MKK3 and MKK4 cDNAs labeled by random priming with [α-^{32}P]dATP (deoxyadenosine triphosphate) (Amersham International PLC).

26. GST-ATF2, GST–c-Jun, GST-JNK1, and GST-ERK2 have been described (4, 19, 20). GST-p38 MAP kinase was prepared from the expression vector pGSTag [H. Dressler et al., Biotechniques 13, 866 (1992)] and a PCR fragment containing the coding region of the p38 MAP kinase cDNA. GST-MKK3 and GST-MKK4 were prepared with pGEX-3X (Pharmacia–LKB Biotechnology) and PCR fragments containing the coding region of the MKK3 and MKK4 cDNAs. The GST fusion proteins were purified by affinity chromatography with the use of GSH-agarose [S. B. Smith and K. S. Johnson, Gene 67, 31 (1988)]. The expression vectors pCMV-Flag-JNK1 and pCMV-MEK1 have been described (4, 20). The plasmid pCMV-Flag-p38 MAP kinase was prepared with the expression vector pCMV5 [S. Andersson, D. L. Davis, H. Dahlbäck, H. Jörnvall, D. W. Russell, J. Biol. Chem. 264, 8222

(1989)] and the p38 MAP kinase cDNA. The expression vectors for MKK3 and MKK4 were prepared by subcloning of the cDNAs into the polylinker of pCDNA3 (Invitrogen). The Flag epitope (-Asp-Tyr-Lys-Asp–Asp-Asp-Aps-Lys-; Immunex, Seattle, WA) was inserted between codons 1 and 2 of the kinases by insertional overlapping PCR [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, Gene 77, 51 (1989)]. A similar PCR procedure was used to replace Thr^{180} and Tyr^{182} of p38 MAP kinase, and Thr^{183} and Tyr^{185} of JNK1, with Ala and Phe, respectively. The sequence of all plasmids was confirmed by automated sequencing on an Applied Biosystems model 373A machine.

27. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (5%) (Gibco-BRL). The cells were transfected with the lipofectamine reagent according to the manufacturer's recommendations (Gibco-BRL) and treated with UV radiation or EGF as described (4). The cells were solubilized with lysis buffer [20

mM tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml)] and centrifuged at 100,000g for 15 min at 4°C. The epitope-tagged protein kinases were immunoprecipitated by incubation for 1 hour at 4°C with the M2 antibody (IBI-Kodak) bound to protein G–Sepharose (Pharmacia–LKB Biotechnology). The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (21).

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Association Between X-Linked Mixed Deafness and Mutations in the POU Domain Gene *POU3F4*

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Deafness with fixation of the stapes (DFN3) is the most frequent X-linked form of hearing impairment. The underlying gene has been localized to a 500-kilobase segment of the Xq21 band. Here, it is reported that a candidate gene for this disorder, *Brain 4* (*POU3F4*), which encodes a transcription factor with a POU domain, maps to the same interval. In five unrelated patients with DFN3 but not in 50 normal controls, small mutations were found that result in truncation of the predicted protein or in nonconservative amino acid substitutions. These findings indicate that *POU3F4* mutations are a molecular cause of DFN3.

Severe, inherited childhood deafness occurs in about 1 out of 1000 births and presents a serious worldwide public health problem (1). In 70% of these cases, deafness is not associated with other clinically recognizable features (2). To date, genes for nonsyndromic sensorineural deafness have been mapped to five different autosomes (3), but none of these has been isolated yet. The most frequent cause of X-linked hearing impairment, X-linked mixed deafness (DFN3) (McKusick catalog number 304400), is characterized by a conductive hearing loss that results from stapes fixation and progressive sensorineural deafness (4, 5). However, a profound sensorineural deaf-

ness sometimes masks the conductive element (6). Computerized tomography (CT) studies in people with DFN3 demonstrated an abnormal dilatation of the internal acoustic canal (IAC) as well as an abnormally wide communication between the

