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RESEARCH ARTICLE

The Molecular Basis of X-Linked Deafness Type 3 (DFN3) in Two Sporadic Cases: Identification of a Somatic Mosaicism for a *POU3F4* Missense Mutation

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We have investigated two unrelated males with X-linked deafness type 3 (DFN3) for mutations in the *POU3F4* gene. In one patient, we observed a mutation that is predicted to result in an Arg330Ser amino acid substitution. In another DFN3 patient, a somatic mosaicism for an Arg323Gly amino acid substitution was found. This mosaicism was detected in two independently established EBV immortalized B cells and peripheral blood lymphocytes (PBLs). Semiquantitative analysis showed that approximately 50% of the PBLs of this patient carry the mutation. We hypothesize that the Arg323Gly mutation occurred very early in embryogenesis, before the differentiation of cells involved in hematopoiesis and inner ear development. In both patients, the missense mutations are situated in the POU homeodomain and are predicted to disrupt the DNA binding of the POU3F4 protein. All nine point mutations thus far described were found in the POU domains of *POU3F4*. Since these domains constitute only 35% of the open reading frame of *POU3F4*, there is a statistically significant preference for mutations in the POU-specific and POU homeodomain. Hum Mutat 10:207–211, 1997.

KEY WORDS: X-linked deafness type 3; *POU3F4* missense mutation; POU homeodomain

INTRODUCTION

X-Linked deafness type 3 (DFN3) is characterized by an abnormally wide opening in the bone separating the basal turn of the cochlea and the inner auditory meatus, a fixation of the stapes, and a perilymphatic gusher upon stapedectomy (Cremers et al., 1983; Phelps et al., 1991). Affected males either have mixed deafness or, less commonly, only sensorineural deafness. In the latter situation, the conductive element is masked by the sensorineural deafness. An accurate physical localization of the DFN3 gene was achieved by the molecular characterization of various deletions associated with DFN3 (Huber et al., 1994). Recently, positional candidate gene cloning resulted in the identification of the DFN3 gene, *POU3F4* (de Kok et al., 1995). *POU3F4* is a member of a gene family of transcription factors that contain two conserved domains, a POU-specific and a POU homeodomain. Small mutations were found in the human *POU3F4* gene in 7 unrelated patients with familial DFN3 (de Kok et al., 1995; Bitner-Glindzicz et al., 1995). Here, we report on the identification of point mutations in the *POU3F4* gene in two sporadic cases of DFN3, one of which shows a somatic mosaicism.

MATERIALS AND METHODS

DFN3 Patients

Patient 1186 (F.B.) is a 44-year-old man who suffered from a progressive hearing loss. At the age of 7 he was obese, had growth retardation, and experienced intermittent diarrhea. He had an exploratory tympanotomy at the age of 18 because of presumed otosclerosis (Cremers et al., 1983). Upon stapedectomy, a perilymphatic gusher was encountered. Polytomography showed dilation of the semicircular canals and of the lateral part of the IAM. In addition to the hearing loss, patient 1186 shows hyperparathyroidism and subclinical hypothyroidism. The latter symptoms are probably due to the multiple endocrine adenomatosis type 1 syndrome found in his father, two of his aunts, three nieces, and a cousin. No other members of his family exhibited hearing loss.

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Patient 1185 (A.M.) had a history of mixed hearing loss dating back to the age of 6 years. At the age of 56 years, he underwent a stapedectomy, upon which a perilymph gusher was encountered (Cremers et al., 1983). Polytomography of the petrous bones in the axial-pyramidal and the Guillen view was normal. The patient died in 1994 because of a lung carcinoma. Genealogical examination did not display any evidence for consanguinity in his family for five generations.

DNA Sequence Analysis

Genomic DNA isolation from Epstein-Barr virus (EBV)-immortalized lymphocytes and peripheral blood lymphocytes (PBLs) was performed as described (Huber et al., 1994). For patients 1185 and 1186, segments of the *POU3F4* gene were amplified by PCR, gel purified, and sequenced as published elsewhere (de Kok et al., 1995).

