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-
puterized tomodography (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, 1/10, 11/7, and G8314 (8,9). Yeast artificial chromosome (YAC) clones spanning the critical region were isolated and a 850 kb cosmid contig spanning DXS26 and DXS995 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 cosmid 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 (STSs) 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 STSS with respect to our 850 kb cosmid contig which was constructed from YACs 4893 and 5045. Three STSS were located in cosmids derived from YAC 4893. STSS 24:5 and 71:15 hybridize to the same 1.9 kb EcoRI fragment of cosmid 4893F4; STSS 34:71:2 detects two adjacent EcoRI fragments of 1.3 and 7.2 kb in cosmid 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; Y. 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 STSS 71:2 and 71:21. As expected from previous mapping data both STSS 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 cosmid contig of YAC 813a2.

Construction of a cosmid 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 cosmid library consisting of approximately 20 000 independent clones. Three hundred and fifty cosmids containing human inserts were isolated from 5000 clones, and grided on nylon membranes. The remaining clones were plated at moderately high density and replicated on nylon membranes. Initially, small cosmid contigs were established by hybridization of cosmid grids with pX104f (DXS169), cosmid 4893D12, and the STSSs 71:21, 24:17, 71:3, 34:2, and 71:2. Next, cosmid grids and Southern blots containing EcoRI digested cosmid DNAs were hybridized with cosmid inserts. The orientation of cosmid 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 cosmids, gaps remained in two regions, i.e. between cosmids 813F4 and 813A1, and between cosmids 813S9 and 813G6. Inserts from these cosmids were used to screen the larger cosmid library, and the most proximal gap could be closed with cosmid 813I13. The distal gap between 813S9 and 813G6 was narrowed, but not yet closed, with the identification of two cosmids, i.e. 813D18 and 813I19. In this way, an EcoRI restriction map was constructed spanning an additional 650 kb. On average, our cosmid coverage is 6-fold and the map does not contain any inconsistencies. The five proximal STSS were hybridized to EcoRI digested cosmid DNAs, and positioned in the contig. STSS 71:2 and 34:2 hybridize to 15 and 7.1 kb EcoRI fragments of cosmids 813I19 and 813A1, respectively (Fig. 2). STSS 71:21, 24:17, and 71:3 recognize the same 9 kb EcoRI fragment of cosmids 813b5 and 813Q5 (Figs 2, 5). A comparison of the STSS location in our cosmid 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 cosmid clones, as well as Southern blot analysis of DFN3 patients with...
The cosmid contig enabled us accurately to map the proximal deletion/paracentric inversion breakpoint in patient II/7 (Fig. 3) which were analyzed in detail. We did not find deletions in the DNA of patients 1187 and 6371 employing all cosmids of 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.

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 POU3F4 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 cosmids 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 POU3F4 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 analyzed in detail. We did not find deletions in the DNA of patients 1187 and 6371 employing all cosmids of 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 EcoRI fragment to 5 kb. Flanking EcoRI fragments (Fig. 5) showed normal sizes in this patient, indicating that the deletion is confined to the 13 kb EcoRI fragment (data not shown).

Employing the cosmids from the proximal part of this contig, Southern blot analysis of the DNA of patients 2540 and GL revealed deletions at different positions. Cosmid 813C4 identified a deletion which apparently encompasses a 6 kb EcoRI fragment (Fig. 3b). To confirm this finding, the 6 kb EcoRI fragment was hybridized to the same Southern blot as shown in Figure 3a,b. Indeed, as shown in Figure 4b, the 6 kb EcoRI fragment is 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 813T4, a 2.7 kb deletion in 813T13, a 6.5 kb deletion in 813H9 (Fig. 3a), a 7 kb deletion of the neighboring 4.1 and 3.0 kb EcoRI fragments in 813Z1, and a 4.4 kb deletion in 813T4 (Figs. 2, 5).

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

Figure 2. A 1500 kb cosmid and deletion map of Xq21.1. The extent of the YACs is given with solid bars; non-X-chromosomal YAC segments are indicated with stippled lines. The cosmids given in the right part (4893D12 to 5045H5) have been described previously (9). The cosmids at the left side (813C3 to 813Q1) were subcloned in this study from YAC 813a2. Cosmid IC4 (Gil 185) was isolated from the ICRFX-chromosomal cosmid library. All structural abnormalities depicted here are associated with classical DFN3; only patient TD in addition shows hypogonadism. Stippled lines represent deletions.
Pol and pol mutations were determined by DNA sequencing in the Pol region and are included in the text (

In conclusion, the Pol region is a critical component in the transcription of the Pol gene and is involved in the regulation of the Pol gene expression. The results of this study indicate that mutations in the Pol region can lead to an increased expression of the Pol gene.

The significance of these findings is that they provide a basis for understanding the role of the Pol gene in transcription and its potential involvement in disease processes. Further research is needed to clarify the mechanisms underlying these effects and to explore potential therapeutic interventions.
Figure 5. Detailed EcoRI restriction and deletion map of the proximal region. On top, the location of cosmids is depicted. Stippled lines represent deletions. Interrupted lines represent DNA segments in patients that have not yet been tested employing Southern blot analysis.

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>
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<td>5086</td>
<td>+</td>
<td>NS</td>
<td>sensorineural</td>
<td>(14,15)</td>
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<td>EP</td>
<td>NI</td>
<td>+</td>
<td>mixed</td>
<td>this study</td>
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<tr>
<td>VS</td>
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<td>+</td>
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<td>6371</td>
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<td>+</td>
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<td>(20,24)</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 μM 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 71:21 and 71:2 in addition consists of 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM MgCl₂, 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 MgCl₂ (STS 7:3) or 3.0 mM MgCl₂ (STSs 71:21, 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 813a2 have been described elsewhere (25). Yeast cell culturing and DNA isolation was performed as described previously (37). A cosmid library of YAC 813a2 was constructed essentially as described (38), using partially Sau3A-digested DNA. DNA fragments with a size of 35–40 kb were ligated into the BamHI site of scOGH2. scOGH2 was derived from scCOH1 (39) by deleting an AatII-SphI fragment containing part of the 3’ non-coding region of the human growth hormone gene including an Alu-repeat element. The primary library consisted of 20 000 cosmids, 5000 of which were plated on 10 LB-agar plates supplemented with 50 μg/ml ampicilline. The colonies were replica-lifted onto hybond-N filters which were hybridized with a32P labeled human DNA. Three hundred signals of positive clones were positioned on the master filters, and fifty positive clones were picked and gridded on four nylon membranes and screened with the respective probe.

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