IAC and the inner ear compartment (7). As a result, there is an increased perilymphatic pressure that is thought to underlie the observed "gusher" during the opening of the stapes footplate. The gene underlying DFN3 has been mapped to Xq21 by linkage analysis (8, 9) and through molecular characterization of large and submicroscopic deletions (10, 11). Yeast artificial chromosome (YAC) clones that span the critical region were isolated, and an 850-kb cosmid contig was constructed (12). This enabled us to identify and characterize two additional microdeletions, as well as a 150-kb duplication in patients with DFN3, and to assign the gene underlying DFN3 to a 500-kb interval of Xq21.1 (12, 13) (Fig. 1B).

Recently, the gene *Brain 4* (*Pou3f4*), which codes for a transcription factor, was mapped between the proteolipid protein locus *Plp* and the *DXMit6* marker near the phosphoglycerate kinase 1 (*Pgk1*) gene on the murine X chromosome (14). The chromosomal region between *Pgk1* and *Plp* is evolutionarily conserved between humans and mice, which suggests that the human *POU3F4* gene is located in the Xq13-q22
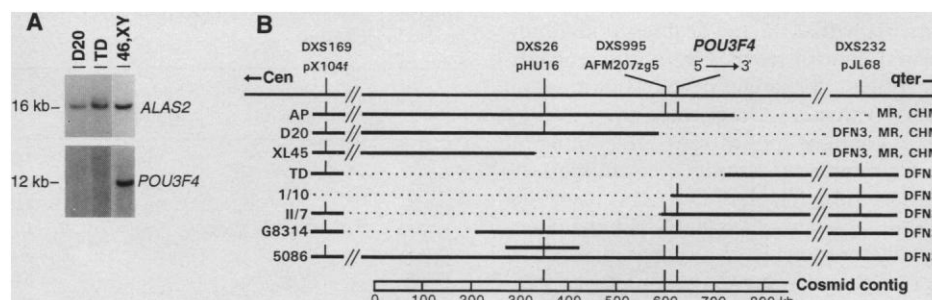
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Fig. 1. Localization of the human *POU3F4* gene. (**A**) Southern blot analysis with the use of a mouse *Pou3f4* probe and a control probe, δ-aminolevulinate synthetase (*ALAS2*), with genomic DNAs from a control male, D20, and TD (16). Patient D20 has choroideremia (CHM), mental retardation (MR), and X-linked mixed deafness (DFN3); patient TD shows evidence of DFN3 (12). (**B**) Physical map of the DFN3 critical region. All patients with deletions have been described elsewhere (12). Patient 5086 carries a 150-kb duplication spanning DXS26. The extent of the cosmid contig is given at the bottom. *POU3F4* is located on cosmids IC2 (ICRFc104L0131) and IC3 (ICRFc104B1939), approximately 20 kb distal to DXS995 (12). Restriction mapping of the cosmid containing the *POU3F4* gene indicated that the *POU3F4* gene is oriented with its 5' end toward the centromere.

interval. The rat homolog of *POU3F4*, *RHS2*, is expressed during embryonic development in the brain, the neural tube, and the otic vesicle at 15.5 and 17.5 days after conception (*15*).

Thus, both its map position and its temporal and spatial expression pattern in early embryogenesis rendered *POU3F4* an attractive candidate gene for DFN3. To confirm and refine the localization of the human *POU3F4* gene, we amplified a murine genomic *Pou3f4* gene fragment by polymerase chain reaction (PCR) and hybridized it to Southern (DNA) blots containing Eco RI–digested DNA from patients with Xq21 deletions (*16*). A 12-kb Eco RI fragment was seen in the male control but not in the DFN3 patients TD and D20 who carry variably sized deletions in Xq21 (Fig. 1A). The deletions overlap in a small chromosomal segment encompassing DXS995 (Fig. 1B), which positions the *POU3F4* gene in a 120-kb region of the previously constructed 850-kb cosmid contig that spans the DFN3 locus. By hybridizing the *Pou3f4* probe to cosmids from this contig, we could localize the *POU3F4* gene 20 kb distal to DXS995 (Fig. 1B).

