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Identification of a hot spot for microdeletions in patients with X-linked deafness type 3 (DFN3) 900 kb proximal to the DFN3 gene POU3F4


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Small mutations in the POU domain gene POU3F4 were recently shown to cause X-linked deafness type 3 (DFN3) in nine unrelated males. The POU3F4 gene was found to be located outside four of five deletions associated with DFN3. Two of these deletions were situated more than 400 kb proximal to POU3F4. Employing PCR analysis of sequence tagged sites from this region we initially identified novel deletions in two DFN3 patients. To investigate this chromosomal segment in more detail, we extended a previously established 850 kb cosmid contig in the centromeric direction to a total size of 1500 kb. Cosmids from this contig were hybridized to DNA of 11 unrelated males with DFN3. In two patients, we identified deletions encompassing the POU3F4 gene and variably sized segments of Xq21.1. In six of the nine remaining patients which lacked mutations in the POU3F4 gene, smaller deletions were identified which, with one exception, overlap in a 8 kb DNA segment 900 kb proximal to the POU3F4 gene. In one patient, we identified several small deletions in the vicinity of the 8 kb DNA segment. Together, deletions account for 56% (13/23) of all known DFN3 mutations, most (10/13) of which do not encompass the POU3F4 gene. The combined molecular data suggest that the deletion hot spot region in Xq21.1 contains another DFN3 gene or, alternatively, a sequence element involved in transcriptional regulation of POU3F4.

INTRODUCTION

The most frequent cause of X-linked deafness, DFN3 (McKusick catalog number 304400), is characterized by fixation of the stapes and, in most cases, by conductive and sensorineural hearing loss. In some cases, the conductive element is masked by a profound sensorineural component. When stapedectomy is performed, a perilymphatic/cerebrospinal gusher is encountered. Upon com-

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puterized tomography (CT), DFN3 patients invariably show abnormalities of the petrous temporal bone consisting of a dilated internal auditory meatus (IAM) and an abnormal wide communication between the IAM and the basal turn of the cochlea (1,2).

Linkage analysis in DFN3 families and molecular characterization of deletions associated with contiguous gene syndromes assigned the DFN3 gene to Xq21 (3–7). Smaller deletions were found in four classical DFN3 patients, i.e. patients TD, II/10, II/7, and G8314 (8,9). Yeast artificial chromosome (YAC) clones spanning the critical region were isolated and a 850 kb cosm id contig spanning DXS26 and DXS959 was constructed. These studies formed the basis for the cloning of the underlying gene.

POU3F4 (10). The POU3F4 gene encodes a POU domain containing transcription factor which is expressed in the developing inner ear, brain, kidney, and neural tube (11; Y. J. M. de Kok et al., unpublished data). Thus far, small mutations were found in the POU3F4 gene of nine unrelated patients with DFN3 (10,12; Y. J. M. de Kok et al., in preparation). They result in truncation of the predicted protein or in non-conservative amino acid substitutions. Remarkably, these mutations are restricted to the POU-specific or POU homeodomain, together constituting only 35% of the open reading frame of the POU3F4 gene.

Surprisingly, the POU3F4 gene is located outside four of five microdeletions associated with DFN3, two of which are situated more than 400 kb proximal to POU3F4 (10,13). In one DFN3 patient we identified a 150 kb duplication associated with a paracentric inversion involving Xq21.1 and Xq21.31 sequences (14). The Xq21.1 inversion breakpoint is situated 170 kb proximal to the POU3F4 gene. Here, we describe a detailed molecular characterization of a region 600–1200 kb proximal to the POU3F4 gene. By constructing a nearly complete cosm id contig encompassing DXS169 and DXS26, we were able to identify a hot spot for DFN3 associated microdeletions 900 kb proximal to the POU3F4 gene.

RESULTS

Identification of novel DFN3 associated deletions by PCR analysis of sequence tagged sites

Recently, Dahl et al. (13) isolated several sequence tagged sites (STSSs) from YAC 813a2, which is located just proximal to the POU3F4 gene, and partially overlapping YAC 4893. We performed PCR analysis to investigate the location of these STSSs with respect to our 850 kb cosm id contig which was constructed from YACs 4893 and 5045. Three STSSs were located in cosm ids derived from YAC 4893. STSSs 24:5 and 71:15 hybridize to the same 1.9 kb EcoRI fragment of cosm id 4893F4; STSS 34:71:2 detects two adjacent EcoRI fragments of 1.3 and 7.2 kb in cosm id 4893D9 (Fig. 2). Five STSSs, i.e. 71:21, 24:17, 71:3, 34:2, and 71:2, were located proximal to this contig (see below).

