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A duplication/paracentric inversion associated with familial X-linked deafness (DFN3) suggests the presence of a regulatory element more than 400 kb upstream of the *POU3F4* gene

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X-linked deafness with stapes fixation (DFN3) is caused by mutations in the *POU3F4* gene at Xq21.1. By employing pulsed field gel electrophoresis (PFGE) we identified a chromosomal aberration in the DNA of a DFN3 patient who did not show alterations in the open reading frame (ORF) of *POU3F4*. Southern blot analysis indicated that a DNA segment of 150 kb, located 170 kb proximal to the *POU3F4* gene, was duplicated. Fluorescence in situ hybridization (FISH) analysis, PFGE, and detailed Southern analysis revealed that this duplication is part of a more complex rearrangement including a paracentric inversion involving the Xq21.1 region, and presumably the Xq21.3 region. Since at least two DFN3-associated minideletions are situated proximal to the duplicated segment, the inversion most likely disconnects the *POU3F4* gene from a regulatory element which is located at a distance of at least 400 kb upstream of the *POU3F4* gene.

**INTRODUCTION**

The most frequent form of X-linked deafness, DFN3, is characterized by fixation of the stapes and a perilymphatic gusher upon stapedectomy. By employing computerized tomography, Phelps et al. (1) identified a unique abnormality of the petrous temporal bone consisting of a dilatation of the internal auditory meatus (IAM) and an abnormal wide communication between the basal turn of the cochlea and the IAM. The gene underlying DFN3 was mapped to Xq21 by linkage analysis and through molecular characterization of large deletions associated with choroideremia, mental retardation, and DFN3 (2–7). Smaller deletions were found in five patients with typical DFN3 (8–11). Yeast artificial chromosome (YAC) clones that span the critical region were isolated, and patients with typical DFN3 (8–11).

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**RESULTS**

**Identification of a duplication associated with DFN3 by PFGE and Southern blot analysis**

We performed pulsed field gel electrophoresis analysis of genomic DNA of several DFN3 patients using a cosmid (4893F6; Fig. 5) located in the Xq21.1 region proximal to the *POU3F4* gene. In the DNA of DFN3 patient 5086, we observed a *SfiI* fragment of 575 kb instead of the normal 675 kb, suggestive of a microdeletion or a *SfiI* restriction fragment length polymorphism (Fig. 1a). Southern blot analysis of EcoRI digested DNA of patient 5086 employing all cosmids from a previously established 850 kb contig did not yield a deletion. In contrast, the hybridization signals of several cosmids near the DXS26 locus suggested the presence of a duplicated DNA fragment (data not shown). To investigate this possibility in more detail, we constructed EcoRI and *TaqI* blots containing equal amounts of DNA from a control female, a control male, DFN3 patient 5086, and patient XL45. The latter patient carries a microscopically visible deletion com-
The endonucleases of the duplicated segment in patient 5086.

**Family studies**

Detailed mapping of the duplicated endonucleases and

**Results (data not shown)**

All cosmids hybridized to cosmid 48934 in the absence of normal hybridization results, as illustrated by cosmid 48934 in the presence of 48934 clones. The absence of hybridization to cosmid 48934 in the absence of normal hybridization is well correlated with a normal intensity, a double intensity, or a normal intensity, as well.

**Figure 2.** Southern blot analysis of genomic DNA from a control female.

**Figure 4.** Southern blot analysis of EcoRI digested DNA from patient 5086.
Figure 1. PCR amplification of 5'flanking and 3'-untranslated regions of the duplication/inversion endpoints.

**Figure 2.** Molecular characterization of the duplication/inversion endpoints.
kb EcoRI and 6.0 kb TaqI breakpoint fragments (Fig. 3a). Since we were unable to identify aberrantly sized fragments with cosmid 4893C4 in EcoRI and TaqI digested DNA of patient 5086, blots were constructed containing HindIII and BamHI digested DNAs. The distal endpoint of the duplication could be detected with a 1.7 kb EcoRI fragment from cosmid 4893C4 which, in addition to normally sized restriction fragments, clearly hybridizes to novel HindIII and BamHI fragments (Fig. 3b). Based on the signal intensities observed, the distal duplication breakpoint is located in a 2.8 kb HindIII and a 18 kb BamHI fragment. From the previously established EcoRI restriction map of the cosmid contig, we estimate that the duplicated DNA segment measures 150 kb (Fig. 5).

