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**Key Words**

Sensorineural progressive  
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DFNA5

Chromosome 7p15

Refinement

Candidate gene CG1

**Introduction**

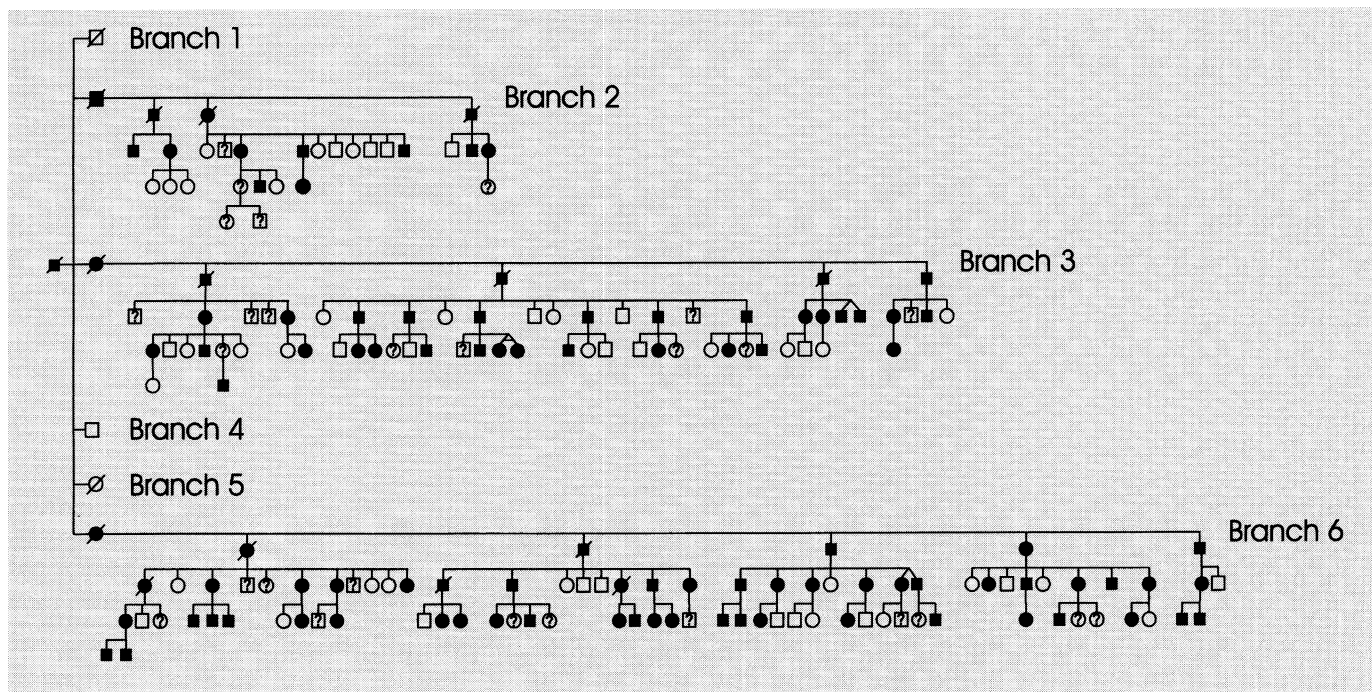
Hearing loss is the most common sensory impairment in developed countries. Approximately 1 child in every 1,000 is born deaf or develops profound prelingual hearing loss in early childhood, and it is estimated that in half of these cases the hearing impairment is caused by genetic factors [1]. Progressive late-onset hearing loss is much more frequent than prelingual deafness. By the age of 65 years, 1 person in 6 has functionally significant hearing impairment, increasing to 1 person in 2 by the age of 80 years [2]. This progressive late-onset hearing loss probably is the result of an interaction of various environmental factors such as infection (otitis), acoustic trauma, ototoxic

# Refined Mapping of a Gene for Autosomal Dominant Progressive Sensorineural Hearing Loss (DFNA5) to a 2-cM Region, and Exclusion of a Candidate Gene That Is Expressed in the Cochlea

**Abstract**

A gene for an autosomal dominant form of progressive sensorineural hearing loss (DFNA5) was previously assigned by us to a 15-cM region on chromosome 7p15. In this study, the DFNA5 candidate region was refined to less than 2 cM, and completely cloned in a YAC contig. The HOXA1 gene located in 7p15 was considered to be a good candidate gene for DFNA5 as it harbours mutations leading to developmental defects of the inner ear in mice. However, the refinement of the candidate region of DFNA5 excludes the HOXA1 gene as a candidate for DFNA5. We cloned a novel candidate gene (CG1, candidate gene 1), which is expressed in human fetal cochlea, from the DFNA5 candidate region. The complete cDNA sequence of CG1, encoding a 423 amino acid protein of unknown function, was determined. Mutation analysis of the CG1 gene in DFNA5 patients, however, could not reveal a disease-causing mutation.