We used PCR primers complementary to the murine *Pou3f4* gene sequences to amplify a human *POU3F4* fragment from cosmid DNA, which was then used as a probe to screen a human fetal brain complementary DNA (cDNA) library (*17*). Six overlapping cDNAs were identified and partially characterized. In total, we isolated 1.4 kb of the human *POU3F4* cDNA sequence, which contained the complete protein coding region of 1083 base pairs as inferred from the rat *RHS2* and mouse *Pou3f4* sequences (*15, 18, 19*) (Fig. 2). The rat and mouse proteins are completely identical, and the human protein contains only four conservative amino acid substitutions (Fig. 2).

To search for mutations in the *POU3F4* gene, we examined DNA from 14 unrelated patients with X-linked deafness and 50 unrelated control females from different ethnic origins for single-strand conformation (SSC) variants (*20*). Because the *POU3F4* gene contains no introns, five different PCR primer sets were sufficient to span the entire coding region of *POU3F4*. Six of the deafness patients (Table 1) had characteristic features of DFN3—that is, a bony defect observed during CT scanning or a perilymphatic gusher encountered upon stapedectomy (*4–7*). In one case (patient 5823), audiologic data were consistent with a progressive mixed type of deafness, but neither CT scan nor stapes surgery were performed. SSC shifts indicative of sequence alterations were found in four DFN3 patients, two of which are shown in Fig. 3A, but not in the 50 control females.