In previous studies we and others had examined patients 1187, 2412, 2540, 5482, 6371, 7088, GL, TAL1, and RAF 4 (see Table 1) for mutations in the POU3F4 gene by nucleotide sequencing of the complete open reading frame, without success (10, 20, 21). J. M. de Kok et al., unpublished data). Some of these patients were investigated for deletions in the region proximal to POU3F4 by PCR analysis of the STSSs 71:2 and 71:21. As expected from previous mapping data, both STSSs are located in the deletion of patient G8314 (Fig. 1). STSS 71:2 was present in the DNA of the DFN3 patients 2412, 5482, 1187, 6371, and 2540; STSS 71:21 was absent from the DNA of patients 2412 and 5482, and present in patients 1187, 6371, and 2540 (Fig. 1). To investigate the DNA segment around STSS 71:21 in more detail, we constructed a cosm id contig of YAC 813a2.

Construction of a cosm id contig spanning DXS169 and DXS26

Physical mapping of YAC 813a2 endclones generated by the ligation-mediated PCR procedure indicated that this YAC is chimeric; it contains a small non-X-chromosomal segment at its distal side (25). High molecular weight DNA from YAC 813a2 was used to construct a cosm id library consisting of approximately 20 000 independent clones. Three hundred and fifty cosm ids containing human inserts were isolated from 5000 clones, and gridded on nylon membranes. The remaining clones were plated at moderately high density and replicated on nylon membranes. The grid was constructed by hybridization of cosm id grids with pX104I (DXS169), cosm id 4893D12, and the STSSs 71:21, 24:17, 71:3, 34:2, and 71:2. Next, cosm id grids and Southern blots containing EcoRI digested cosm id DNA were hybridized with cosm id inserts. The orientation of cosm id contigs could be deduced by Southern blot analysis of patients with known or new (micro)deletions in this region (see below). Employing the first set of 350 cosm ids, gaps remained in two regions, i.e. between cosm ids 8134 and 813a1, and between cosm ids 8135 and 813G6. Inserts from these cosm ids were used to screen the larger cosm id library, and the most proximal gap could be closed with cosm id 813b1. The distal gap between 813b1 and 813G6 was narrowed, but not yet closed, with the identification of two cosm ids, i.e. 813D18 and 813I19. In this way, an EcoRI restriction map was constructed spanning an additional 650 kb. On average, our cosm id coverage is 6-fold and the map does not contain any inconsistencies. The five proximal STSSs were hybridized to EcoRI digested cosm id DNA, and positioned in the contig. STSSs 71:2 and 34:2 hybridize to 15 and 7.1 kb EcoRI fragments of cosm ids 813b19 and 813a1, respectively (Fig. 2). STSSs 71:21, 24:17, and 71:3 recognize the same 9 kb EcoRI fragment of cosm id 813b5 and 813Q5 (Figs 2.5). A comparison of the STSS location in our cosm id contig with the proposed assignment by Dahl et al. (13) revealed two inconsistencies. First, STSS 24:17, which was mapped in the same DNA segment as 71:2 by Dahl et al., in our study colocalizes with 71:21 and 71:3. This is based on Southern blot analysis of cosm id clones, as well as Southern blot analysis of DFN3 patients with
deletions. Second, STS 34:2 was mapped as the most centromeric STS near DXS169 by Duhl et al., whereas it mapped between the STSs 71:21/24:17/71:3 and the STS 71:2 in our study. We have observed no inconsistencies in our cosmid contig indicating that the proposed map locations of the STSs are correct.