Hybridization of Allele-Specific Oligonucleotides

PCR products generated from DNA of patient 1185 by primers Va (5′-CATTGACAAGATCGCTGCAC-3′) and Vb (5′-GCCTCCTCGCTTCCTCCA-3′) were cloned in the *Sma*I site of pBluescript (SK–). Eight clones were sequenced, three of which showed the mutant sequence. Next, segment V (nucleotides 804–1105) of the *POU3F4* gene (de Kok et al., 1995) was amplified employing DNA isolated from PBLs of patient 1185, a control male, and from cloned mutant segment V. The PCR products were analyzed on an agarose gel and quantitated spectrophotometrically. Serial twofold dilutions (4, 2, and 1 ng) of the 302-bp fragment were applied to a nylon filter (GenescreenPlus, NEN) according to the manufacturer's protocol using a dot-blot apparatus. Duplicate filters were prepared for each sample, prehybridized for 4 hr in 5× SSPE/0.3% sodium dodecyl sulfate (SDS), and then hybridized overnight at 52°C in the same buffer with either the mutant (5′-GGAAGTGGTGGGTGTCTGGTT-3′) or the normal (5′-GGAAGTGGTGCGTGTCTGGTT-3′) oligonucleotides end-labeled with γ-^{32}P-ATP to a specific activity of 5×10^8 dpm/μg. The filters were washed 2 × 15 min in 5 × SSPE/0.3% SDS at 68°C and exposed to 3–6 hr. Quantitation of the signal was carried out employing the Hewlett Packard Scanjet IIc densitometer. The relative frequencies of the mutant and wild-type *POU3F4* alleles in patient 1185 were calculated by comparing the densities of the dots with those of mutant and normal PCR products in four independent experiments. Next, the filters were stripped in 0.1 × SSC/0.1% SDS and hybridized with primer Va as described above for the other oligonucleotide.

RESULTS

Identification of *POU3F4* Point Mutations in DFN3 Patients 1185 and 1186

Genomic DNA of EBV immortalized B cells of the DFN3 patients 1185 and 1186 was amplified using five primer pairs that together permit analysis of the complete open reading frame of the intronless *POU3F4* gene (de Kok et al., 1995). Employing primers Va and Vb, which amplify a 302-bp fragment (segment V) encompassing the POU homeodomain, we found an A-to-T transversion at nucleotide position 990 in patient 1186. This mutation is predicted to result in a substitution of a serine for an arginine residue at amino acid position 330 (results not shown). In patient 1185, we found a mixture of the normal C and a mutant G at nucleotide position 967 (Fig. 1a). The mutation replaces a glycine for an arginine residue at position 323. This mutation was also identified in the DNA of another independently established EBV cell line of DFN3 patient 1185, as well as in his PBLs (data not shown).

To rule out the possibility of a duplication of the *POU3F4* gene followed by a point mutation in *POU3F4*, we hybridized *POU3F4* on a Southern blot containing *Eco*RI-digested PBL DNA of patient 1185 and a control male. The hybridization signal of *POU3F4* in patient 1185 was comparable to that in the control male, excluding a duplication (data not shown). Since the mutant/wild-type nucleotide ratio cannot be estimated by comparing the respective fluorescence peaks, we performed dot-blot analysis employing allele-specific oligonucleotides.