drugs, and presbycusis with predisposing genetic factors [3]. A first requirement to unravel these interactions between genetic and environmental factors is a better understanding how single gene mutations lead to progressive hearing loss [1]. Large pedigrees showing autosomal dominant hearing loss, which is nearly always progressive and postlingual, offer an opportunity to identify these genes by positional cloning, and therefore represent excellent working models to unravel the complex process of progressive late-onset hearing loss. The genes involved in autosomal dominant hearing loss are given a DFNA prefix followed by a consecutive number, in the order of discovery. Recessive deafness genes are given a DFNB prefix in an analogous manner. Up to now, 13 DFNA loci and



**Fig. 1.** Pedigree of the DFNA5 family. Affected individuals are represented by solid symbols. Individuals whose affection status is uncertain are represented with ? in the respective symbols. Spouses were omitted from the figure. Family branches 1, 4 and 5 are excluded from the figure, as these branches contain no additional patients.

17 DFNB loci have been localised [4]. A list of all published loci, including detailed genetic and clinical information, and their respective references can be found online in the Hereditary Hearing loss Homepage (<http://www.dnalab.uia.ac.be/dnalab/hhh>; downloaded in August 1997). Although 13 DFNA loci and 17 DFNB loci have already been found, only a single gene for autosomal dominant and 2 genes for autosomal recessive hereditary hearing loss have been identified. Recently, mutations in the connexin 26 gene were found in dominant as well as in recessive families, identifying connexin 26 as the DFNA3 as well as the DFNB1 gene [5]. Mutations in the myosin VIIA gene were proven to be the cause of DFNB2 recessive non-syndromic deafness [6, 7].

In a previous study we have reported the localisation of the DFNA5 gene in branch 6 of an extended Dutch family to a 15-cM candidate region between D7S493 and D7S632 on chromosome 7p15 [8]. This DFNA5 family has been followed during the past decades, and the DFNA5 hearing loss has been characterised as a progressive hereditary perceptive deafness, starting in the high frequencies at an age between 5 and 15 years [9–14]. In the present study, linkage analysis was performed in

branches 2 and 3 of the DFNA5 family. This enabled us to reduce the DFNA5 candidate interval to a 2-cM region, which was completely cloned in a YAC contig. Furthermore, we identified a novel gene that is expressed in the cochlea from the DFNA5 candidate region. Mutation analysis could not reveal a disease-causing mutation in this DFNA5 candidate gene.

## Materials and Methods

### Clinical Studies

The pedigree of the extended Dutch family linked to DFNA5, and the clinical aspects of the hearing loss have been reported before [8–14]. Briefly, the hearing loss in the Dutch family is non-syndromic and starts at the high frequencies at an age between 5 and 15 years. The hearing loss is progressive and with increasing age also the middle and lower frequencies become affected. The pattern of inheritance is autosomal dominant. For the initial localisation of the DFNA5 gene to chromosome 7p15, 88 members of branch 6 were examined [8]. In this study, 32 members of branch 2, 71 members of branch 3, and 6 additional members of branch 6 were examined (fig. 1). Otologic examination, pure-tone audiometry, and clinical diagnosis were performed as described before [8]. Blood samples for DNA extraction were collected after informed consent.

**Table 1.** Primer sequences to amplify CG1 cDNA

Primer	Sequence	Nucleotide position
CG1-R1	5'-CGAGAAGATGACGTCACAGTAGC-3'	-119 → -97
CG1-R2	5'-CGAACGGTGCAGACTGAAGACG-3'	-46 → -25
CG1-R3	5'-CTGGAGCTTCAACTAACAGGAAGG-3'	254 → 277
CG1-R4	5'-CCAGAGATATCCAATGTCATCCAG-3'	153 → 177
CG1-R5	5'-CCAGAGGAATTGAGGCTTGAATACC-3'	463 → 487
CG1-R6	5'-GAAAAGAACTTCTGGAAGGAATTG-3'	337 → 361
CG1-R7	5'-CTAAATATATCAACTAAAAGTAGCTTGC-3'	583 → 610
CG1-R8	5'-GCTGCAGCCTCTACCAGTTCAG-3'	817 → 838
CG1-R9	5'-CCTTTGGAGGTGGCAGTTCTGTG-3'	1004 → 1026
CG1-R10	5'-CCACTTCTCTGTCAGCCTCAAGC-3'	1109 → 1131
CG1-R11	5'-CGAACATCCCGGTGCTAGG-3'	60 → 78
CG1-S1	5'-CGTGCATAATTTTTTCAGTTCATTTC-3'	1510 → 1486
CG1-S2	5'-CATCTCTGTATGAATCACTCAAAGC-3'	1338 → 1314
CG1-S3	5'-GGTGTGAATAACACATTATCTGTTGC-3'	1166 → 1141
CG1-S4	5'-GGCTCCCAAATCCAAAAGCTGG-3'	878 → 856
CG1-S5	5'-GCAGGTGCTGCTTGATTTACTCC-3'	650 → 628
CG1-S6	5'-GAGAATGTGAGCCCGGACTACC-3'	1057 → 1036
CG1-S7	5'-CTGTAAGTTATTGCTGGTTAAGAAG-3'	516 → 492
CG1-S8	5'-CCCTGATGATTCCCAAACCTCC-3'	393 → 372
CG1-S9	5'-GCAAATGGGTTCTCAGACAATCC-3'	308 → 286
CG1-S10	5'-CCCCATGGTGTGGATTTGGAGA-3'	209 → 188
CG1-S11	5'-CCTGAAGGCTGCTGCTGC-3'	122 → 105