To investigate whether this rearrangement segregates with the DFN3 phenotype in the family of patient 5086, we hybridized the 6.5 kb EcoRI fragment of cosmid 4893A1 to a Southern blot containing EcoRI digested DNAs from several family members. Clearly, the breakpoint fragment indicative of the chromosomal rearrangement can be seen in all DFN3 patients and female carriers (Fig. 4).

**FISH and PFGE analysis**

To investigate whether the two copies of the duplicated segment are located next to each other near the POU3F4 gene, a cosmid located on the duplicated segment, 4893H12, was hybridized to metaphase chromosome preparations from an EBV-immortalized lymphoblastoid cell line of patient 5086. In most chromosome spreads, the X-chromosome showed two specific, but rather diffuse signals (Fig. 6a). To our surprise, we observed four discrete signals in approximately one-third of the metaphases investigated, indicating that cosmid 4893H12 hybridizes to two different regions of the X-chromosome of patient 5086 (Fig. 6b,c). In a control metaphase X-chromosome, 4893H12 identified one distinct locus in the Xq21 band (data not shown). The most straightforward explanation for the FISH and Southern blotting results is a duplication-paracentric inversion event which moved one of the copies of the duplicated segment away from the endogenous copy of cosmid 4893H12 (Fig. 7).

To test this hypothesis, cosmid 4893H12 and cosmid IC2, the latter of which contains the POU3F4 gene, were successively employed as probes on the PFGE blot described above. As expected, cosmid 4893H12 detected the 575 kb SfiI fragment identified by cosmid 4893F6, corresponding to one of the inversion breakpoints, and an additional 475 kb SfiI fragment (Fig. 1b). Since the latter fragment is also identified by cosmid IC2 (Fig. 1c), it most likely spans the other inversion-breakpoint as indicated in Figure 7. To investigate whether the inversion involves chromosomal sequences proximal or distal to the POU3F4 gene, FISH analysis was performed with differently labeled cosmids from the duplication (4893H12) and the choroidelemia (CHM) gene (cosmid U98B5) located at Xq21.2. In only one X-chromosome, the CHM cosmid could be localized between the duplicated sequences; in all other metaphase chromosome spreads analyzed, the 4893H12 and U98B5 signals were not resolved (data not shown).
POU3F4 analysis in patient 5086

DNA of patient 5086 was examined for single strand conformation (SSC) variants by employing PCR primer sets defining five overlapping DNA segments that span the entire coding sequence of the POU3F4 gene (14). All five DNA segments could be readily amplified indicating no apparent structural abnormality in the protein coding region of POU3F4. No SSC shifts indicative of sequence alterations were found. The entire ORF of POU3F4 was analysed by DNA sequencing but no abnormalities were found.

DISCUSSION

We have identified and characterized a complex rearrangement in a patient with DFN3. The results of PFGE and Southern blot analysis are not consistent with a simple tandem duplication event, i.e. insertion of the new copy adjacent to the endogenous sequence proximal to the POU3F4 gene. Since SfiI restriction sites are known to flank the duplicated segment (Fig. 7), this event would generate a 825 kb SfiI band and not the observed 575 kb SfiI band. FISH analysis using a cosmid from the duplicated segment showed that the duplicated segments are separated by several megabases of DNA. Results from the duplicated segment showed that the duplicated segment and 320 kb proximal to the distal inversion breakpoint. To explain the DFN3 phenotype in patient 5086, we favour a model in which the proposed inversion separates a control element, most likely an enhancer element, from the POU3F4 transcription unit. A similar situation was reported for the α- and β-globin gene clusters in which deletions remove important control regions (21,22). To account for the clinical findings in all patients with minideletions [patients 1/10, 11/7, G8314 (Fig. 4) and patient ML (11)] that do not span the POU3F4 gene, the putative enhancer sequence should be located more than 400 kb upstream of the gene. Since in none of these patients mutations were found in the ORF of the POU3F4 gene (14; Y.J.M.de K. and F.P.M.C., unpublished data), the deletions must be causative for the observed phenotype. It is noteworthy that the deletion in patient II/7 is accompanied by a paracentric inversion. The breakpoints in
this familial case are in Xq13.1 and Xq21.2 (23). Thus far, we were unable to test our hypothesis directly since reverse transcription-PCR analysis of the POU3F4 mRNA isolated from control lymphoblast failed, indicating that POU3F4 expression in lymphoblasts is very low.

