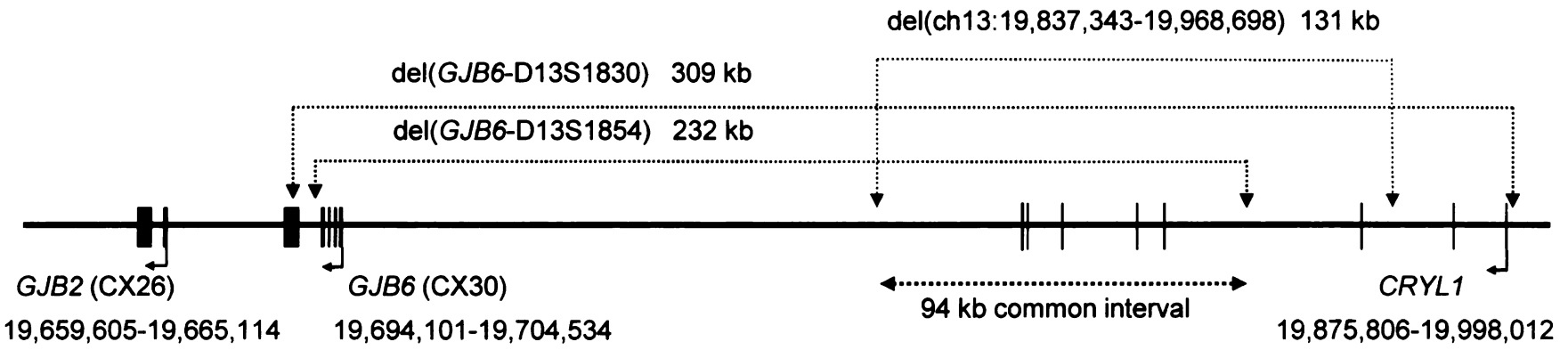
del(ch13:19,837,343-19,968,698) 131 kb
del(GJB6-D13S1830) 309 kb
del(GJB6-D13S1854) 232 kb
GJB2 (CX26)
19,659,605-19,665,114
GJB6 (CX30)
19,694,101-19,704,534
94 kb common interval
CRYL1
19,875,806-19,998,012

Figure 3-2C

(data not shown). This discrepancy may indicate that regulation of *GJB2* and *GJB6* differs with tissue type, and that expression assayed in lymphocytes may not be relevant to expression in cochlear cells.

Common et al. (2005) also demonstrated loss of normal Cx26 expression from the deletion-bearing allele in stratified epithelial skin cells in an individual with hearing loss and a 35delG/del(*GJB6*-D13S1830) genotype. These results are consistent with hypotheses of complex *cis*-regulation of *GJB2* and loss of an important *GJB2* regulatory element as a result of these deletions. Qualitative loss of *GJB2* expression from alleles bearing del(GJB6-D13S1830) in compound heterozygosity with *GJB2* mutations/variants E47X, M34T, and V27I/E114G has since been demonstrated in three individuals (Rodriguez-Paris & Schrijver, 2009), further supporting this mechanism of action.

Feldmann et al. (2009) have provided additional evidence against the *GJB2/GJB6* digenic hypothesis. They have documented a >920 kb deletion encompassing *GJB2*, *GJB6*, *GJA3* and several other genes that is present in *trans* with the V84M mutation of *GJB2* in a deaf individual. Three other persons with normal hearing in this pedigree carry this contiguous gene deletion, indicating that loss of single copies of *GJB2* and *GJB6*, per se, cannot explain the pathogenicity of the two *GJB6*-truncating deletion alleles, del(*GJB6*-D13S1830) and del (*GJB6*-D13S1854).

Del(chr13:19,837,344-19,968,698) is of particular interest as it leaves both *GJB2* and *GJB6* completely intact, has a proximal breakpoint substantially farther from *GJB2* than the *GJB6*-truncating DFNB1 deletion alleles, and segregates with reduced expression of both *GJB2* and *GJB6*. These findings suggest that time- and tissue-specific enhancer elements for both genes may lie a considerable distance upstream, and/or that a

locus control region exists for these two genes. We hypothesize a similar mechanism of pathogenesis for all three deletion alleles that involves, in addition to loss or reduction in expression of *GJB6*, the loss of a critical *cis*-regulatory element for *GJB2* located within the common 95.4 kb genomic interval that is deleted in all three alleles. Sequence conservation between species is one metric by which candidate non-coding regulatory DNA may be identified. Human-mouse sequence conservation and rank VISTA (rVISTA) calculations of potential regulatory function (Brudno, et al., 2003; Loots, Ovcharenko, Pachter, Dubchak, & Rubin, 2002) (Figure 3-2D), reproduced from the VISTA browser) show numerous candidate regulatory elements within this interval.

Because we found del(chr13:19,837,344-19,968,698) in only one extended family, as an alternate, less parsimonious hypothesis, del(chr13:19,837,344-19,968,698) could be in linkage disequilibrium with a pathogenic single nucleotide substitution or other undetected DNA lesion closer to both *GJB2* and *GJB6* that disrupts *cis*-regulatory function of both genes coordinately. This possibility also permits a common mechanism of pathogenesis of the three deletion alleles which leaves *GJB2* intact, and accommodates the evidence of loss of *GJB2*/Cx26 expression observed from the del(*GJB6*/D13S1830) allele by Rodriguez-Paris et al. (2009) and by Common et al. (2005).

The identification of additional deletions is possible with the increasing accessibility of array CGH and other techniques to assess CNV. Although patients with severe-to-profound hearing loss and monoallelic *GJB2* mutation are candidates for

Figure 3-2D. Upper VISTA track shows sequence conservation between human (March 2006) and mouse (July 2007) genomes over the 95.4-kb common deletion interval indicated in Fig. 2C; pink-colored peaks represent regions of 100+ bp of non-coding sequences conserved at ≥70% identity. Lower track shows rankVISTA (rVISTA) predictions ($-\log_{10}$[P-value]) of potential regulatory function based on multispecies sequence conservation combined with identification of transcription factor binding sites (http://genome.lbl.gov/vista/index.shtml).

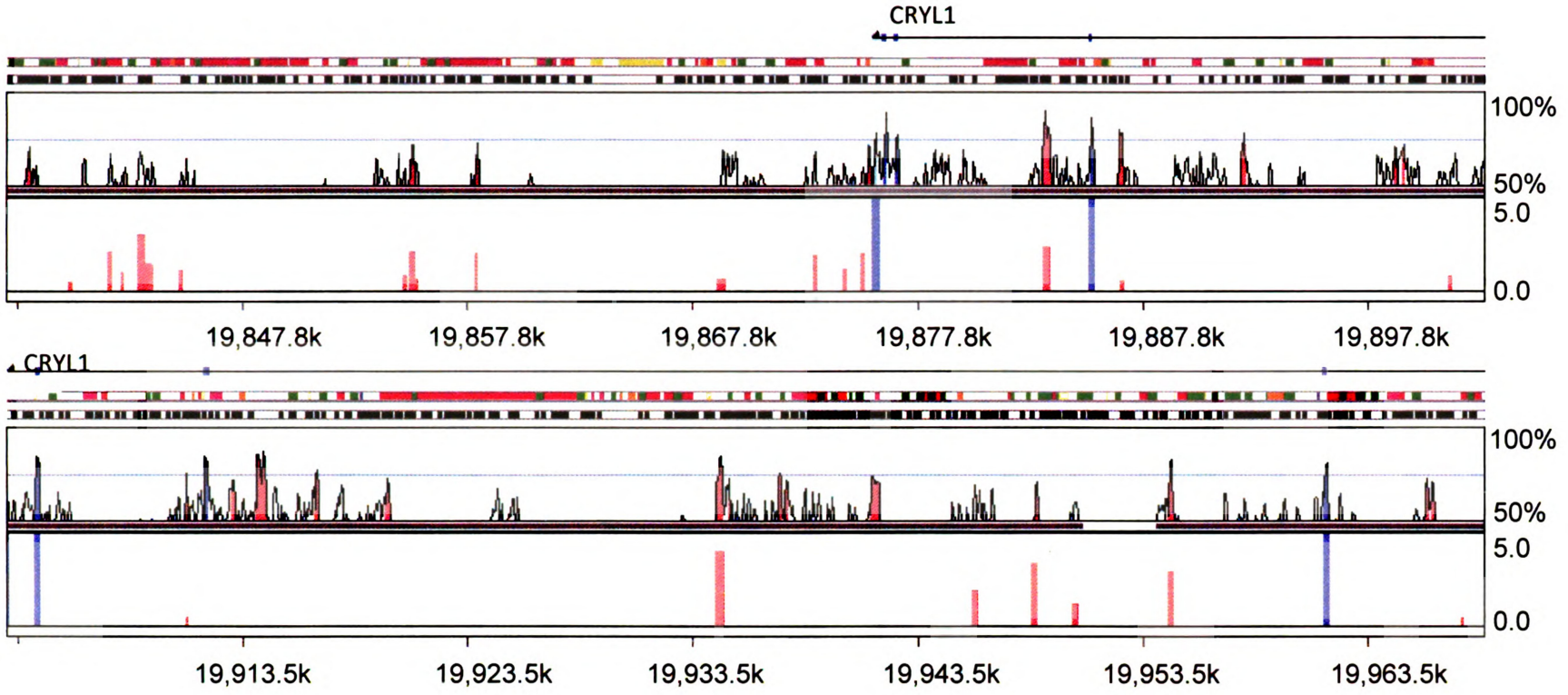
CRYL1
100%
50%
5.0
0.0
19,847.8k
19,857.8k
19,867.8k
19,877.8k
19,887.8k
19,897.8k
CRYL1
100%
50%
5.0
0.0
19,913.5k
19,923.5k
19,933.5k
19,943.5k
19,953.5k
19,963.5k