#### Microsatellite Analysis

Polymorphic markers of chromosome 7p were analysed radioactively using standard procedures [15]. PCR consisted of 27 cycles of 1 min denaturation (94°C), 1 min annealing at temperatures ranging from 55 to 60°C depending on the marker, and 1 min extension (72°C), followed by a final 10-min extension step. PCR products were separated on a 6% polyacrylamide gel, and subjected to autoradiography.

#### Linkage Analysis

Two-point lod scores were calculated using the LINKAGE software package version 5.1 [16]. The frequency of hearing loss due to DFNA5 mutations was set at 1/100,000. Penetrance was assumed to be 98%, and the risk of phenocopies was set at 2%. Recombination frequencies were assumed equal for males and females. The number of alleles was set at the observed number of alleles in the pedigree (N), and allele frequencies were set equal at 1/N.

Haplotypes were constructed assuming a minimum number of recombinational events.

#### Construction of a YAC Contig

A YAC contig between D7S2496 (centromeric side) and D7S1838 (telomeric side) spanning part of the DFNA5 linkage interval [17] was expanded to the telomeric side using the same methodology. Basically, 21 polymorphic markers from a 7-cM region between D7S493 and D7S2449 on chromosome 7p15 were selected from the GENETHON genetic map [18], the CHLC genetic map [19], and the WHITEHEAD/MIT database (<http://www-genome.wi.mit.edu>; downloaded in January 1997). One non-polymorphic marker (D7S1429), originating from the National Human Genome Research

Institute (NIH), was included. YACs for the region were selected from the WHITEHEAD/MIT database (YAC contig WC-633 and YAC contig WC7.3, downloaded in January 1997). YAC clones were obtained from the UK HGMP Resource Centre. Thirty-five PCR cycles were performed for all selected polymorphic markers on all YACs.

#### RNA Isolation and Nested RT-PCR

Total RNA was isolated from EBV-transformed cell lines using Trizol (Life Technologies), according to the Manufacturer's Guidelines. cDNA synthesis was performed using the random hexamers provided in the SuperScript Preamplification System following the Instruction Manual (Life Technologies). Resulting cDNAs were directly used as templates in the PCR reactions. A first PCR (30 cycles) was performed using CG1 primer pair S1-R1 (table 1). The resulting PCR product was diluted 50-fold and used in a nested PCR consisting of 25 cycles. The nested PCR was performed using various combinations of CG1-R and CG1-S primers (table 1).

#### Single-Stranded Conformation Polymorphism

Nested RT-PCR was performed in the presence of  $\alpha$ -<sup>32</sup>P-dCTP (ICN; 3,000 Ci/mmol). PCR products were separated on a Hydrolink MDE gel (FMC BioProducts) at room temperature in the presence of 10% (v/v) glycerol.

#### Sequencing

Sequencing reactions, based on the dideoxy sequencing method, were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Fragments were analysed using an ABI-373 Automated Sequencer. Either 500 ng plasmid DNA or 50 ng nested RT-PCR fragments were used in the

sequencing reactions. Plasmid DNA was prepared using the QIAprep Spin Plasmid Miniprep Kit (Qiagen). Nested PCR products were separated on a 2% (w/v) agarose gel, and purified from the gel using the Sephaglas BandPrep Kit (Pharmacia Biotech).