In three patients with DFN3 we were unable to find causative mutations in or outside the DFN3 gene. If the expression of this gene depends on the presence of an enhancer situated proximal to the POU3F4 gene, small mutations or chromosomal abnormalities might be found in the chromosomal region centromeric to the cosmid contig. To investigate this region in more detail, a YAC clone from this particular region was recently isolated (20) and the construction of a cosmid contig is underway. Elucidation of the molecular mechanism responsible for the DFN3 phenotype in patients with structural abnormalities at a large distance from the POU3F4 gene will yield important new insights into the regulation of this gene.

MATERIALS AND METHODS

DFN3 patients

Patient 5086 is the youngest member of a multigeneration deafness family (24; Fig. 4). Audiologic examination showed a profound sensorineural hearing loss. Two maternal uncles of the proband showed a total hearing loss. Radiological examination using computerized tomography in patient 5086 and two maternal uncles revealed dilated internal auditory canals and structural lesions of the cochlea that cause an incomplete separation of perilymphatic and cerebrospinal fluids. The mother of the proband showed a moderate mixed hearing loss in a pure tone audiogram (24). Together, these findings suggest that the deafness in this family can be classified as DFN3. In most patients with DFN3, both sensorineural and conductive hearing loss is found. In this case the conductive element is probably masked by the profound sensorineural component. Patient XL45 suffers from DFN3, mental retardation and choroideremia. In this patient, both sensorineural and conductive hearing loss is found. Together these findings suggest that the deafness in this family can be classified as DFN3. In most patients with DFN3, both sensorineural and conductive hearing loss is found. In this case the conductive element is probably masked by the profound sensorineural component. Patient XL45 suffers from DFN3, mental retardation and choroideremia, and has been described in more detail elsewhere (7,25,26).

Pulsed field gel electrophoresis and Southern blotting

PFGE and Southern blot analysis were performed as described by Bach et al. (7) and Huber et al. (9), respectively. Cosmid IC2 corresponds to the ICRF clone c104L0131. Clone 50:17 (DXS6673E) is a cDNA constituting part of the POU3F4 gene. A second cosmid, c1005 (DXS6671E), was isolated from the same region. Clone c104L0131. Clone 50:17 (DXS6673E) is a cDNA constituting part of the POU3F4 gene. A second cosmid, c1005 (DXS6671E), was isolated from the same region.

Single strand conformation analysis and nucleotide sequencing

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSC) analysis (27) was performed employing five partially overlapping PCR segments spanning the ORF of POU3F4 as described elsewhere (14).

Fluorescence in situ hybridization

All fluorescence in situ hybridization (FISH) procedures used were essentially as described previously (28–30). Briefly, cosmid 4893H12 was labeled with dig-11-UTP (Boehringer) and the X centromere probe pBAMX5 with Fluorolink Cy3-dCTP (BDS inc. Pittsburgh) using a nick-translation kit (Gibco, Life Technologies). 100 ng labeled 4893H12 probe DNA and 5 μg Cot-1 DNA (Gibco, Life Technologies) was dissolved in 6 μl of a hybridization solution (50% v/v deionized formamide, 10% w/v dextran sulphate, 2XSSC, 1% v/v Tween-20, pH 7.0). Prior to hybridization, the probe was denatured at 80°C for 10 min, chilled on ice, and incubated at 37°C for 30 min allowing preannealing. For pBAMX5 20 ng DNA in 6 μl was used per reaction and no competitor DNA was added. Metaphase spreads were prepared using standard procedures. After denaturation of the slides, probe incubations were carried out under an 18×18 mm coverslip in a moist chamber for 45 h. Immunochemistry detection of the hybridizing probes was achieved using FITC conjugated sheep-anti-digoxigenin (1:20, Boehringer Mannheim). For evaluation of the chromosomal slides a Zeiss epiphraphscope microscope equipped with appropriate filters for visualization of Texas Red, DAPI and FITC fluorescence was used. Digital images were acquired using a high-performance cooled CCD camera (Photometrics, Tucson, USA), interfaced to a Macintosh Ici computer. All digital image-acquiring, processing and analysis functions were accomplished by means of the BDS-Image software package of Biological Detection Systems Inc., Rockville, USA.