Figure 3-2D

Figure 3-2D. Upper VISTA track shows sequence conservation between human (March 2006) and mouse (July 2007) genomes over the 95.4-kb common deletion interval indicated in Fig. 2C; pink-colored peaks represent regions of 100+ bp of non-coding sequences conserved at ≥70% identity. Lower track shows rankVISTA (rVISTA) predictions ($-\log_{10}$[P-value]) of potential regulatory function based on multispecies sequence conservation combined with identification of transcription factor binding sites (http://genome.lbl.gov/vista/index.shtml).

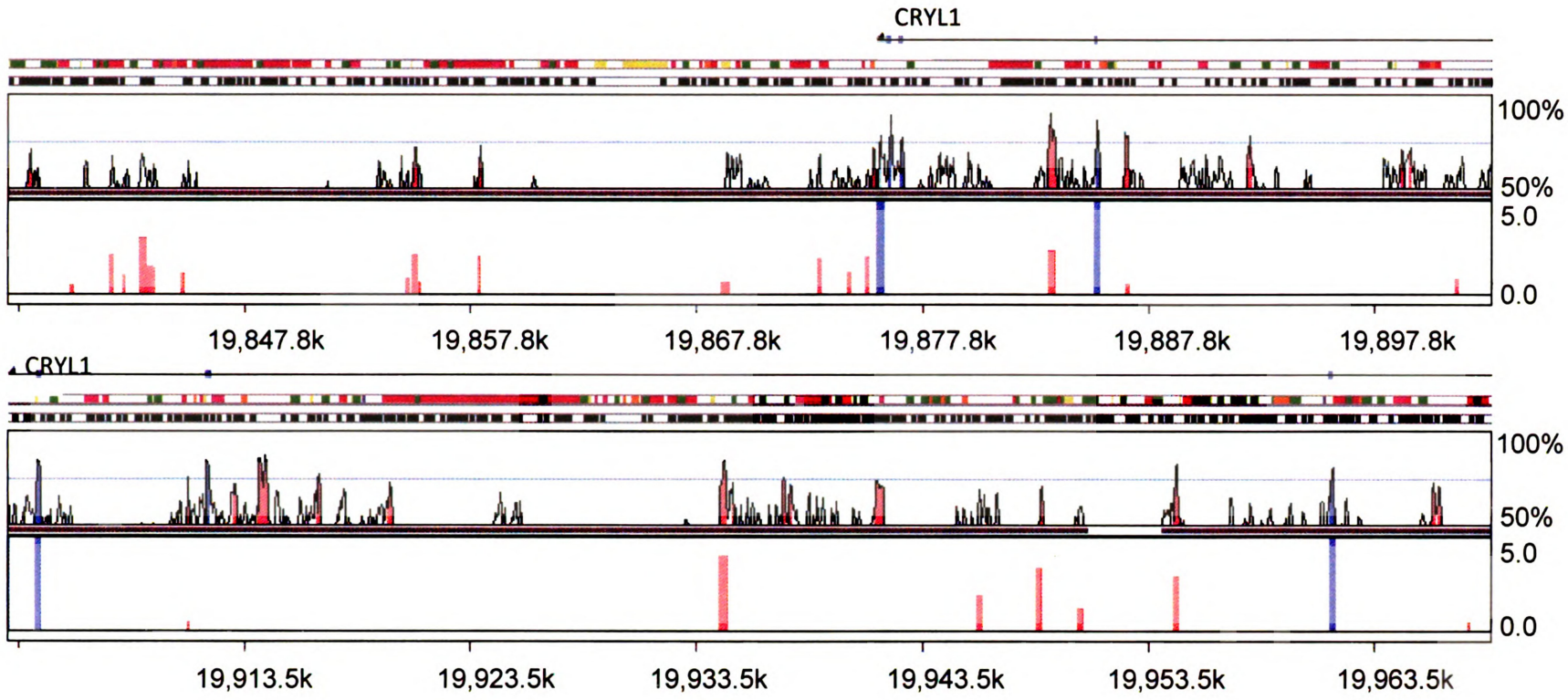
CRYL1
100%
50%
5.0
0.0
19,847.8k
19,857.8k
19,867.8k
19,877.8k
19,887.8k
19,897.8k
CRYL1
100%
50%
5.0
0.0
19,913.5k
19,923.5k
19,933.5k
19,943.5k
19,953.5k
19,963.5k

Figure 3-2D

pathogenic CNV, our findings from 10 such patients suggest that while CNV is common, identifying the regulatory regions may require an alternative approach such as enhancer-reporter assays. The CNVs we found among our 10 patients were all found among CEPH controls at frequencies higher than 20%.

Sequence variation within regulatory elements can give rise to allele-specific quantitative differences in expression of message that may yield phenotypic variation (Cowles, Hirschhorn, Altshuler, & Lander, 2002; Knight, 2004). Variability in degree of hearing loss is a well-documented feature of *GJB2* deafness, even among persons with biallelic null (protein-truncating) mutations that completely abolish Cx26 protein expression (Azaiez, et al., 2004; Snoeckx, et al., 2005). No modifier genes have yet been identified to explain this phenotypic variability (Hilgert, Huentelman, et al., 2009). We hypothesize that sequence variation in *cis* that regulates *GJB6* expression from a distance may be partially responsible for some of this variability.

In summary, our studies of del(chr13:19,837,344-19,968,698) support the presence of distant *cis*-regulation of *GJB2* and substantially reduce the span of chromosome 13 that is most strongly implicated in this function. We also provide the first evidence of distant *cis*-regulation of *GJB6*. Additional efforts to elucidate and characterize distant *GJB2* and *GJB6 cis*-regulatory regions are clearly warranted.