Semiquantitative Analysis of the Somatic Mosaicism Employing Allele-Specific Oligonucleotide Hybridization

We amplified segment V of the *POU3F4* gene employing DNA derived from PBLs from patient 1185, a male control, and cloned mutant DNA. Serial dilutions of these three samples were immobilized on nylon membranes hybridized with ^{32}P-labeled 21-mers containing the mutant or the normal sequence (Fig. 1b, top). Subsequently, the membranes were stripped and rehybridized with ^{32}P-labeled primer Va, one of the primers used to amplify the PCR products (Fig. 1b, bottom). Hybridization with primer Va clearly shows that equal amounts of the PCR products were blotted on the membrane. Under the conditions employed, the mutant and wild-type oligonucleotides hybridized specifically to their homologous PCR products (Fig. 1b, top). As expected from the nucleotide sequence analysis, both primers hybridize to PCR products from patient 1185. Next, by employing densitometry, we compared the inten-

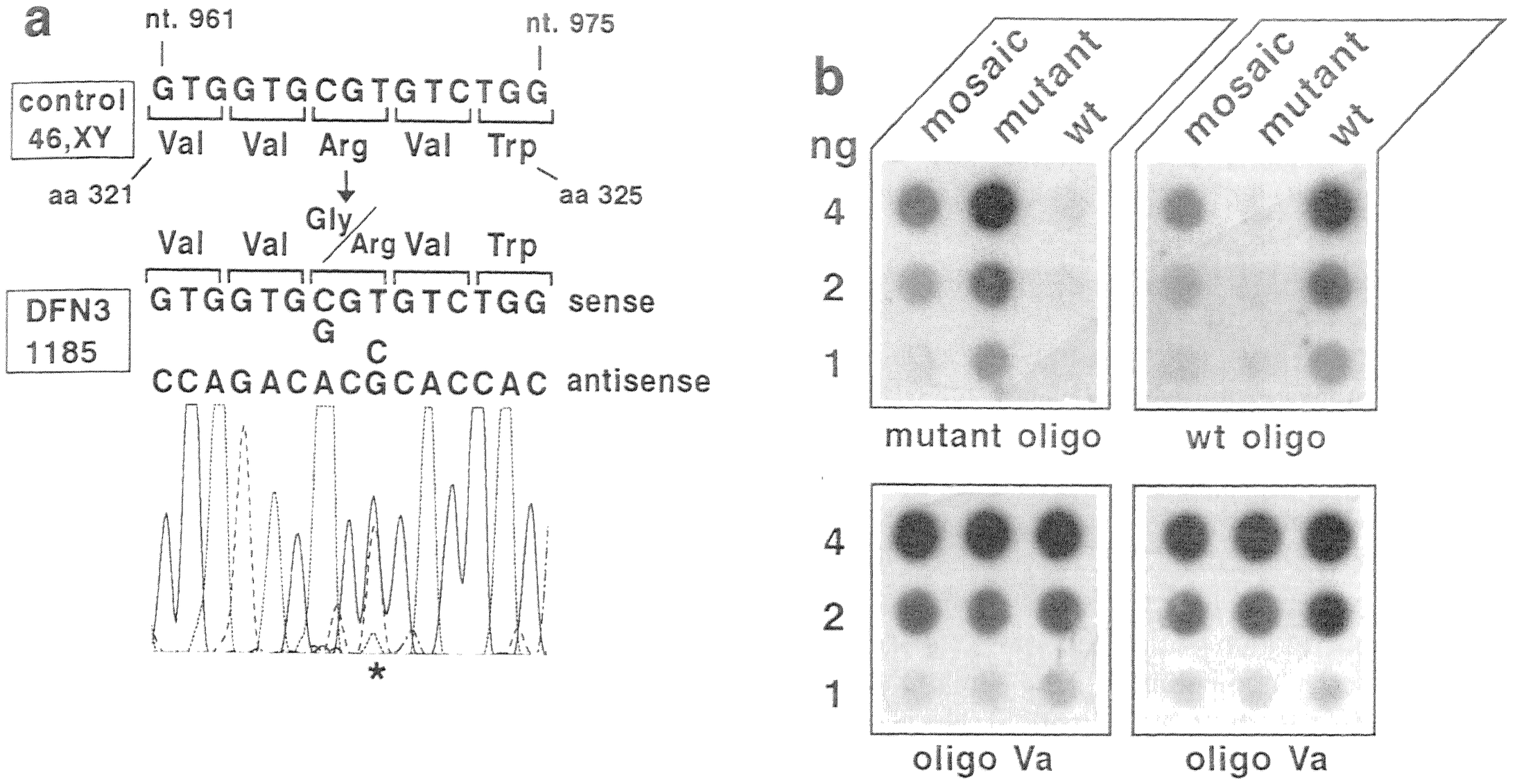

FIGURE 1. Mosaicism for a *POU3F4* homeodomain missense mutation. **a:** Sequence analysis of DNA from an EBV-immortalized B cell line of patient 1185 using the antisense primer Vb shows a mixture of a normal G and a mutant C residue at nucleotide position 967. For reasons of clarity, the amino acid sequence is given above the mutant sense sequence. The altered nucleotide is marked with an asterisk. **b:** Allele-specific oligonucleotide hybridization to duplicate filters containing serially diluted amplified DNA isolated from PBLs of patient 1185 (mosaic), recombinant mutant DNA (mutant), and control male DNA (wt). Membranes were hybridized with either the mutant or wild-type oligonucleotides (top). Stripping of the membranes and rehybridization with a control primer Va shows that equal amounts of PCR products were loaded (bottom).

sity of the signals observed in the 2 and 4-ng PCR products of patient 1185 (mosaic), with those observed for mutant and wild-type PCR products in four independent experiments. The mean percentage of mutant 1185 DNA, compared to mutant recombinant DNA was 50.0 ± 1.1%; the mean percentage of wild-type 1185 DNA compared to the wild-type control was 52.0 ± 0.5%. From these calculations, we estimate that approximately 50% of the PBLs in patient 1185 carry the mutant sequence.

DISCUSSION