#### *Homology Searches and Computer Analysis*

Homology searches using the BLAST computer program [20] were performed on different computers over the World-Wide Web, either at the Institute for Genomic Research (TIGR; <http://www.tigr.org>), The National Center for Biotechnology Information (GenBank; <http://www.ncbi.nlm.nih.gov>), or at the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp>). Searches for putative functional sites were performed with the PC GENE version (Intelligenetics) of PROSITE [21], and at the PROSITE web site (<http://www.ebi.ac.uk/searches/prosite.html>). TMPred ([http://ulrec3.unil.ch/software/TMPRED\\_form.html](http://ulrec3.unil.ch/software/TMPRED_form.html)) was used for the prediction of the transmembrane helices.

#### *Northern, Southern and Zoo Blots*

A commercially available Northern blot (Clontech) containing RNA of heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was hybridised with a CG1 R2-S2 PCR product (1,384 bp). A Northern blot containing 15 µg of total RNA derived from EBV-transformed cell lines and fibroblasts from patients and controls was prepared and hybridised with the same PCR product. Three Southern blots were prepared using 5 µg of respectively *EcoRI*-, *MspI*- or *TaqI*- digested genomic DNA of 3 affected and 2 unaffected family members, and hybridised with the same PCR product. Two zooblots containing *EcoRI*- and *HindIII*-digested genomic DNA of human, cat, chicken, rat, rabbit, pig, cow and horse were hybridised with the same PCR product.

#### *Analysis of CG1 Polymorphisms*

The 12-bp duplication was investigated by PCR amplification with primers R11 and S11 (table 1). The G1046A substitution, which destroys an *Eco24I* restriction site, was analysed by PCR amplification using primers R9 and S3 (table 1), followed by *Eco24I* (Fermentas) digestion. PCR fragments were analysed on a 12% polyacrylamide gel.

## **Results**

#### *Further Study of the Family*

Only part of the family (branch 6) had been used for the initial localisation of the DFNA5 gene [8]. In the current study, branches 2 and 3, and 6 additional members of branch 6 were collected (fig. 1). In total, 197 DNA samples of the Dutch family including 87 affected patients and 51 unaffected individuals, were available for linkage analysis. The disease status of 27 persons was uncertain. DNA samples of 32 spouses were collected in order to simplify haplotype construction.

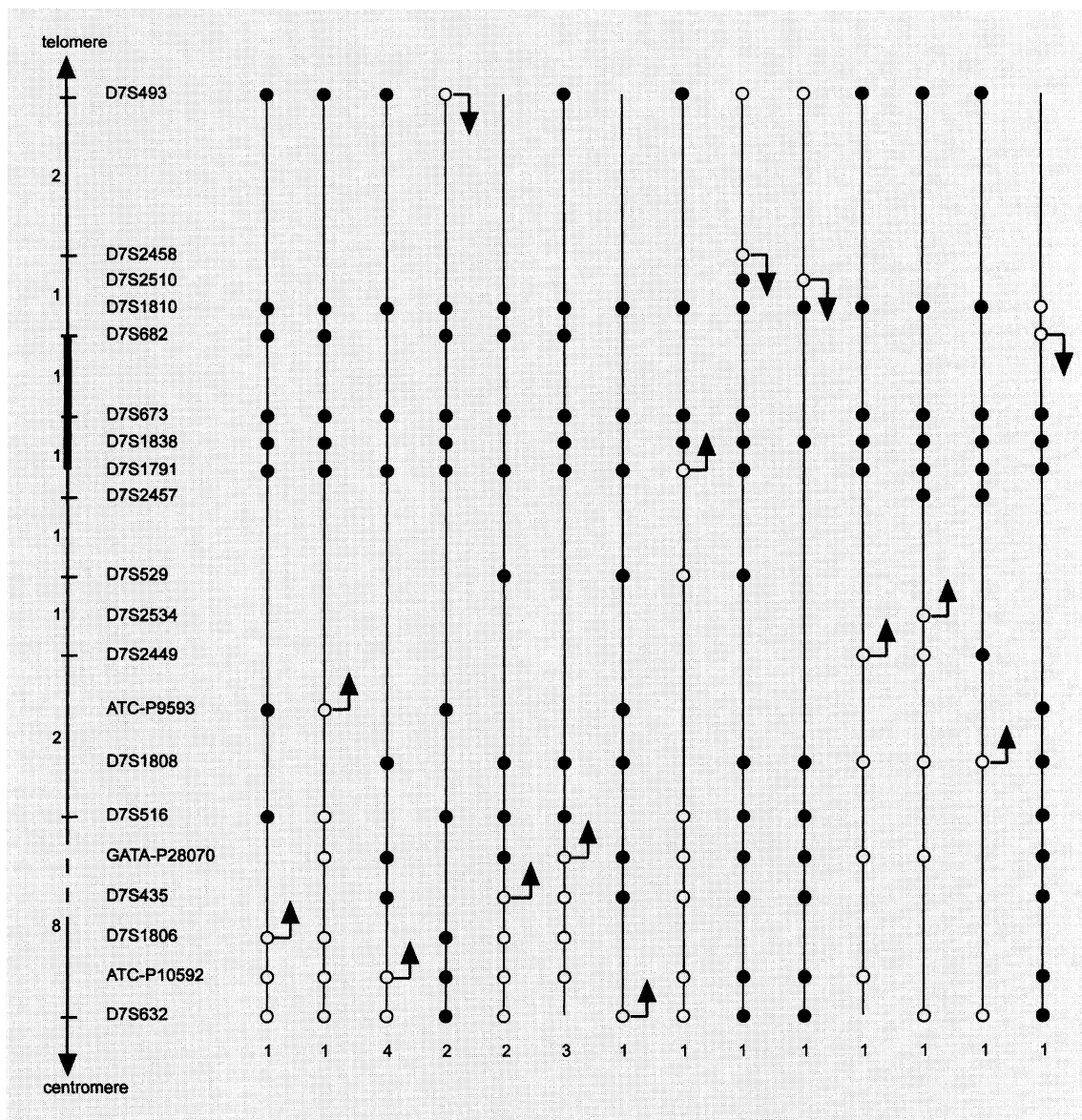
#### *Linkage Analysis and Key Recombinants*