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ABBREVIATIONS

Array CGH	array comparative genome hybridization
CEPH	Centre d'Etude du Polymorphisme Humain
CNV	copy number variant
CX26	Connexin 26
CX30	Connexin 30
DSB	double-strand break
LINE	long interspersed nuclear element
NHEJ	non-homologous end joining
PCR	polymerase chain reaction
SNHL	sensorineural hearing loss
UTR	untranslated region

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CHAPTER 4

Enhancer assays of conserved DNA elements in the 131.4 kb MSU-DF5 deletion interval

INTRODUCTION

Mutations in *GJB2*, the gene encoding the gap junction protein connexin 26 (Cx26), are the most frequent cause of congenital deafness globally. *GJB2* is a small gene that is easy to screen for coding region, splicing, and basal and proximal promoter mutations by direct sequencing. Occupying about 5 kb of genomic sequence on human chromosome 13q12, the gene consists of a 116 bp noncoding exon, a 3.2 kb intron, and a second exon containing 22 bp of 5' UTR sequence, the entire 678 bp coding sequence, and 1.4 kb of 3' UTR sequence. Over 200 mutations causing recessively inherited hearing loss have been identified in *GJB2* (Hilgert, Smith, & Van Camp, 2009); fewer than two dozen other mutations are reported to cause dominantly inherited hearing loss or syndromic forms of hearing loss that include various symptoms of skin dysfunction.

The DFNB1 locus also includes two other connexin genes, *GJA3* (Cx46) and *GJB6* (Cx30). Cx46 is expressed in lens only (Gerido & White, 2004) and several mutations of *GJA3* have been associated with dominantly inherited cataract. Like Cx26, Cx30 is also expressed in the cochlea and in stratified skin epithelial cells. In the cochlea, the two connexins have overlapping, although non-identical, expression patterns, and are known to co-assemble into heteromeric connexons (Ahmad, Chen, Sun, & Lin, 2003; Zhao & Yu, 2006). Hemichannels of heteromeric Cx26/Cx30 connexons have been shown to have gating and transport properties that differ from homomeric connexons of either Cx26 or Cx30. In spite of this intimate co-expression in the cochlea, the mutation spectrum of *GJB6* differs considerably from that of *GJB2*. A single missense mutation of *GJB6* is associated with dominantly inherited hearing loss; three

missense mutations cause Clouston syndrome (MIM129500), a hair-nail dystrophy without hearing loss. With the notable exception of the two deletions discussed below, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), no mutations of *GJB6* are associated with recessively inherited nonsyndromic hearing loss.

It has been apparent for some time that many people with hearing loss have only monoallelic mutation of *GJB2*, more than can be expected to be incidental carriers of a *GJB2* mutation with hearing loss of a different etiology. The carrier frequency of *GJB2* mutations varies with population, but can be as high as 3-4%; the proportion of individuals with hearing loss who are found to have monoallelic *GJB2* mutation is not uncommonly 10% or higher (Kenneson, Van Naarden Braun, & Boyle, 2002). As it is relatively straightforward to thoroughly screen the *GJB2* gene itself, it is generally believed that additional, regulatory mutations of *GJB2* must exist within the DFNB1 locus, but at some distance from the gene itself; that is, beyond the one to several kilobases of upstream sequence that is typically interrogated when sequencing efforts fail to turn up a coding region mutation. For other genes, the catalog of such mutations has been growing substantially over the last several years.

Four large deletions have been identified that segregate as DFNB1 mutations. Two of these, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1824), are deletions of 309 kb and 232 kb, respectively, that both truncate the 5' end of *GJB6* and extend telomerically to include several 3' exons of *CRYL1*, and leave *GJB2* sequence intact (F. J. del Castillo, et al., 2005; I. del Castillo, et al., 2002; Lerer, et al., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002). A hypothesis of digenic inheritance with *GJB2* was

made on the basis of results of allele-specific expression assayed in peripheral leukocytes (Lerer, et al., 2001). Those authors detected expression of *GJB2* from the del(*GJB6*-D13S1830) allele, contradicting the hypothesis that this allele leads to loss of function of *GJB2*.

The characterization of two additional deletions at this locus (Feldmann, et al., 2009; Wilch, et al., 2010) along with two studies of the del(*GJB6*-D13S1830) mutation in particular (Common, et al., 2005; Rodriguez-Paris & Schrijver, 2009) together contradict the hypothesis of digenic inheritance of *GJB2* and *GJB6*. Firstly, the 131.4 kb deletion characterized by our lab (Wilch, et al., 2010; Wilch, et al., 2006) overlaps with the two deletions previously described, but has a proximal breakpoint more than 140 kb distant from the transcriptional start site of *GJB6*, thus leaving both *GJB6* and *GJB2* sequences intact. We demonstrated that this deletion segregates with low expression of both *GJB2* and *GJB6* mRNA, by qualitative allele-specific expression assays on buccal cell cDNA. This finding suggests that one or more regulatory elements for *GJB2* are disrupted within this deleted interval, at least 170 kb upstream of the transcriptional start site of *GJB2*. Similar assays on persons bearing the del(*GJB6*-D13S1830) allele heterozygously with coding-region variants of *GJB2* also indicated reduced expression of *GJB2* mRNA from the deletion allele (Rodriguez-Paris & Schrijver, 2009). This result is consistent with that of Common et al. (2005), who showed by immunohistochemistry lack of Cx26 protein expression in certain stratified epithelial skin cells in a person with hearing loss who is a compound heterozygote for del(*GJB6*-D13S1830) and the 35delG null mutation of *GJB2*. Finally, a >920 kb deletion that removes all three of the connexin genes at this locus was recently reported (Feldmann, et al., 2009). In the pedigree in which this deletion was

identified, the proband carried the deletion in *trans* with the known pathogenic V84M mutation of *GJB2*, and had been profoundly deaf since infancy. Three family members with normal hearing were also found to be carriers of the deletion. This finding contradicts a model of digenic inheritance, as these carriers have lost one allele of *GJB2* and one allele of *GJB6*, albeit in *cis*, but maintain normal hearing. Expression in leukocytes assayed by Lerer *et al.* (2001) may not be relevant to expression in cochlear or skin cells, and may explain this single experimental result supportive of digenic inheritance.

Thus, it is appropriate to identify and test candidate enhancer elements within the deleted intervals of the three deletions that leave *GJB2* intact. Here, I report on the results of luciferase enhancer assays on several conserved elements within the 131.4 kb deletion region. Among the elements assayed, two were found to have enhancer activity, driving expression of the luciferase reporter under the control of an SV40 promoter. Three elements showed silencer activity. Two constructs, differing only by one nucleotide within the 702 bp sequence, yielded allele-specific differences in expression. These two constructs also showed cell-specific differences in expression.

METHODS

Construction of pGL3 luciferase reporter plasmids

Six sets of primers were designed to amplify 700-1200 bp candidate sequence elements (Table 4-1). Candidate sequences were chosen from among a set of human-mouse conserved sequences (at least 100 bp at 68% or greater identity, over a 100bp sliding window) tabled from Vista (http://pipeline.lbl.gov) (Table 4-2). Ideally, all

Construct	Location	Length	F primer	R primer
A	chr13:19883070-19883944	875 bp	CCGCTCGAGCGTCTTTCAGGGGTGTTGTT	CGACGCGTGAAGGGAAGAGGGGAATCTG
B	chr13:19891915-19892616	701 bp	CCGCTCGAGGAATTATGAGTTTGTTCCCTCCA	CGACGCGTGCCTCAGTGAGCCTCTATGC
C	chr13:19898807-19899785	978 bp	CCGCTCGAGCCTTTGAATAAGTTTGCCACAA	CGACGCGTGCAGCCGAGCATTAAAGAGA
D	chr13:19913615-19914792	1178 bp	CCGCTCGAGATAAACAAGGTGCCCTGACG	CGACGCGTGCCCCTGAAAGACAGAGTTG
E	chr13:19934417-19935414	998 bp	CCGCTCGAGAGGGGATGGCAGTACAGATG	CGACGCGTGAGGTGGAGTTTGGTTGAGG
F	chr13:19954092-19954882	791 bp	CCGCTCGAGCTCCAGTTTCCTCGCACATT	CGACGCGTCACAGACTGCTTGCCACAAT

Table 4-1. Candidate enhancer element locations and lengths, and primers used to amplify elements from genomic DNA.

Table 4-2. Human-mouse most-conserved sequences (>100 bp) in the region spanned by the three DFNB1 deletions that leave *GJ2B* intact.