The mutation in patients 1186 and 1185 affects arginine residues that are conserved among all POU domain proteins (Okamoto et al., 1993). These amino acids are presumed to contact the DNA backbone in the major groove in the proposed crystal structure of the Oct-1 POU domain–octamer complex (Klemm et al., 1994). Thus, both mutations are predicted to give rise to the DFN3 phenotype. These point mutations were found in sporadic cases, and thus represent *de novo* mutations. In patient 1186, who does not show a mosaicism in the EBV cell line analyzed, the mutation probably arose in the germline of his mother or grandmother. In patient 1185, the somatic mosaicism suggests that the mutation occurred early in embryonic development before the differentiation of the cells involved in hematopoiesis and temporal bone development. In this respect, it is interesting to note that hematopoietic cells and osteoclasts, which are involved in bone resorption, are believed to be derived from the same stem cells (Grigoriadis et al., 1995).

Somatic mosaicism is well established for chromosomal abnormalities, for mitochondrial inherited disorders, and for disorders caused by expansion of triplet repeats. Somatic mosaicisms of mutations in three collagen genes (Wallis et al., 1990; Kontasaari et al., 1992; Winterpacht et al., 1993), the neurofibromatosis type 1 gene (Colman et al., 1996), the adenosine deaminase gene (Hirschhorn et al., 1994), the factor IX gene (Taylor et al., 1991), and the ornithine transcarbamylase gene (Maddalena et al., 1988), have been found in mild or late-onset variants of known diseases. Also, a postzygotic mutation in $G_s\alpha$, the gene encoding a guanine nucleotide-binding regulatory protein, has been described in McCune-Albright syndrome (Schwindinger et al., 1992). Here, we report on a somatic mosaicism in a patient which, based on audiometric and surgical findings, displays two out of three hallmarks of X-linked deafness type 3 (i.e., mixed deafness and a

perilymphatic gusher) during stapes mobilization. The fact that no temporal bone defect was found in patient 1185 might be due to the inadequate resolution of the polytomography technique that was used to investigate the temporal bone compared to the high-resolution computed tomography (CT) studies performed more recently. CT scanning cannot be performed, as the patient is deceased.

