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). Yeast artificial chromosome (YAC) clones that span the critical region were isolated, and in the DNA of DFN3 patient 5086, we observed a SfiI restriction fragment of 575 kb instead of the normal 675 kb, suggestive of a microdeletion or a SfiI restriction fragment 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 DXS24 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 con-

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 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-

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end, 6.5 Kbp EcoRI fragment of cosmid 4893A1 detected. 4.0
Kbp EcoRI fragment of cosmid 4893A4 was detected. 4.0
Kbp EcoRI fragment of cosmid 4893A1 and 4893A4. All the
fragments from cosmid 4893A1 and 4893A4, plus control
DNA, were mapped in detail by Southern hybridization
DNA from patient 5086 and in unaffected control individual with EcoRI
The labelling of the duplicated segment in patient 5086

Family analysis

Detailed mapping of the duplication endpoints and the
results (data not shown). All cosmid clones 4893C4 and 4893E4 showed normal
hybridization. Results obtained with normal hybridization
DNA (Fig. 2d) are shown in Table 3. No abnormalities were detected.
A single band appeared to be of double intensity compared to control DNA.
A double intensity band appeared in patient 5086 compared to a
single intensity band. The 4.0 Kbp EcoRI fragment only in 4893A4
For cosmid 4893C4, only a single fragment of double intensity
was observed (Fig. 2b). All cosmid clones (Fig. 2a) showed
hybridization with a normal intensity, a double intensity, as well
as an apparent single band of EcoRI (4.0 Kbp) and 191 (6.0 Kbp).

Figure 4. Southern blot analysis of EcoRI digested DNA from a control female

BP = breakpoint. WT = wildtype. bp = basepairs. The restriction
endonuclease site of the reference cosmid 4893A1 was compared to the
sequence of cosmid 4893A1. The results of this analysis are shown
in Table 3. The 6.5 Kbp EcoRI fragment of cosmid
4893A1 was observed for cosmid 4893A4 (Fig. 2c).

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

DNA was detected in the patients. In patient 5086, a
double intensity of EcoRI and 191 bands compared to control
DNA. The region investigated here in patient 5086.
kb EcoRI and 6.0 kb TaqI breakpoint fragments (Fig. 3a). Since we were unable to identify aberrantly sized fragments with cosmide 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 cosmide 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 cosmide 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 cosmide 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 cosmide located on the duplicated segment, 4893H12, was hybridized to metaphase chromosomes prepared 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 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 cosmide 4893H12 (Fig. 7).

To test this hypothesis, cosmide 4893H12 and cosmide IC2, the latter of which contains the POU3F4 gene, were successively employed as probes on the PFGE blot described above. As expected, cosmide 4893H12 detected the 575 kb SfiI fragment identified by cosmide 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 cosmide 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 choroideremia (CHM) gene (cosmid U98B5) located at Xq21.2. In only one X-chromosome, the CHM cosmide could be localized between the duplicated sequences; in all other metaphase chromosome spreads analysed, the 4893H12 and U98B5 signals were not resolved (data not shown).
could be readily amplified indicating no apparent structural 
DNA of patient 5086 was examined for single strand conforma­
tion (SSC) variants by employing PCR primer sets defining 
the ORF of POU3F4. We have identified and characterized a complex rearrangement 
shifts indicative of sequence alterations were found. The entire 
abnormality in the protein coding region of POU3F4, and Xq13.3 or Xq21.3. In agreement with this, 
cosmid IC2, spanning the POU3F4 gene, detected an additional 
SfiI fragment corresponding to the other breakpoint of the 
inversion (Fig. 7). How this mechanism plays a role in the Xq21 region, it is 
directional in nature, since a large deletion 120 kb distal to 
the POU3F4 gene (patient AP; Fig. 4), is associated with 
mental retardation and choroideremia, but not with hearing 
impairment (20). It remains to be investigated whether tran­
scriptional silencing of the respective genes is due to posi­
tion effects.

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 
\(\alpha\) - and \(\beta\) -globin gene clusters in which deletions remove 
important control regions (21,22). To account for the clinical 
findings in all patients with minideletions \(\text{patients 1/10, 11/7, G8314 (Fig. 4) and patient ML (11)}\) 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 pheno­ 
type. It is noteworthy that the deletion in patient II/7 is 
accompanied by a paracentric inversion. The breakpoints in 
The duplication/inversion event proposed here does not affect the 
POU3F4 gene proper, which, in the rearranged situation (Fig. 7), is situated 170 kb proximal to one copy of 
the duplicated segment and 320 kb proximal to the distal 
inversion breakpoint. To explain the DFN3 phenotype in 
patient 5086, as well as the aforementioned minideletions 
situated proximal to the POU3F4 gene, a few possibilities are 
considered. First, in the normal situation, the 5' part of the 
POU3F4 gene, including its promoter, might be situated farther 
centromeric. In this situation, the POU3F4 gene would contain 
a single, unusually large intron (>400 kb) in its 5' untranslated 
region (Fig. 5). We have located the 3' end of the POU3F4 
mRNA 2.4 kb downstream of the ORF. Since the POU3F4 mRNA was estimated to be 3.5 kb in size, we can deduce that 
the 5' untranslated region measures less than 200 bp 
(unpublished data). From these findings we deduce that most 
probably, there is no large 5' intron in the POU3F4 gene. 
Second, another gene involved in DFN3 might be situated in the 
chromosomal segment proximal to POU3F4. If so, this 
gene would be predicted to span a region of more than 
200 kb, the distance between the proximal inversion-breakpoint 
observed in patient 5086 and the DFN3 associated deletion 
mapping farthest centromeric (G8314; Fig. 5). We believe that 
this is an unlikely possibility, too, since this chromosomal 
segment was found to be devoid of sequences transcribed in 
fetal brain tissue. In a third model, the three minideletions 
and the duplication/inversion would juxtapose heterochromatic 
sequences located more than 50 kb away from the SOX9 
gene (17,18). Similarly, translocations up to 85 kb from the 
PAX6 gene are causally related to the aniridia phenotype (19). 

Figure 7. Model for the duplication/paracentric inversion event that gave rise 
to the observed rearrangement in DFN3 patient 5086. The upper drawing 
reflects the normal situation; the middle shows an intermediate structure 
resulting from a DNA duplication event; the lower schematic illustrates the 
proposed final rearrangement observed in patient 5086. The locations of the 
SfiI (S) sites flanking the duplicated segment were derived from the cosmid 
contig (unpublished data). The locations of the SfiI sites in the Xq21.3 region 
were deduced from the observed SfiI fragment sizes (see Fig. 1).

| DNA | 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 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 band. FISH analysis using a cosmid 
spanning the POU3F4 gene, located on the duplicated segment, and 
the dosage-sensitive region (DSR) of the X-chromosome suggests locations at Xq21.1 and Xq21.3, 
also reverse mapping near the duplicated segment were derived from the cosmid 
contig (unpublished data). The locations of the SfiI sites in the Xq21.3 region 
were deduced from the observed SfiI fragment sizes (see Fig. 1).
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 POU3F4 mRNA isolated from control lymphoblasts 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 cosm id 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. 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).

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REFERENCES


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 Photorlink Cy3-ACP (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 dextrane sulphate, 2× SSC, 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 pronase pre-treatment. 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.

Immunocytochemical 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 epi-fluorescence 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 IICi computer. All digital image-acquiring, processing and analysis functions were accomplished by means of the BIDS-Image® software package (Biological Detection Systems Inc., Rockville, USA).