Location	Length, bp	% identity, human-mouse
Conserved elements common to del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854)		
19730166-19730257	100bp	70.00%
19730790-19730905	120bp	71.70%
19737446-19737741	297bp	75.10%
19740981-19741092	114bp	72.80%
19741260-19741361	103bp	71.80%
19742396-19742479	85bp	75.30%
19779910-19780063	158bp	74.10%
19780200-19780292	94bp	75.50%
19828527-19828667	141bp	76.60%
Conserved elements common to all three deletions, intergenic		
19838242-19838339	98bp	70.40%
19855245-19855410	166bp	68.70%
19858147-19858257	111bp	73.90%
19875532-19875648	117bp	69.20%
Conserved elements common to all three deletions, intronic (*CRYL1*)		
19883338-19883590 (included in A)	254bp	81.10%
19886680-19886878	201bp	74.60%
19892138-19892264 (included in B)	127bp	71.70%
19898889-19899008 (included in C)	124bp	69.40%
19899273-19899387 (included in C)	116bp	71.60%
19910910-19911007	98bp	75.50%
19912961-19913076	117bp	70.10%
19914056-19914459 (included in D)	405bp	76.80%
19916684-19916796	114bp	75.40%
19919824-19919988	165bp	70.30%
Conserved elements common to MSU-DF5 deletion and del(*GJB6*-D13S1830), intronic (*CRYL1*)		
19934455-19934749 (included in E)	298bp	73.20%
19935030-19935118 (included in E)	89bp	80.90%
19937216-19937399	189bp	69.80%
19937556-19937663	113bp	70.80%
19941365-19941585	225bp	70.20%
19941620-19941709	97bp	71.10%
19948667-19948762	98bp	71.40%
19954587-19954745 (included in F)	159bp	74.80%
19966012-19966115	105bp	71.40%
19966281-19966370	100bp	70.00%

conserved sequences would have been included in candidate enhancer constructs; however, difficulty with both amplification and cloning of candidate elements redefined this as a pilot project. Primers were designed with XhoI (each forward primer) or MluI (each reverse primer) recognition sequence added to the 5' ends, in order to clone the amplified product, in genomic orientation, into the multiple cloning site of the pGL3-Promoter vector (Promega Corporation). This vector (Figure 4-1) contains the SV40 promoter and modified coding sequence of firefly luciferase, as well as the *E. coli* ampicillin-resistance gene. Inserts were amplified with *Pfu* polymerase with template DNA from MSU-DF5 family members. Amplified DNA was digested overnight with XhoI and MluI, purified and concentrated by QiaQuick (Qiagen), ligated with T4 DNA ligase to XhoI/MluI-digested and QiaQuick-purified pGL3-Promoter at room temperature for 2 hours (NEB), and transformed into competent *E. coli* DH5α cells. Colonies grown on LB agar with ampicillin were screened by whole colony PCR for insert. Correct sequence was confirmed, and plasmid DNA for all transfections was purified by a single Promega Pure-Yield midiprep.

The pGL3-Basic vector lacks the SV promoter, and was used in these experiments as a negative control. The pGL3-Control vector contains an SV40 enhancer element upstream of the SV40 promoter, and was used in these experiments as a positive control. The pRL-TK vector contains the coding sequence of *Renilla* luciferase. Cotransfection of pRL-TK with the pGL3 plasmid allows expression from the pGL3 vector to be corrected for transfection efficiency.

Figure 4-1. Schematic of pGL3-Promoter vector (Promega)

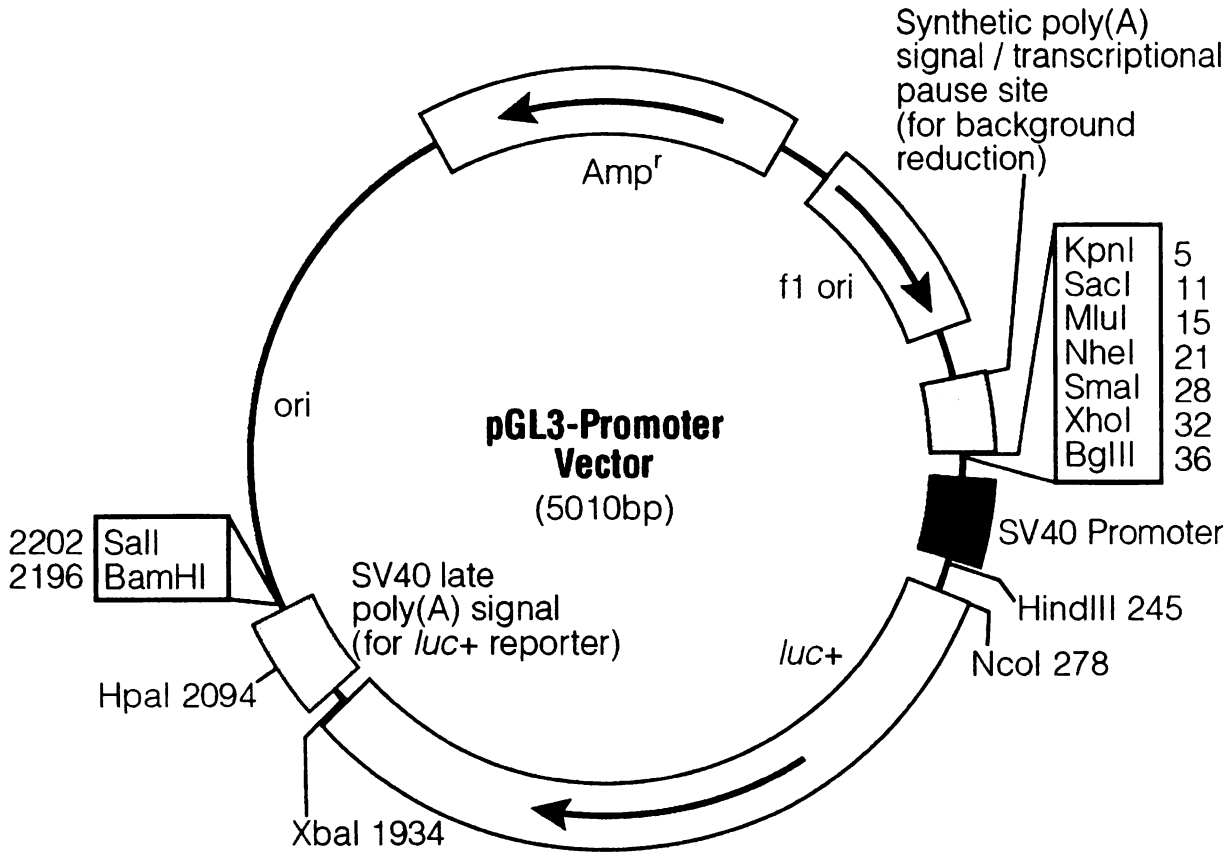
Synthetic poly(A)
signal / transcriptional
pause site
(for background
reduction)
Amp^r
f1 ori
ori
pGL3-Promoter
Vector
(5010bp)
KpnI 5
SacI 11
MluI 15
NheI 21
SmaI 28
XhoI 32
BglII 36
SV40 Promoter
HindIII 245
NcoI 278
luc+
SV40 late
poly(A) signal
(for luc+ reporter)
2202 SalI
2196 BamHI
HpaI 2094
XbaI 1934

Figure 4-1

Cell lines and cell culture conditions

Human vaginal stratified epithelial cell line VK2/E6E7 (ATCC Catalog No. CRL-2616) was obtained from ATCC. Cells were grown in keratinocyte serum-free medium (Gibco-BRL 17005-042) with 0.1 ng/ml human recombinant epidermal growth factor, 0.05 mg/ml bovine pituitary extract, and 44.1 mg/L calcium chloride. HaCaT cells, human skin keratinocytes, were obtained from a neighboring lab. Cells were grown in DMEM (Gibco 11965) with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin at 37°C and 5% CO_2.