Subsequently, we sequenced the protein coding region of the *POU3F4* gene in all patients with X-linked hearing impairment. In all four patients with SSC shifts as well as in patient 5823, who was not studied by PCR-SSC analysis, mutations were observed in the *POU3F4* gene. In three DFN3 patients, we found small deletions in the POU-specific and POU homeodomains of *POU3F4* that would result in frameshifts and
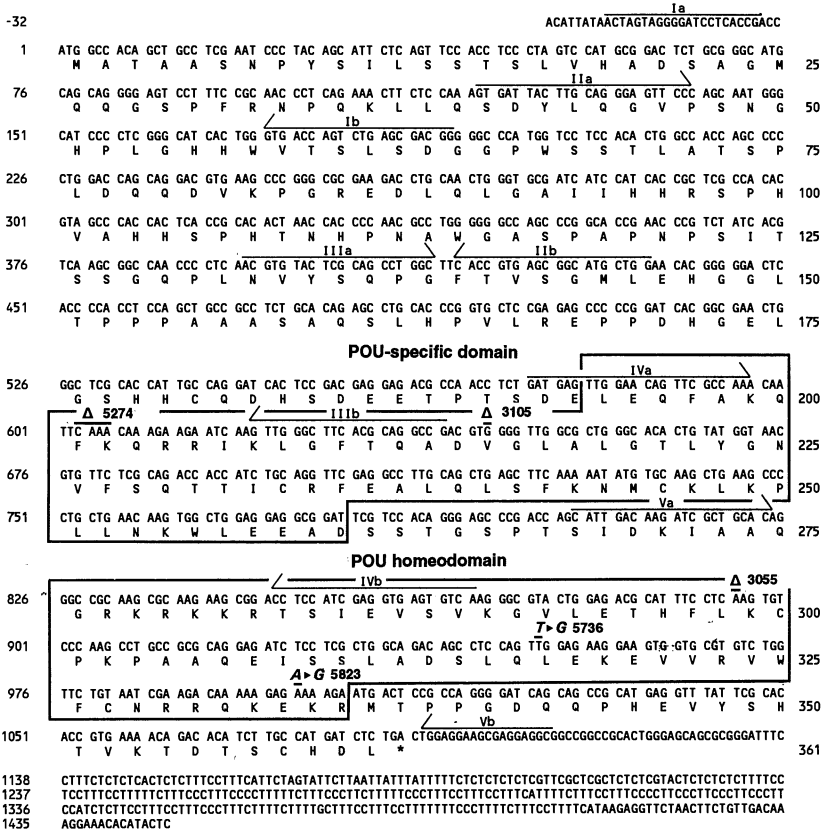
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                                                                            Ia
-32                                                   ACATTATAACTAGTAGGGGATCCTCACCGACC
  1  ATG GCC ACA GCT GCC TCG AAT CCC TAC AGC ATT CTC AGT TCC ACC TCC CTA GTC CAT GCG GAC TCT GCG GGC ATG
      M   A   T   A   A   S   N   P   Y   S   I   L   S   S   T   S   L   V   H   A   D   S   A   G   M   25
                                                                                          IIa
 76  CAG CAG GGG AGT CCT TTC CGC AAC CCT CAG AAA CTT CTC CAA AGT GAT TAC TTG CAG GGA GTT CCC AGC AAT GGG
      Q   Q   G   S   P   F   R   N   P   Q   K   L   L   Q   S   D   Y   L   Q   G   V   P   S   N   G   50
                            Ib
151  CAT CCC CTC GGG CAT CAC TGG GTG ACC AGT CTG AGC GAC GGG GGC CCA TGG TCC TCC ACA CTG GCC ACC AGC CCC
      H   P   L   G   H   H   W   V   T   S   L   S   D   G   G   P   W   S   S   T   L   A   T   S   P   75
226  CTG GAC CAG CAG GAC GTG AAG CCC GGG CGC GAA GAC CTG CAA CTG GGT GCG ATC ATC CAT CAC CGC TCG CCA CAC
      L   D   Q   Q   D   V   K   P   G   R   E   D   L   Q   L   G   A   I   I   H   H   R   S   P   H   100
301  GTA GCA CAC CAC TCA CCG CAC ACT AAC CAC CCC AAC GCC TGG GGG GCC ACC CCG GCA CCG AAC CCG TCT ATC ACG
      V   A   H   H   S   P   H   T   N   H   P   N   A   W   G   A   S   P   A   P   N   P   S   I   T   125
                        IIIa                                          IIb
376  TCA AGC GGC CAA CCC CTC AAC GTG TAC TCG CAG CCT GGC TTC ACC GTG AGC GGC ATG CTG GAA CAC GGG GGA CTC
      S   S   G   Q   P   L   N   V   Y   S   Q   P   G   F   T   V   S   G   M   L   E   H   G   G   L   150
451  ACC CCA CCT CCA GCT GCC GCC TCT GCA CAG AGC CTG CAC CCG GTG CTC CGA GAG CCC CCG GAT CAC GGC GAA CTG
      T   P   P   P   A   A   A   S   A   Q   S   L   H   P   V   L   R   E   P   P   D   H   G   E   L   175

                            POU-specific domain                    IVa
526  GGC TCG CAC CAT TGC CAG GAT CAC TCC GAC GAG GAG ACG CCA ACC TCT GAT GAG TTG GAA CAG TTC GCC AAA CAA
      G   S   H   H   C   Q   D   H   S   D   E   E   T   P   T   S   D   E   L   E   Q   F   A   K   Q   200
      ── Δ 5274 ──        ── IIIb ──          ── Δ 3105 ──
601  TTC AAA CAA AGA AGA ATC AAG TTG GGC TTC ACG CAG GCC GAC GTG GGG TTG GCG CTG GGC ACA CTG TAT GGT AAC
      F   K   Q   R   R   I   K   L   G   F   T   Q   A   D   V   G   L   A   L   G   T   L   Y   G   N   225
676  GTG TTC TCG CAG ACC ATC TGC CGG TTC GAG GCC TTG CAG CTG AGC TTC AAA AAT ATG TGC AAG CTG AAG CCC
      V   F   S   Q   T   T   I   C   R   F   E   A   L   Q   L   S   F   K   N   M   C   K   L   K   P   250
                                                                                       Va
751  CTG CTG AAC AAG TGG CTG GAG GAG GCG GAT TCG TCC ACA GGG AGC CCG ACC AGC ATT GAC AAG ATC GCT GCA CAG
      L   L   N   K   W   L   E   E   A   D   S   S   T   G   S   P   T   S   I   D   K   I   A   A   Q   275