Females carrying a DFN3 mutation show a variable phenotype, ranging from asymptomatic to a moderate hearing loss (Nance et al., 1971; Thorpe et al., 1974; Cremers and Huygen, 1983). This suggests a not yet formally proven functional mosaicism due to random X-inactivation of the normal *POU3F4* gene. On average, X-inactivation in female carriers results in inactivation of the *POU3F4* gene in 50% of somatic cells, comparable to the somatic mosaicism observed in the PBLs of patient 1185. However, since patient 1185 shows at least two of the characteristic features of DFN3, it seems more likely that the mesodermal precursor cells of the temporal bone contain a higher percentage of cells with the Arg323Gly mutation.

Including the mutations reported in this study, nine point mutations have been identified in DFN3 patients, invariably located in the POU domains of *POU3F4*. Four stop mutations reside in the POU specific or POU homeodomain; five missense mutations are located in the POU homeodomain (de Kok et al., 1995; Bitner-Glindzicz et al., 1995). The complete POU domain constitutes 35% of the ORF, indicating a statistically significant preference for mutations in these regions ($P < 0.0001$ for the POU domain). There should be no a priori preference for stop mutations in a given domain of *POU3F4*, since any stop mutation upstream of the POU domains would be predicted to have the same result as stop mutations in the POU domains, i.e. loss of function of the protein. The clustering of missense mutations in the POU homeodomain might be explained by the assumption that mutations of this type in other parts of the protein result in a completely different phenotype or in no phenotype at all. The latter possibility seems less probable, since orthologous *POU3F4* genes in mouse, rat, and human are highly conserved (Hara et al., 1992; Le Moine and Young, 1992; de Kok et al. 1995). Apart from the developing inner ear, the *POU3F4* gene is transiently expressed in the fetal rat brain and developing neural tube (Le Moine and Young, 1992), mouse pituitary gland (Schonemann et al., 1995), and human fetal brain and fetal kidney (S.M. van der Maarel, Y.J.M. de Kok, and F.P.M. Cremers, unpublished results). As shown for Oct-1 and Oct-2, POU proteins cooperate with a myriad of transcription factors including other POU domain proteins, in order to confer cell type-specific expression of target genes (Veenstra et al., in press). Possibly, POU3F4 proteins carrying missense mutation outside the POU homeodomain might interact erroneously with vital transcription factors in some of these tissues, resulting in embryonic lethality.

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REFERENCES

Bitner-Glindzicz M, Turnpenny P, Höglund P, Kääriäinen H, Sankila E-M, van der Maarel SM, de Kok YJM, Ropers H-H, Cremers FPM, Pembrey M, Malcolm S (1995) Further mutations in Brain 4 (*POU3F4*) clarify the phenotype in the X-linked deafness, DFN3. Hum Mol Genet 4:1467–1469.

Colman SD, Rasmussen SA, Ho VT, Abernathy CR, Wallace MR (1996) Somatic mosaicism in a patient with neurofibromatosis type 1. Am J Hum Genet 58:484–490.

Cremers CWRJ, Huygen PLM (1983) Clinical features of female heterozygotes in the X-linked mixed deafness syndrome (with perilymphatic gusher during stapes surgery). J Pediatr Otorhinolaryngol 6:179–185.

Cremers CWRJ, Hombergen GCJH, Wentges RThR (1983) Perilymphatic gusher and stapes surgery. A predictable complication? Clin Otolaryngol 8:235–240.

de Kok YJM, van der Maarel SM, Bitner-Glindzicz M, Huber I, Monaco AP, Malcolm S, Pembrey ME, Ropers H-H, Cremers FPM (1995) Association between X-linked mixed deafness and mutations in the POU domain gene *POU3F4*. Science 267:685–688.

Grigoriadis AE, Wang Z-Q, Wagner EF (1995) Fos and bone cell development: Lessons from a nuclear oncogene. Trends Genet 11:436–441.

Hara Y, Rovescalli AC, Kim Y, Nirenberg M (1992) Structure and evolution of four POU domain genes expressed in mouse brain. Proc Natl Acad Sci USA 89:3280–3284.