Transfections and enhancer assays

Cells were grown to 50-80% confluency in 40 ml medium in 96-well half-area tissue culture plates (Corning 3885). Each transfection mix consisted of 900 ng of the appropriate pGL3 plasmid, 90 ng pRL-TK plasmid, and 40 ul OptiMEM, in a final volume of 50 ul. This yielded a final concentration of 18 ng/ul of pGL3 plasmid. FuGENE6 transfection reagent (Roche) was added in a 6:1 ratio to each transfection mix, and each well transfected with 2.5 ul mix (~50 ng plasmid total). Transfections of all constructs were done at the same time, and all wells contained cells plated from the same cell preparation. Transfections of the pGL3-Basic and pGL3-Control vectors, as well as empty pGL3-Promoter vector were also made at the same time. Cells were incubated for 24 hours, and firefly and *Renilla* luciferase expression assayed by DualGlo luciferase assay system (Promega), according to the manufacturer's protocol. Six to nine replicate wells were transfected with each construct. Background luminescence was assayed from three to six untransfected wells.

The results given and discussed below are based on a single experiment, so do not account for variation introduced by error introduced in pipetting small volumes of plasmid into the transfection mix. The concentrations of plasmid stocks were determined by Nanodrop, and volumes of between 1.6 ul and 4.7 ul pipetted into each transfection mix to yield 18 ng pGL3 plasmid per ul of mix. Following one set of experiments, the same volumes of plasmid stocks were pipetted into water to make a final volume of 50 ul, and 5 ul of this run on a 5% agarose gel. The mass of DNA in each well was assessed on the Storm Imager (GE). Results are calculated and shown using both sets of mass calculations.

RESULTS

Over the genomic interval of our deletion, chr13:19,837,344-19,968,698, there are 24 intergenic or intronic, non-exonic, non-UTR conserved sequences (Table 4-2). Twenty of those sequences are located in *CRYL1* introns; four are intergenic. Fourteen sequences are common to the three DFNB1 deletion alleles that leave *GJB2* sequence intact. Our deletion defines the proximal boundary of the ~94 kb common interval, at chr13:19,837,344, and the del(*GJB6*-D13S1854) deletion defines the distal boundary of the common interval, around chr13:19,935,000 (Figure 4-2). There are nine conserved sequences in the 130 kb between *GJB6* and the proximal boundary of the common deleted interval.

Nine candidate enhancer constructs were made by cloning PCR-amplified sequence into the multiple cloning site of the pGL3-Promoter vector. Two constructs were made for each of three of the six genomic regions investigated. Two identical

Figure 4-2. Locations of candidate enhancer constructs.

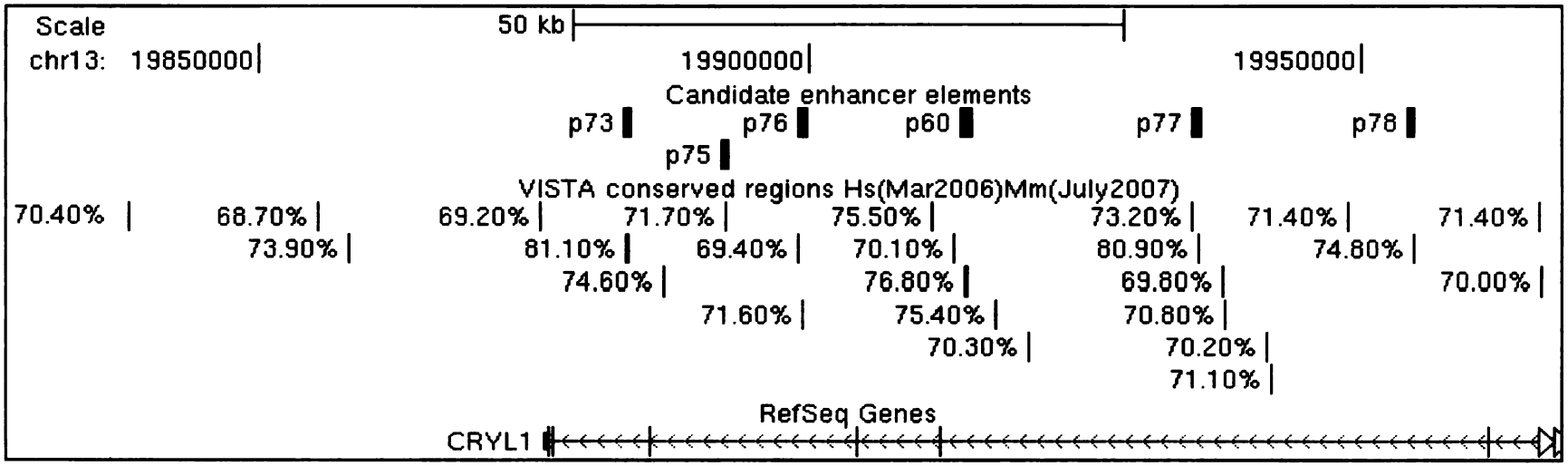
Scale
50 kb
chr13: 19850000
19900000
19950000
Candidate enhancer elements
p73
p76
p60
p77
p78
p75
VISTA conserved regions Hs(Mar2006)Mm(July2007)
70.40%
68.70%
73.90%
69.20%
81.10%
74.60%
71.70%
69.40%
71.60%
75.50%
70.10%
76.80%
75.40%
70.30%
73.20%
80.90%
69.80%
70.80%
70.20%
71.10%
71.40%
74.80%
71.40%
70.00%
RefSeq Genes
CRYL1

Figure 4-2

constructs were cloned separately for regions C and F; these two pairs of constructs (C1 and C2; F1 and F2) serve as technical replicates. The two constructs B1 and B2 are an allelic pair; the 702 bp insert sequence is identical except for the nucleotide at SNP rs7320760. This is a highly polymorphic SNP that lies within the 127 bp human-mouse conserved sequence element. Expression assay results are shown in Figure 4-3, and p values are shown in Table 4-3. Student's t-test was used for all statistical comparisons, and starred comparisons are significant at $p<0.001$. Stars above columns in Figure 4-3 indicate significant difference between the mean expression observed for that construct compared to mean expression from the empty pGL3-Promoter plasmid ("promoter control").

The region D element is 1168 bp in length. This element contains the longest human-mouse conserved sequence in 250 kb of sequence upstream of *GJB6*, 405 bp conserved at 76.8%. This is the only element that contains >100 bp of sequence conserved at >70% in chicken; about 250 bp are conserved above 70%. This element is located in the third intron of *CRYL1*, about 2 kb upstream of intron 4. In both cell lines assayed, this element displayed a repressor effect, with about a five-fold reduction in luciferase expression relative to the promoter-only construct in HaCaT cells, and a two-fold reduction in VK2/E6E7 cells.

The region C construct is 979 bp in length, and its sequence is located in the fifth intron of *CRYL1*. This construct contains about 240 bp of conserved sequence. This element also displayed a repressor effect in both cell lines, a greater than two-fold reduction relative to the promoter-only constructs. Plasmid preps from two separate

Figure 4-3. Expression assay results.