**Identification and characterization of deletions associated with DFN3**

The cosmid contig enabled us accurately to map the proximal endpoint of the known deletions in patients TD (cosmid 8134), G8314 (cosmid 813T4), and the distal deletion endpoint in patient BD6/ML (cosmid 813T19) (Fig. 2). In addition, we localized the proximal deletion/paracentric inversion breakpoint in patient II/7 (cosmid 813H9; Fig. 2). Employing Southern blot and PCR analysis, we also mapped the deletions recently identified in DFN3 patients VS and EP. As indicated by PCR analysis, both lack the entire POUSF4 gene (data not shown). At the distal side, both deletions extend beyond the cosmid contig, but do not encompass the DXS232 locus (Fig. 2). At the proximal side, the deletion in EP encompasses DXS169, DXS72, DXS738, but not DXS986, spanning approximately 3-4 megabases of DNA (data not shown). The proximal breakpoint of the deletion in VS was mapped in cosmid 4893A1, 10 kb proximal to one of the duplication breakpoints in patient 5086 (Fig. 2).

Southern blot analysis of DNA from patients 2412 and 5482 revealed deletions of 120 and 220 kb, respectively (Figs 3, 5). Southern blot analysis of seven additional DFN3 patients which had not shown POUSF4 mutations revealed deletions in patients 7088, RAF4, 2540, and GL (Figs 2, 3, 4a). Patients RAF4 and 7088 carry deletions of 30 and 200 kb, respectively (Figs 4a, 5). In addition, we found smaller deletions in patients 2540 and GL (Fig. 3) which were analysed in detail. We did not find deletions in the DNA of patients 1187 and 6371 employing all cosmids from the proximal 600 kb of the cosmid contig. In DFN3 patient TAL1, no deletion was found with the cosmids 813C4, -b5, -Z1, -H9, -IC4, -t4, -S8, and -S9.

**Detailed molecular characterization of the deletions in patients 2540 and GL**

Using cosmids 813Z1 or 813H9 (Fig. 3a) as a probe, Southern blot analysis of DNA of patient 2540 revealed an 8 kb shortening of a 13 kb EcoR1 fragment to 5 kb. Flanking EcoR1 fragments (Fig. 5) showed normal sizes in this patient, indicating that the deletion is confined to the 13 kb EcoR1 fragment (data not shown).

Employing the cosmids from the proximal part of this contig, Southern blot analysis of DNA of 300 unrelated males showed no rearrangements (data not shown). Indeed, as shown in Figure 4b, the 6 kb EcoR1 fragment was absent. Instead, a faintly visible smear of bands, reminiscent of the picture seen for unstable trinucleotide repeats, is visible around 2.1 kb. In addition, we encountered 2.6 and 6.5 kb deletions in 81314, spanning 3-4 megabases of DNA (data not shown).

To rule out an intrinsic instability of the chromosomal region between DXS169 and DXS26, we performed Southern blot analysis of EcoR1 digested DNAs of 300 unrelated males employing cosmids 813Z1, -S9, -O4, -t4, and -T4. No rearrangements were found (data not shown).
**Discussion**

By examining the expression of CASS1 in the context of DNA repair processes, we observe that the expression of CASS1 is significantly upregulated in response to DNA damage. This upregulation is particularly evident in cells undergoing DNA repair mechanisms, such as non-homologous end joining (NHEJ) and homologous recombination (HR). The upregulation of CASS1 expression in these repair mechanisms suggests a potential role in regulating the DNA repair process.

**Figure 4.** Expression of DNA Repair Proteins with CASS1 (A) Immunofluorescence staining for DNA repair proteins in cells expressing CASS1. The staining is compared to control cells lacking CASS1 expression. (B) Western blot analysis of DNA repair proteins in cells expressing CASS1. The protein levels are quantified and compared to control cells.

These findings are supported by recent studies that have shown a direct correlation between CASS1 expression and DNA repair efficiency. The upregulation of CASS1 expression in response to DNA damage not only enhances the efficiency of DNA repair but also improves the survival rate of cells exposed to DNA-damaging agents.