                            POU homeodomain
                    ── IVb ──                                            Δ 3055
826  GGC CGC AAG CGC AAG AAG CGG ACC TCC ATC GAG GTG AGT GTC AAG GGC GTA CTG GAG ACG CAT TTC CTC AAG TGT
      G   R   K   R   K   K   R   T   S   I   E   V   S   V   K   G   V   L   E   T   H   F   L   K   C   300
                                                                          T▸G 5736
901  CCC AAG CCT GCC GCG CAG GAG ATC TCC TCG CTG GCA GAC AGC CTC CAG TTG GAG AAG GAA GTG GTG CGT GTC TGG
      P   K   P   A   A   Q   E   I   S   S   L   A   D   S   L   Q   E   K   E   V   V   R   V   W   325
                                      A▸G 5823
976  TTC TGT AAT CGA AGA CAA GAG AAG AGA ATG ACT CCG CCA GGG GAT CAG CAG CCG CAT GAG GTT TAT TCG CAC
      F   C   N   R   R   Q   K   E   K   R   M   T   P   P   G   D   Q   Q   P   H   E   V   Y   S   H   350
                                                      Vb
1051 ACC GTG AAA ACA GAC ACA TCT TGC CAT GAT CTC TGA CTGGAGGAAGCGAGGAGGCGGCCGGCCGCACTGGGAGCAGCGCGGGATTTC
      T   V   K   T   D   T   S   C   H   D   L   *                                                       361