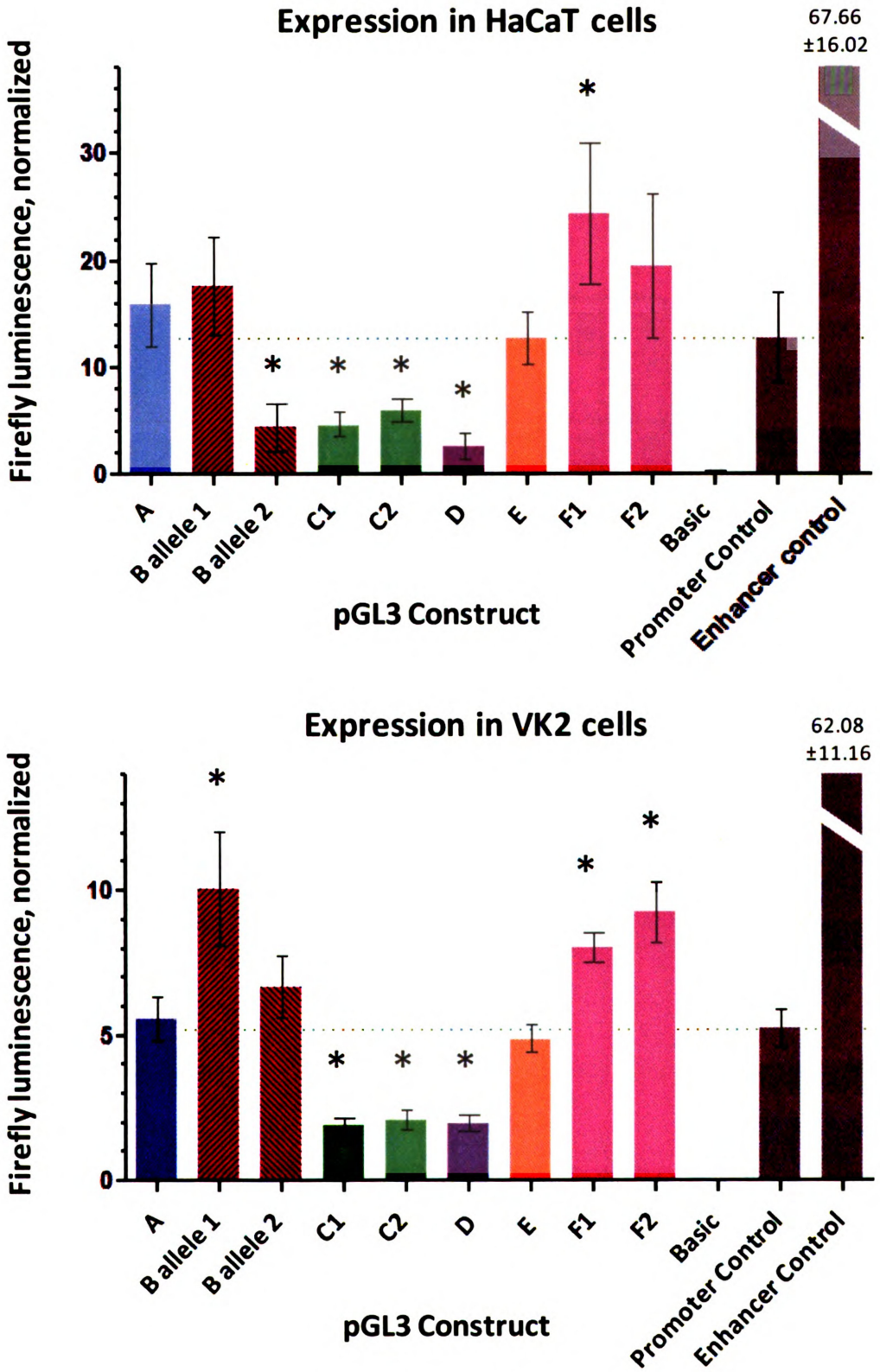
Expression in HaCaT cells
67.66
±16.02
Firefly luminescence, normalized
30
20
10
0
A
B allele 1
B allele 2
C1
C2
D
E
F1
F2
Basic
Promoter Control
Enhancer control
pGL3 Construct
Expression in VK2 cells
62.08
±11.16
Firefly luminescence, normalized
10
5
0
A
B allele 1
B allele 2
C1
C2
D
E
F1
F2
Basic
Promoter Control
Enhancer Control
pGL3 Construct

Figure 4-3

	Expression in HaCaT cells			Expression in VK2/E6E7 cells		
Construct	Average luminescence, normalized	Standard deviation	p value, vs. Promoter	Average luminescence, normalized	Standard deviation	p value, vs. Promoter
A	15.86	3.94	0.5009	5.57	0.77	0.3669
B allele 1	17.62	4.56	0.0371	10.05	1.99	<0.0001
B allele 2	4.42	2.23	0.001	6.67	1.08	<0.0001
C1	4.66	1.17	<0.0001	1.93	0.24	<0.0001
C2	6.00	1.11	0.0001	2.10	0.35	<0.0001
D	2.54	1.23	<0.0001	1.97	0.27	<0.0001
E	12.71	2.46	0.8571	4.88	0.50	0.4821
F1	24.36	6.53	0.0001	8.04	0.50	<0.0001
F2	19.42	6.71	0.0143	9.24	1.07	<0.0001
Basic	0.15	0.21		0.04	0.02	
Promoter control	12.78	4.22		5.23	0.65	
Enhancer control	67.66	16.02		62.08	11.16	

Table 4-3. Average expression levels of tested candidate enhancer constructs, and p-values calculated by Student's t-test, in comparison to the Promoter control.

region C clones were assayed in this experiment, and t-tests indicate that their means are not significantly different from each other. T-tests comparing expression mean of each region C construct against that of the promoter-only construct all yielded highly significant p values. The region C and region D constructs were the only constructs to show repressor activity consistently in both cell lines.

The 791 bp region F construct contains conserved sequence that is located beyond the distal breakpoint of del(*GJB6*-D13S1854), so is deleted in only two of the three DFNB1 deletion alleles. Two region F constructs were cloned, prepped and the sequences of the inserts confirmed to be identical. Both constructs showed enhancer activity. A nearly two-fold increase in luciferase expression was significant for both constructs in VK2 cells. Both constructs also showed enhancer activity in HaCaT cells, although the mean of expression of one construct, at about 1.5 times the level of the promoter-only construct, was only significant at $p<0.03$, which is not a sufficiently small p value for multiple testing.

The most interesting pattern of activity was that of the region B constructs. These two constructs are an allelic pair, differing by a single nucleotide out of the 702 bp sequence. rs7320760 is a transition single nucleotide polymorphism located within the 127 bp of human-mouse conserved sequence. The B1 construct displays the major C allele, which is calculated to have a population allele frequency of 0.6-0.7, based on a set of European, Chinese, and Japanese genotypes. In HaCaT cells, the B1 construct had no effect on luciferase expression. However, the B2 construct had a significant repressor effect, with a 2 to 3-fold reduction relative to the promoter-only construct. Conversely,

in VK2/E6E7 cells, the B1 construct showed enhancer activity, with a 2-fold increase over the promoter-only mean. The B2 construct also showed enhancer activity, significant but perhaps modest; the p-value for one set of calculations (ImageQuant quantified plasmid amount) is 0.0035, above the cutoff for significance for multiple comparisons. In sum, these data indicate a real difference in expression between the two constructs, in each cell type. The data also indicate, for each of these two constructs, a real difference in expression between cell types.

Two constructs, those of regions A and E, demonstrated no effect in either cell line. The conserved sequences represented in these constructs are among the longest and most conserved in the MSU-DF5 deletion interval. The region A conserved sequence, 254 bp conserved at 81.1%, is common to all three deletions, and is located in intron 6 of *CRYL1*. The region E sequence is just telomeric to the distal breakpoint of del(*GJB6*-D13S1854), so is only included in two of the three deletion regions. The region E construct contains two conserved sequences, 298 bp at 73.2% and 89 bp at 80.9%, separated by about 250 bp of intervening sequence.

DISCUSSION

Hearing loss has complex etiology, and much congenital or prelingual hearing loss cannot be diagnosed based on any distinguishable audiological or anatomical characteristic. Determining the genetic or environmental etiology of hearing loss is important to decisions regarding therapies and to the early detection and management of other conditions that may present later than hearing loss in some syndromes.