DFNA5 had previously been localised to a 15-cM region on chromosome 7p15 between D7S493 (telomeric

side) and D7S632 (centromeric side) [8]. Two point lod scores for these 2 flanking markers and 8 additional markers (D7S1810, D7S673, D7S1838, D7S1791, D7S529, D7S1808, GATA-P28070, and ATC-P10592) located between D7S493 and D7S632 were calculated for the entire family, resulting in maximum lod scores up to 32. Haplotypes of all family members were constructed using the 10 polymorphic markers, and 21 recombinational events between D7S493 and D7S632 were discovered in family members with a certain affection status. Additional polymorphic markers whose relative position was already known or determined in our YAC contig (see below), were analysed in these 21 recombinants (fig. 2). This panel of recombinants defines a DFNA5 candidate region between D7S682 (telomeric side) and D7S1791 (centromeric side). The exact genetic distance between the 2 flanking markers is unknown, as they were selected from different genetic maps (D7S682 from the GENETHON map, and D7S1791 from the CHLC map). As the genetic distance between D7S682 and D7S2457 (the GENETHON marker which is located near D7S1791) is 2 cM, and D7S1791 was positioned at the telomeric side from D7S2457 in our YAC contig, we assume that the DFNA5 candidate region measures less than 2 cM.

#### *Construction of a YAC Contig*

Previously, a YAC contig from chromosome 7p14-p15 covering the region between D7S1838 (telomeric side) and D7S2496 (centromeric side) has been described [17]. This YAC contig was expanded at the telomeric side (fig. 3), because one of the recombinational events positioned the DFNA5 gene telomeric from D7S1791. Our YAC contig contains four additional polymorphic markers (D7S1795, D7S2458, D7S2463, and D7S2190), as compared to the WHITEHEAD/MIT YAC contigs WC7.3 and WC-633. The relative order of the other genetic markers was identical in both YAC contigs with one exception for the relative position of D7S2510 and GCT16H03. We believe that our order (D7S2510 centromeric from GCT16H03) is correct for two reasons. First, the same relative order of markers was established independently in the National Human Genome Research Institute (NIH) using a slightly different set of YACs. Second, our analysis consists of YACs without internal deletions, whereas the WHITEHEAD/MIT YAC contigs contain several YACs with internal deletions. The relative order of the sets of markers whose position was not determined accurately in the GENETHON genetic map (D7S2458, D7S2510 and D7S629; D7S2463 and D7S673; D7S2444, D7S2493 and D7S2457; D7S2525,



**Fig. 2.** Schematic representation of the chromosomes with recombinations between DFNA5 and closely linked chromosome 7 markers. Informative alleles are indicated by circles: the circles are filled in the region containing the DFNA5 gene, and open in the regions from which DFNA5 is excluded. The number below each chromosome indicates how often each particular recombination was observed. Arrows indicate the position of DFNA5. The DFNA5 candidate region is indicated in bold. Genetic distances are given in centimorgans.