Hirschhorn R, Ruixi Yang D, Israni A, Huie ML, Ownby DR (1994) Somatic mosaicism for a newly identified splice-site mutation in a patient with adenosine deaminase-deficient immunodeficiency and spontaneous clinical recovery. Am J Hum Genet 55:59–68.

Huber I, Bitner-Glindzicz M, de Kok YJM, van der Maarel SM, Ishikawa-Brush Y, Monaco AP, Robinson D, Malcolm S, Pembrey ME, Brunner HG, Cremers FPM, Ropers H-H (1994) X-Linked mixed deafness (DFN3): Cloning and characterization of the critical region allows the identification of novel microdeletions. Hum Mol Genet 3:1151–1154.

Klemm JD, Rould MA, Aurora R, Herr W, Pabo CO (1994) Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. Cell 77:21–32.

Kontasaari S, Tromp G, Kuivaniemi H, Stolle C, Pope FM, Prockop DJ (1992) Substitution of aspartate for glycine 1018 in the type III procollagen (COL3AI) gene causes type IV Ehlers-Danlos syndrome: The mutated allele is present in most blood leukocytes of the asymptomatic and mosaic mother. Am J Hum Genet 51:497–507.

Le Moine C, Young WS,III (1992) RHS2, a POU domain-containing gene, and its expression in developing and adult rat. Proc Natl Acad Sci USA 89:3285–3289.

Maddalena A, Sosnoski DM, Berry GT, Nussbaum RL (1988) Mosaicism for an intragenic deletion in a boy with mild ornithine transcarbamylase deficiency. N Engl J Med 319:999–1003.

Nance WE, Setleff R, McLeod A, Sweeney A, Cooper C, McConnell F (1971) X-Linked mixed deafness with congenital fixation of the stapedial footplate and perilymphatic gusher. Birth Defects 7:64–69.

Okamoto K, Wakamiya M, Noji S, Koyama E, Taniguchi S, Takemura R, Copeland NG, Gilbert DJ, Jenkins NA, Muramatsu M, Hamada H (1993) A novel class of murine POU gene predominantly expressed in central nervous system. J Biol Chem 268:7449–7457.

Phelps PD, Reardon W, Pembrey M, Bellman S, Luxon L (1991) X-Linked deafness, stapes gushers and a distinctive defect of the inner ear. Neuroradiology 33:326–330.

Schonemann MD, Ryan AK, McEvilly RJ, O'Connell SM, Arias CA, Kalla KA, Li P, Sawchenko PE, Rosenfeld MG (1995) Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2. Genes Dev 9:3122–3135.

Schwindinger WF, Francomano CA, Levine MA (1992) Identification of a mutation in the gene encoding the α subunit of the stimulatory G protein of adenyl cyclase in McCune-Albright syndrome. Proc Natl Acad Sci USA 89:5152–6156.

Taylor SAM, Deugau KV, Lillicrap DP (1991) Somatic mosaicism and female-to-female transmission in a kindred with hemophilia B (factor IX deficiency). Proc Natl Acad Sci USA 88:39–42.

Thorpe P, Sellars S, Beighton P (1974) X-Linked deafness in a South African kindred. S Afr Med J 48:587–590.

Veenstra GJC, Van der Vliet PC, Destrée OHJ (1997) POU domain transcription factors in embryonic development. Mol Biol Rep 24 (in press).

Wallis GA, Starman BJ, Zinn AB, Byers PH (1990) Variable expression of osteogenesis imperfecta in a nuclear family is explained by somatic mosaicism for a lethal point mutation in the αI(I) gene (COLIAI) of type I collagen. Am J Hum Genet 46:1034–1040.

Winterpacht A, Hilbert M, Schwarze U, Mundlos S, Spranger J, Zabel BU (1993) Kniest and Stickler dysplasia phenotypes caused by collagen type II gene (COL2A1) defect. Nature Genet 3:323–326.