Mutations in *GJB2* as the cause of hearing loss must be reliably ruled out in order to justify searching for less prevalent mutations in other genes associated with deafness. However, the inheritance and pathogenesis of DFNB1 hearing loss is not completely understood. Many people with hearing loss carry monoallelic mutation of *GJB2* at a significantly higher rate than the population allele frequency of those mutations. Two hypotheses can be made to explain this observation. The first is that unidentified mutations exist in *cis*-regulatory DNA, that some of these *cis*-regulatory elements may exist at a considerable distance from *GJB2* itself, and disruption of one or more general or ear-specific enhancer elements by wholesale deletion or by small deletion or substitution of one or more critical nucleotides results in lost or reduced transcription of *GJB2*. Very little of the regulatory landscape of *GJB2* is known, beyond the proximal and basal promoter regions (Matos, et al., 2007; Tu & Kiang, 1998). The evidence of several large deletions that segregate as DFNB1 mutations now clearly shows that a *cis*-regulatory region exists, at a considerable distance from both *GJB2* and *GJB6*. The alternative hypothesis is that hearing loss due to mutations in *GJB2* may rarely be inherited as a dominant trait with incomplete penetrance. Since the 35delG null mutation is commonly the monoallelic mutation identified in this circumstance, this hypothesis requires that *GJB2* is occasionally haploinsufficient in these heterozygous individuals. This hypothesis is conceivable, given the number and sophistication of molecular interactions involving Cx26 in hearing that are coming to be understood. These two hypotheses are not necessarily mutually exclusive. In fact, allelic variation in expression of *GJB2* resulting from distant functional *cis*-regulatory variants on a non-mutant allele may contribute to this hypothesized occasional haploinsufficiency, in combination with

variation at other loci that encode proteins that interact with Cx26, or that function in the same cell biological pathways.

We have demonstrated sequence-specific and cell-type-specific enhancer and repressor activity in genomic sequences distal to *GJB2* and *GJB6*, utilizing a luciferase reporter system and two epithelial cell lines that are confirmed to express endogenous Cx26 and Cx30. These experiments provide three important findings. First, there are sequences within the interval common to the three DFNB1 deletions leaving *GJB2* intact that are capable of enhancer activity, and whose loss might therefore explain the pathogenicity of these deletions. Second, cross-species conservation is an appropriate criterion to use to identify candidate *cis*-regulatory elements. Four of six 700-1200 bp regions assayed demonstrated statistically significant up- or down-regulation of the luciferase reporter in one or both cell lines. Third, the experimental assay system is able to reproducibly detect enhancer or repressor activity in our hands. The pGL3 family of vectors includes three vectors for control transfections. The pGL3-Control vector includes SV40 enhancer and promoter elements in front of the luciferase coding region. Expression from this vector was on the order of 6-fold (in HaCaT cells) to 14-fold (in VK2/E6E7 cells) that of the promoter-only construct (pGL3-Promoter). Expression from the pGL3-promoter vector was robust compared to that of the pGL3-Basic vector, which lacks both enhancer and promoter sequences. Expression from this vector was always reliably close to zero.

Assaying six to nine technical replicates transfected with each experimental construct (same transfection mix and same cell suspension in each of the six to nine

replicate wells) yielded reasonable standard deviations. T-tests comparing the means of expression of each construct against the mean of expression of the promoter-only construct were generally highly significant ($p<0.0001$). The expression means were sometimes statistically different between duplicate constructs (C1 and C2 are identical constructs that were cloned separately, as are F1 and F2); however, the direction (up- vs. down-regulation) was never different, and significance of effects relative to the promoter-only construct was maintained in three of four comparisons.

This experiment leaves some important questions unanswered, but suggests means of redress. First, of the sequences with identified transcriptional regulatory effect, do any interact with the *GJB2* or *GJB6* promoter in vivo? Our lab has been supplied with the PGL3-Basic vector with the *GJB2* promoter cloned in (gift of Dr. Hela Azaiez and Dr. Richard Smith, University of Iowa), so that these and other candidate regulatory elements can be assessed on their ability to drive expression of the luciferase gene under the control of the *GJB2* promoter. Ultimately, an experimental approach such as 3C (chromosome conformation capture) may be best suited to answer this question. This technique preserves and visualizes interactions that bring regions of chromatin into close contact. First, DNA and proteins are cross-linked in cells expressing the protein of interest, and the DNA is then fragmented by restriction digest and religated; PCR products that result from amplification across ligation junctions represent close interactions between those genomic regions, such as looping and contact via transcription factors between enhancer elements and basal or proximal promoter elements.

Connexins 26 and 30 are each expressed in a number of tissues. In both inner ear and skin, where each of these proteins has been most closely investigated, expression patterns are intricate and very cell-type specific. An important question is whether any sequence shown to have transcriptional regulatory activity drives expression of either protein in any cells of the cochlea itself. As no human cochlear-derived immortalized cell line has yet been developed, this is a question that cannot be answered in cell culture experiments. One approach is that of recombineering of BAC transgenes containing putative regulatory elements, such as that used to localize tissue-specific enhancers of *GDF6* (Mortlock, Guenther, & Kingsley, 2003; Portnoy, et al., 2005).

In summary, these experiments suggest that some elements within the 131.4 kb deletion interval may have regulatory function. We have not yet demonstrated specific, in vivo interactions between these elements and the *GJB2* and *GJB6* promoters.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Mutations in *GJB2* are the most common cause of hearing loss globally. More than 200 recessively-acting hearing-loss mutations of *GJB2* have been identified since *GJB2* was found to be the causative gene in DFNB1 hearing loss in 1997. The discovery of a novel deletion mutation that segregates as a DFNB1 allele in a large Michigan kindred is the major finding of this dissertation. Although this mutation has so far been identified only in this extended family, its characterization strongly supports the existence of a previously-unknown, distant *cis*-regulatory region for *GJB2*, and for *GJB6*. Our enhancer assays of candidate cross-species conserved sequences within the deleted interval show that some non-exonic sequences up- or down-regulate expression of a reporter gene driven by a generic promoter.

Globally, mutations within the DFNB1 locus have not been fully elucidated. In many populations, many persons with hearing loss are found to bear monoallelic mutation of *GJB2*. This phenomenon has two explanations. The first is that unidentified, fully penetrant DFNB1 mutations exist. Since the coding-, splice site-, promoter-, and untranslated-regions of *GJB2* are easily sequenced, this argues for distant, *cis*-regulatory mutations that abrogate the expression of *GJB2*, such as the deletion that we have found in the MSU-DF5 kindred. Although this particular deletion has so far not been identified outside of this kindred, it is important because it narrows the interval within which variation in the DNA of other persons with presumed DFNB1 hearing loss may be evaluated for pathogenicity.

The second explanation is that some pathogenic alleles of *GJB2* may occasionally be dominantly-acting, with reduced penetrance. Penetrance is a measure of the sufficiency of a mutation, at a single locus, to confer a phenotype. Fully-penetrant alleles

confer phenotype in perfect accordance with the rules of single-gene inheritance. So, under the assumption of full penetrance, a DFNB1 allele segregates with hearing loss only when present in homozygosity or in compound heterozygosity with another DFNB1 allele. Most DFNB1 alleles result in loss of function of the connexin 26 protein; as the phenotype is recessively inherited, these alleles are thus usually haplosufficient with respect to the trait of hearing—the protein product that is the translated result of transcription from only one active locus is sufficient to maintain normal hearing. Conceptualize a continuum, with one end corresponding to no expressed Cx26 protein at all and a hearing-loss phenotype. The other end of the continuum is set a bit arbitrarily, but corresponds to a 'usual' amount of Cx26 protein expressed from two active, 'normal' loci, and a normal-hearing phenotype. Somewhere along that continuum— between 50% and 0% of the 'usual' dose of Cx26 protein—will be a point at which the transition between the normal-hearing phenotype and the hearing-loss phenotype occurs. Where does this point exist? For Cx26 and hearing loss, this will depend not only on some minimal, sufficient level of the Cx26-specific biological tasks accomplished by some minimal, sufficient number of Cx26 molecules expressed in the relevant cells in the cochlea, but also on all sorts of other tasks being accomplished by other molecules in those cells that will determine what the minimal, sufficient number of Cx26 molecules must be. One can imagine a cochlea where dysfunction of one (in a digenic model of inheritance), a few (in an oligogenic model of inheritance) or several (in complex disease) other molecules is such that even 50% of the usual dose of Cx26 is not sufficient to accomplish the Cx26-specific tasks. In this manner, a null, hypofunctional, or

hypomorphic allele, which normally confers no hearing-loss phenotype in *trans* with a normal *GJB2* allele, may, on some genetic backgrounds, be haploinsufficient.