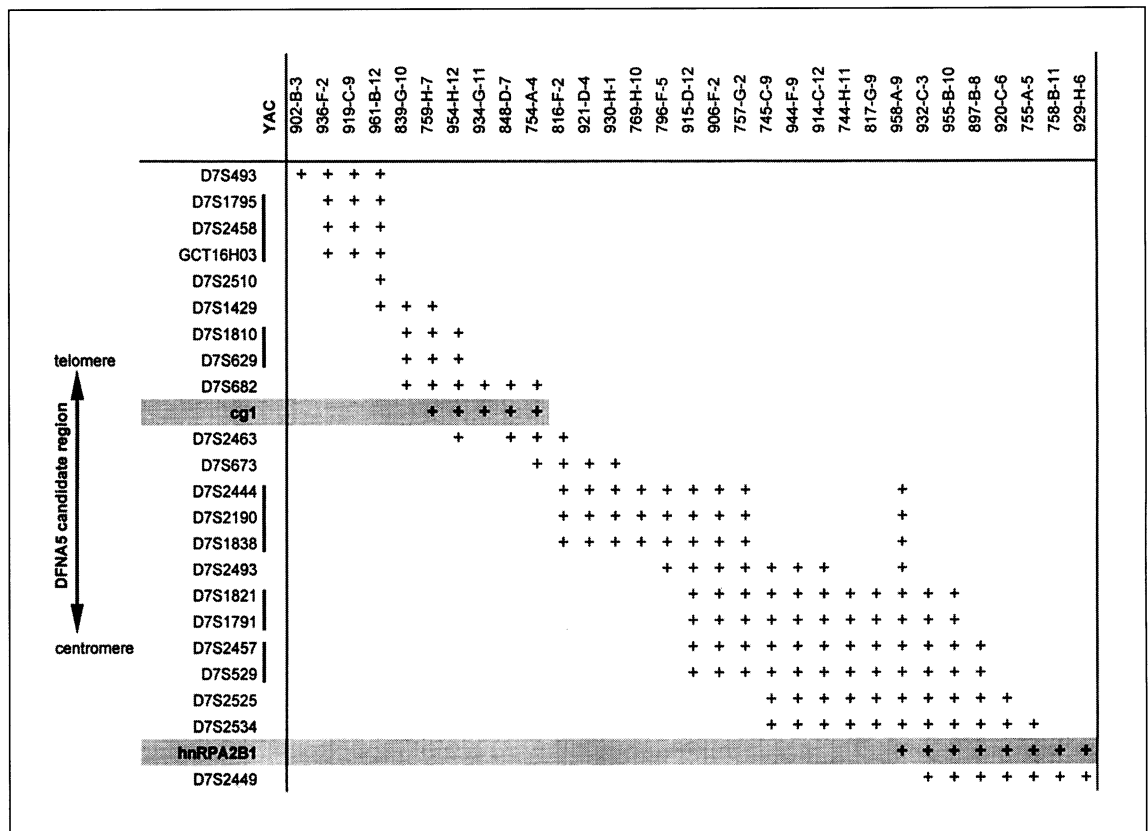
D7S2534 and D7S2449), could be deduced from our YAC contig. In order to close a remaining gap between D7S2510 and D7S1810, one additional non-polymorphic marker (D7S1429) was selected and positioned in our YAC contig.

Two genes known to be located in the initial DFNA5 candidate region could be mapped outside the current

linkage interval. The hnRPA2B1 gene is located between D7S2534 and D7S2449 (fig. 3), and the HOXA1 gene centromeric from D7S2449, as described previously [17].

#### *Full-Length Sequence of the CG1 cDNA*

No DFNA5 candidate genes apart from HOXA1 and hnRPA2B1 are known. We, therefore, searched for ESTs



**Fig. 3.** YAC contig between D7S493 and D7S2449 on chromosome 7p15. The DFNA5 candidate region between D7S682 and D7S1791 is indicated with an arrow. A positive PCR signal for the respective marker on the YAC is indicated with +. Genetic markers whose relative order could not be determined are indicated with a line on the left.

located in the candidate linkage interval in different databases. EST D7S2314 (also reported as WI-6656 in the WHITEHEAD/MIT database, GenBank accession number G05795) was mapped between the polymorphic markers D7S682 and D7S2463 at the National Human Genome Research Institute (NIH). Homology searches were performed at TIGR and Genbank with the 269-bp sequence of EST D7S2314. This resulted in the identification of several cDNA clones whose 3' ends showed very high homology to D7S2314: za27e05, yw33a03, yg04b09, c-0jd01, and c-0ab06. Because one of these clones (yw33a03) was derived from a human fetal cochlear cDNA library [22], the gene was considered to be a good candidate gene for DFNA5, and designated CG1 (candidate gene 1). To obtain more cDNA sequence from the CG1 gene, all the cDNA clones obtained from the UK HGMP Resource Centre were sequenced. The compiled cDNA sequence of CG1 comprises 1,778 bp. The cDNA sequence is available in GenBank (accession number:

U97198). A 5' untranslated region (UTR) of 219 bp precedes a putative initiation codon whose surrounding sequence has homology to the Kozak consensus sequence for 8 out of 10 nucleotides [23]. A CpG island clone of 272 bp (86a8) [24] was found to be homologous to the CG1 cDNA sequence from base -2 to base 123. As CpG islands always cover the transcription start and almost always cover one or more exons [25], this confirms that the ATG codon at position 1-3 is the initiation codon. The 3' UTR has a length of 290 bp, and a polyadenylation signal AATAAA was identified 22 bp upstream of the poly (A) tail. The cDNA sequence contains an open reading frame (ORF) of 1,269 bp, coding for a protein of 423 amino acids, with an estimated molecular weight of 44,871 daltons. PROSITE detected 7 potential glycosylation sites, one tyrosine sulphatation site, and several phosphorylation and myristoylation sites. Whether one of these sites is functional, remains unclear. PC GENE and TMPred predicted the presence of at least 2 transmem-

brane helices in the middle region of the ORF. No secretory signal peptide was detected. No significant homologies with known proteins or functional domains were found when BLAST searches were performed in Genbank, TIGR and DDBJ. A Northern blot (Clontech) was hybridised with a CG1 R2-S2 PCR product, and expression was present in all 8 tissues investigated (results not shown). Zooblots with 2 different restriction enzymes were hybridised with the same PCR product. Only human DNA showed a strong signal. Cat, rat, rabbit and horse showed weaker signals, while chicken, pig and cow showed no signal (results not shown).

### *Mutation Analysis in the CG1 Gene of DFNA5*

#### *Patients*

As the CG1 gene is located in the DFNA5 linkage interval and expressed in the cochlea, it is a good candidate gene for DFNA5. We, therefore, performed mutation analysis of CG1 in patients from the DFNA5 family. Single-stranded conformation polymorphism (SSCP) analysis was performed on 9 nested RT-PCR products covering the complete coding region of the CG1 gene on cDNA of 3 DFNA5 patients from the Dutch family, and 3 controls. Nested PCR was performed using the overlapping primer pairs: R2-S10, R4-S9, R3-S8, R6-S7, R5-S5, R7-S4, R8-S6, R9-S3 and R10-S2 (table 1). No SSCP band shift was observed for any PCR fragment derived from the DFNA5 patients. As SSCP analysis does not detect all mutations, all 9 nested PCR fragments derived from a DFNA5 patient were sequenced. To exclude that mutations would be present in the 5'UTR, an extra hemi-nested PCR using the CG1 primers R1-S10 was performed, and the fragment was sequenced. No mutations were found by sequence analysis. To exclude a deletion of one of the two copies of the gene, Southern blots with 3 different restriction enzymes were analysed, but no differences were detected between lanes derived from affected and unaffected family members (results not shown). Finally, RNA derived from EBV-transformed cell lines and fibroblasts from patients and control persons was analysed on a Northern blot, but no detectable differences in band length or intensity were observed (results not shown).

#### *Polymorphisms in the CG1 Gene*

In the course of the mutation analysis of CG1 in our DFNA5 family, 2 controls were found to have an SSCP band shift, respectively in PCR fragment R2-S10 and in R9-S3. To determine the nature of these SSCP band shifts, the 2 aberrant bands were further investigated. Sequencing of the aberrant S3-R9 PCR fragment revealed

a missense mutation G1046A, leading to a Gly349Asp change on the protein level. This mutation destroys an *Eco24I* restriction site, providing an easy detection method to screen for the mutation. Forty-one controls were investigated by PCR followed by *Eco24I* digestion and 12% polyacrylamide gel analysis. Only 2 heterozygotes with the G1046A mutation were found, and an allele frequency of 2.4% was calculated for the minor allele containing an A at cDNA position 1046.

The R2-S10 nested PCR fragment was also sequenced, and an in-frame duplication of 12 bp, encoding the 4-amino-acid sequence RQQP, was found. Evaluation of the 12-bp duplication on 48 unrelated healthy control individuals was performed on a 12% polyacrylamide gel, using a R11-S11 PCR product. Four controls with the 12-bp duplication were found, and an allele frequency of 4.2% was calculated for the allele containing the 12-bp duplication. To exclude the possibility that there were more alleles with a variable copy number of glutamines such as in a CAG repeat, we analysed all the mutated alleles on a single 6% polyacrylamide sequencing gel. Only 2 alleles, the normal one and the allele with the 12-bp duplication were found (results not shown).