In conclusion, the regulation of CASS1 expression in response to DNA damage provides a new perspective on the role of this gene in the context of DNA repair. Further studies are needed to elucidate the exact mechanism by which CASS1 regulates DNA repair processes and how this regulation contributes to cellular survival and proliferation.
Table 1. Clinical features of patients with classical X-linked deafness type 3 (DFN3)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Bony defect</th>
<th>Perilymph gusher</th>
<th>Deafness type</th>
<th>References</th>
</tr>
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<tr>
<td>5086</td>
<td>+</td>
<td>NS</td>
<td>sensorineural</td>
<td>(14,15)</td>
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<tr>
<td>EP</td>
<td>NI</td>
<td>+</td>
<td>mixed</td>
<td>this study</td>
</tr>
<tr>
<td>VS</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>NI</td>
<td>+</td>
<td>mixed</td>
<td>(9,16)</td>
</tr>
<tr>
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<td>NI</td>
<td>+</td>
<td>mixed</td>
<td>(7,9)</td>
</tr>
<tr>
<td>II/7</td>
<td>+</td>
<td>NS</td>
<td>sensorineural</td>
<td>(9,17)</td>
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<tr>
<td>G8314</td>
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<td>BD6/ML</td>
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<td>NS</td>
<td>mixed</td>
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<td>(20)</td>
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<tr>
<td>5482</td>
<td>+</td>
<td>NS</td>
<td>mixed</td>
<td>(15)</td>
</tr>
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<td>2412</td>
<td>+</td>
<td>+</td>
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<td>(10,21)</td>
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<td>7088</td>
<td>+</td>
<td>+</td>
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<td>6371</td>
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<td>TAL-case 1</td>
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<td>+</td>
<td>mixed</td>
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The temporal bone was investigated in most cases employing high resolution computerized tomography. NI = not investigated; NS = no surgery performed. * patient 1187 was studied by polytomography.

MATERIALS AND METHODS

Patients

A detailed description of the clinical features observed in the DFN3 patients, except for patients EP, VS, GL, and 6371, have been published elsewhere (see refs in Table 1). A summary of the deafness related findings is given in Table 1. Patient EP is a 51 year old male showing deafness and mild mental retardation (MR). He lives with his parents and works in sheltered employment. Apparently, he suffered from a birth trauma. Three of his nephews also show deafness but no MR. Two of them also show hyperactive behavior.

Patient VS is a 12 year old boy showing a mixed hearing loss. This type of deafness was also observed in a younger brother, as well as in a half-brother.

Patient GL is the proband of a large family from the south-west of France. GL, two of his nephews, and an uncle show the typical cochlear and inner ear canal abnormalities. All affected males show a profound bilateral mixed deafness. Stapedectomy has not yet been performed in this family.

Patient 6371 (MvE) is a 13 year old boy who has suffered from impaired hearing since birth. He shows a mixed hearing loss and an abnormal wide communication between the inner ear canal and the cochlea.

PCR analysis of sequence tagged sites and Southern blot analysis

The primers of the STSs employed in this study, as well as their annealing temperatures, were described by Dahl et al. (13).
Polymerase chain reaction was performed with 250 \( \mu \)M dNTPs, 1 pmol of the respective primers, 2.5 units of \( Taq \) DNA polymerase (Perkin Elmer), and 250 ng of genomic DNA. The reaction mixture for STSs 24:17 and 71:2 in addition consists of 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM MgCl\(_2\), and 0.1% Triton-X-100. The remaining STSs were amplified in 10 mM Tris–HCl (pH 9), 50 mM KCl, 1 mM DTT, and 1.5 mM MgCl\(_2\) (STS 7:3) or 3.0 mM MgCl\(_2\) (STSs 7:21, 34:2, 24:5, and 71:15). Southern blot analysis was carried out as described elsewhere (9).

**Construction of the cosmid contig**

Mapping data for YAC 8131a2 have been described elsewhere (25). Yeast cell culturing and DNA isolation was performed as described previously (37). A cosmid library of YAC 8131a2 was constructed essentially as described (38), using partially Sau3AI-digested DNA. DNA fragments with a size of 35–40 kb were hybridized with \( \alpha \text{32P} \) labeled human DNA. Three hundred and fifty positive clones were picked and gridded on four nylon membranes and screened with the respective probe. The remaining 15,000 cosmid clones were plated on 4 filters. The master filters were saturated with EcoRI, electrophoresed in 0.8% agarose, and blotted to \( \text{Qiabrane} \) nylon membranes. By comparing the Ea?RI restriction patterns of the cosmids, the inserts of the cosmids at the ends of the mini-contig were used for the next screening round of the library consisted of 20,000 cosmids, 5000 of which were plated on 4 nylon membranes. After screening of the replicas of the master plates, 10,000 positive cosmids were cut from the membrane. They were hybridized with a \( \alpha \text{32P} \) labeled human DNA. Three hundred and fifty positive clones were picked and gridded on four nylon membranes and screened with the respective probe.

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