Thus, concepts that are applied to describing inheritance of phenotype in human pedigrees, particularly those of penetrance, expressivity, dominance, and recessivity, are perhaps most appropriately thought of, not as properties inherent to any particular allele, but as properties emerging from the interactions between alleles and their genetic/physiological/environmental contexts. Further, the body of literature and the accumulated understanding of the DFNB1 locus—the number of DFNB1 alleles, their nature with respect to the above concepts, the population genetics of DFNB1 hearing loss, the difficulty of the tasks of sorting out pathogenic mutations and benign variants, in particular, the difficulty of interpreting monoallelic mutation segregating with a phenotype that is traditionally understood as recessive—illustrates not only the fluidity of these genetic concepts, but also the interrelationship of the concepts with each other. Thus, penetrance is directly implicated in the assignment of dominance/recessivity. As well, all of this impinges on the traditional distinctions between single-gene and complex disease (Scriver & Waters, 1999). All of the above is, for me, the most important genetic lesson of the DFNB1 locus. With respect to the future directions of this project, this thinking is relevant to the formulation of hypotheses to test in the MSU-DF5 kindred with regard to two phenomena: 1) unexplained congenital and/or prelingual hearing loss in four MSU-DF5 study participants (none of whom segregate any known DFNB1 alleles), and 2) the complex phenotype of age-related hearing loss (presbycusis).

As of the date of this dissertation, there are no MSU-DF5 study participants who have hearing loss and monoallelic mutation of *GJB2*. However, four study participants

have a history of prelingual hearing loss, but no genetic diagnosis at this time. For three of these individuals, we recently sequenced *SLC26A4,* the gene encoding the ion-transporter protein pendrin, mutations of which cause nonsyndromic hearing loss (DFNB4), hearing loss with EVA (enlarged vestibular aqueduct, a finding that can only be discerned radiologically), and Pendred syndrome (hearing loss with thyroid dysfunction and EVA) (Pryor, et al., 2005). Two of our MSU-DF5 study participants were found to harbor the D724G mutation monoallelically. This mutation is thought to be a fully penetrant, recessive mutation causing hearing loss with or without thyroid involvement (Pera, et al., 2008). Monoallelic D724G mutation with hearing loss in these two persons suggests unidentified mutation on each chromosome in *trans*; however, our sequencing efforts have ruled out *SLC26A4* coding region mutation. One MSU-DF5 study participant bears the L597S mutation of *SLC26A4*, also monoallelically. This mutation is associated with some degree of altered protein function *in vitro*, and is found in excess as monoallelic mutation among persons with hearing loss with EVA (Choi, et al., 2009). Although the weight of evidence suggests that this variant is probably not pathogenic, Choi et al. (2009) suggest that it may contribute to hearing loss under some circumstances. Sequencing of *SLC26A4* in the fourth MSU-DF5 subject is not yet complete, but has so far failed to yield any candidate pathogenic mutation. None of the four subjects harbors the 131.4 kb DFNB1 deletion, or the 35delG or any other coding-region mutation of *GJB2*. As in any recessively-inherited phenotype with locus heterogeneity, some people may carry a single mutant allele at an interrogated locus, but display the relevant phenotype due to mutation at a different unknown or uninvestigated locus. Alternatively, the phenotype may have an environmental etiology; this is

sometimes the case in congenital hearing loss, as certain prenatal infections may go unrecognized. In these three cases, further investigation of *SLC26A4* is appropriate, for the following reasons: First, in three of the four subjects, one mutation (or debated variant) of *SLC26A4* has already been identified. Second, two of the four subjects are children with one parent who does not share MSU-DF5 ancestry. Because *GJB2* and *SLC26A4* are the two genes that are by a large margin most commonly responsible for recessively-inherited or spontaneous congenital hearing loss, mutation in one of these two genes should be suspected first.

A more specific lesson of the DFNB1 locus, elucidated in this dissertation, is that some pathogenic alleles may harbor distant *cis*-regulatory mutations. For the four subjects, and any additional subjects with hearing loss and no *GJB2* mutation that may in future be recruited into the MSU-DF5 study, haplotyping of the *SLC26A4* locus may identify a novel DFNB4 allele, shared between some of these subjects, and only in compound heterozygosity with an identified *SLC26A4* mutation in persons who also have hearing loss. This finding would justify looking hard for candidate regulatory mutation, and for working up an expression assay that might yield functional evidence that a regulatory mutation of *SLC26A4* exists on this allele. Cataloguing and evaluation of all variants within a haplotype shared between family members may not be practical; however, array CGH is a means by which potential candidate deletions, at least, may be identified. Chromosomal rearrangements that do not involve copy number variation are more difficult to look for. Paired-end massively-parallel sequencing permits discovery of such rearrangements at the same time that SNP genotyping is accomplished.

Although the existence of additional mutation at the DFNB1 locus is generally accepted, the literature is muddier with respect to *SLC26A4* and the DFNB4 locus, in particular, with respect to the presumed dominance/recessivity of the many missense alleles of *SLC26A4*. It seems that dominance with reduced penetrance may be an assignment of convenience in those situations where a second mutation in *trans* cannot be identified. With respect to MSU-DF5 and monoallelic mutation of *SLC26A4*, it may be important to investigate haplotypes and/or coding-region variants of other cochlear-expressed genes, to assess potential contributions of other candidate loci to digenic or oligogenic inheritance of hearing loss. Evidence exists suggesting digenic interactions between *SLC26A4* and *FOXI1* (Yang, et al., 2007) or *KCNJ10* (Yang, et al., 2009) in some persons with hearing loss and monoallelic mutation of *SLC26A4*. We have largely ruled out this possibility in one of our study subjects, but currently do not have sufficient heterozygous markers typed at these two loci to rule this out in the other two subjects. Expression studies, such as those described for *GJB2* and *GJB6* in this dissertation, are not so easily accomplished for *SLC26A4*, as pendrin is not expressed in tissues that are easily obtained from living subjects. However, pendrin has been found to be expressed in placenta, as well as kidney, thyroid, and cochlea; expression studies in placental tissue may be possible with members of this kindred.

A lesson of the MSU-DF5 kindred is that large-family studies may be particularly useful in guiding investigation into phenotypes with locus- and allelic-heterogeneity. One person with monoallelic mutation of *GJB2* is not remarkable; four family members with monoallelic mutation of *GJB2* is. Elucidation of the shared haplotype on their common 'non-mutant' chromosome justified time- and labor-intensive efforts to search

for a pathogenic variation. Further, had this kindred not also been segregating the 35delG mutation, the deletion allele would never have been associated with a phenotype. To date, we have identified four (perhaps five) hearing-loss alleles segregating in the MSU-DF5 kindred: 35delG and the 131.4 kb deletion for DFNB1/*GJB2*, and L445W and D724G (and L597S) for DFNB4/*SLC26A4*. Of our >200 study participants, more than 40% carry one or more of these five alleles. The potential for identification of significant associations of variation in cochlear-expressed genes with presbycusis and/or noise-induced hearing loss is significant, as is the potential to clarify the status of the L597S allele.

Work with this kindred is moving forward under Drs. Brian Schutte and Debra Schutte of Michigan State University; they seek to expand the number of study participants by more than 10-fold, and to investigate complex disease genetics. This kindred is a population of a size and degree of relatedness that fills an important gap between highly-consanguineous, isolated extended families, that have been particularly valuable for the discovery of mutations in recessive, single-gene disease, and large populations of unrelated persons, that provide cases and controls for association studies, that have yielded some insight into complex disease. Where risk alleles in complex disease become less-penetrant alleles in single-gene disease, study populations such as the MSU-DF5 kindred may be particularly valuable in elucidating pathogenic combinations of alleles of several genes, and in clarifying genotype-phenotype relationships.

This particular community has also been generous with their long-term involvement and dedication to achieving the goals of our research. Our critical

experiments could not have been made without the long-standing, productive working relationships that we have enjoyed with individual participants as well as with community members who have been part of our advisory and ethics committees over the last dozen or so years. We have gone back to participants many times for more cheek swabs, more blood, and more information. We have gained additional participants from among interested friends and relatives who have been put in touch with us by our dedicated committee members. I sincerely thank them for making this dissertation possible, and for making it better.

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