#### *Genetic Mapping of the CG1 Gene*

To confirm the localisation of the CG1 gene on chromosome 7, we performed linkage analysis. As the two CG1 polymorphisms (the 12-bp duplication and G1046A) did not segregate in the DFNA5 family, linkage analysis was performed between the 12-bp duplication polymorphism and a polymorphic marker D7S673 located in the DFNA5 linkage interval, in 35 members of another extended family. A lod score of 3.55 at  $\theta = 0.0$  was obtained, confirming the location of the CG1 gene in the DFNA5 region on chromosome 7.

## **Discussion**

We have previously localised the DFNA5 gene for autosomal dominant non-syndromic hearing loss starting at the high tones to a 15-cM region on chromosome 7p15, between D7S493 and D7S632 [8]. In this study, the DFNA5 candidate region was further refined to a region of less than 2 cM between the flanking markers D7S682 and D7S1791. The new candidate region is defined at each side by a single recombination in a clearly affected patient. As phenocopies for this type of progressive hearing loss are not infrequent [26], the possibility that one of the key recombinants is a phenocopy cannot be excluded.

Therefore, a more conservative estimate of the candidate region, defined by two flanking recombinants at each side, is a 5-cM interval, between D7S2510 and D7S2534.

The HOXA1 and hnRPA2B1 genes have been located in the original 15-cM candidate region of DFNA5 [17]. HOXA1 was considered to be a good candidate gene for DFNA5 because homozygous HOXA1 knockout mice show developmental defects of the inner ear [27]. However, we exclude here the HOXA1 gene, and also the hnRPA2B1 gene as candidate genes for DFNA5, as they both map outside the new candidate region. IL6 was located between D7S1810 and D7S629 at the National Human Genome Research Institute (NIH), which also excludes IL6 as a DFNA5 candidate gene. More genes have been assigned to chromosome 7p15: AHR, PSP, GHRHR, TCRG, RALA, EVX1, INHBA, ACTB, among others. As these genes are not in the close vicinity of the present DFNA5 candidate region according to the CHROMOSOME 7 SUMMARY MAP (<http://cedar.genetics.soton.ac.uk/pub/chrom7/map.html>; downloaded in April 1997), these genes were not considered to be DFNA5 candidate genes. A mouse syntenic region for human chromosome 7p15 does not exist, as the genes listed above have been assigned to various mouse chromosomes.

A novel gene designated CG1 (for candidate gene 1) was identified from the candidate region between D7S682 and D7S2463. Because the CG1 gene is expressed in fetal cochlea, it is considered to be a DFNA5 candidate gene. As analysis of the CG1 gene in DFNA5 patients could not reveal a mutation, it is not very likely that CG1 is the DFNA5 gene. The physiological function of the CG1 gene remains unknown.

During the mutation analysis of the CG1 gene, two polymorphisms with low frequency were found in the CG1 coding region: a point mutation leading to a substitution of a glycine residue (amino acid 349) for an aspartic acid, and an in-frame 12-bp duplication. The Gly349Asp substitution is a change of a small, non-polar and non-charged glycine residue for a larger, polar and charged aspartic acid residue. However, in the Dayhoff scoring matrix [28] a glycine to aspartic acid change has a (+1) score, indicating that this substitution is rather conservative. This mutation which has a frequency of 2.4% in the general population, therefore, most likely represents a polymorphism.

The effect of the 4 amino acid (ArgGlnGlnPro) duplication, resulting from the 12-bp insertion, on the CG1 protein is unclear. None of the putative functional sites found in the CG1 gene is disrupted by the duplication.

The fact that a proline residue is involved, which usually has a role in maintaining the three-dimensional structure, suggests that this duplication may lead to a CG1 variant with altered secondary or tertiary structure. We only found one other example of a polymorphic in-frame insertion of one or more amino acids in other genes. A 12-bp in-frame insertion leading to a duplication of a 4-amino-acid stretch GluGlnGlnGln was reported in human apolipoprotein A-IV (APOA-IV), but the possible physiological implications of this APOA polymorphism are not clear yet [29]. A stretch of 8 nucleotides (from nucleotide 85 to 92: GGAGGAGG), resembling the human variable tandem repeat (VTR) consensus element (GC[A/T]GG[A/T]GG) [30] for 7 out of 8 nucleotides, precedes the duplicated region. The VTR consensus element in turn shows high homology to the Chi element, which constitutes a generalised recombination signal in *Escherichia coli* [31], and which is reported to be the core element in human minisatellites [32]. The presence of this Chi-like element in the immediate vicinity of the 12-bp duplication might therefore explain the origin of this duplication. The possible functional importance of this duplication is unclear, but it is unrelated to the hearing loss.

It is not very likely that CG1 is the DFNA5 gene, but the refinement of the DFNA5 candidate region to less than 2 cM will facilitate the isolation of the gene in the future.

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