1138 CTTTCTCTCTCACTCTCTTTCCTTTCATTCTAGTATTCTTAATTATTTATTTTTCTCTCTCTCTCGTTCGCTCGCTCTCTCGTACTCTCTCTCTTTTCC
1237 TCCTTTCCTTTTTCTTTCCCTTTCCCCTTTTTCTTTCCCTTCTTTTTCCCTTTCCTTTCCTTTCATTTTCTTTCCTTTCCCCTTCCCTTCCCTTCCCTT
1336 CCATCTCTTCCTTTCCTTTCCCTTTCTTTTCTTTTGCTTTCCTTTCCTTTTTTTCCCTTTTCTTTCCTTTTCATAAGAGGTTCTAACTTCTGTTGACAA
1435 AGGAAACACATACTC
```

Fig. 2. Nucleotide sequence and the deduced amino acid sequence encoded by human *POU3F4* (*21*). The oligonucleotides used for PCR-SSC analysis are indicated with arrows. The POU-specific and POU homeodomains are boxed. The human POU3F4 protein is identical to the protein in mice and rats, except for the following conservative amino acid changes from humans to rats and mice: T15S, P122S, A159T, and T356A. The nucleotide changes and deletions (Δ) in the DFN3 patients are indicated; affected nucleotides are marked with a bar. The asterisk indicates a stop codon.

Table 1. Clinical features and *POU3F4* mutations of patients with X-linked deafness (*21*). The deafness type here is based on audiologic examination. The *POU3F4* mutations are named "stop" according to the last wild-type amino acid that remains in the predicted protein. Blank spaces indicate that no CT scan (second column) or stapedectomy (third column) was performed. "–" designates that no mutations were found in *POU3F4*.

Patient	Bony defect	Perilymph gusher	Deafness type	POU3F4 mutation	Reference
2412		Yes	Mixed	–	(29)
2540	Yes	Yes	Mixed	–	(5)
3055		Yes	Mixed	L298 stop	(30)
3105	Yes	Yes	Mixed	D215 stop	(30)
5274	Yes		Sensorineural	K202 stop	(9)
5736	Yes		Mixed	L317W	(9)
5823*			Mixed	K334E	(30)
2418			Sensorineural	–	(30)
2964			Sensorineural	–	(30)
3036			Sensorineural	–	(30)
3056		No	Sensorineural	–	(30)
5106	No		Sensorineural	–	(9)
5347	No		Sensorineural	–	(9)
5943	No		Sensorineural	–	(9)
2266			Conductive	–	(30)

*Some DFN3-affected maternal relatives of patient 5823 also have cataracts at a young age.

premature stops of translation (Table 1 and Fig. 2). Patient 3055 carries a deletion of an A nucleotide at position 895 (Figs. 2 and 3C), patient 3105 has a deletion of one G that is part of a GGGG tetranucleotide stretch at positions 648 to 651, and in patient 5274 a CAAA tetranucleotide is deleted that is present in tandem at positions 603 to 610 of the wild-type POU3F4 sequence. As shown in Fig. 3B by SSC analysis, the mutation found in patient 5274 cosegregates with the DFN3 phenotype in the whole family. Two patients had missense mutations in the POU3F4 gene. In patient 5736, we found a T to G transversion at position 950, whereas patient 5823 showed an A to G transition at nucleotide 1000. These mutations result in the nonconservative L317W and K334E substitutions in the POU homeodomain (21).

The leucine residue at position 317 that is mutated in patient 5736 is located between helices 2 and 3 of the POU homeodomain, as deduced from nuclear magnetic resonance and crystallographic studies (22). It is conserved in six of seven members of the non-POU domain proteins that belong to the homeodomain superfamily (23) and in all POU domain proteins, except Oct-2 and emb. Here, the leucine residue is substituted for a methionine and a tyrosine residue, respectively (24). The wild-type lysine residue at position 334 that is mutated in patient 5823 resides in helix 3 of the POU homeodomain. This residue is conserved among all POU domain proteins and members of the homeodomain superfamily (23, 24) and contacts the DNA backbone in the major groove in the proposed crystal structure of the engrailed homeodomain-DNA complex, but not in the Oct-1 POU domain–octamer complex (22, 25). Thus, these data strongly suggest that all mutations we observed in the POU3F4 gene are clinically relevant and give rise to DFN3. Because DFN3 is a rare disorder, the total number of DFN3 patients that can be investigated will remain limited. Neverthe-

less, the identification of five mutations in seven DFN3 patients as compared to no single mutation in 100 control X chromosomes is statistically highly significant (26).

Unexpectedly, three Xq21 microdeletions (G8314, II/7, and 1/10) and one duplication (in patient 5086) that had been identified previously in patients with DFN3 do not encompass the POU3F4 gene. In all four cases, the rearrangement is located proximal and 5' to POU3F4, with physical distances varying between 15 and 400 kb (Fig. 1B). In none of these patients, nor in two others with either a perilymphatic gusher during stapes surgery or a temporal bone defect (patients 2412 and 2540), were point mutations detected in the POU3F4 gene. Thus, in these cases DFN3 may be caused by mutations that affect 5' noncoding or regulatory sequences mapping farther upstream. Alternatively, these aberrations may affect the gross chromosomal structure and thus may affect expression of POU3F4. Another, less likely explanation might be the presence of other genes in Xq21.1 that can cause DFN3.

In seven patients with X-linked sensorineural deafness and in one with X-linked conductive deafness, mutations were not found in the POU3F4 gene. This is not surprising because previous linkage studies had indicated that X-linked deafness is heterogeneous. In at least one family with sensorineural deafness, the defect has been excluded from Xq21 (9). In two other families, linkage to the Xq21 and Xp11.3-p21.1 regions has been found (27). Here, we demonstrate that DFN3 is correlated with mutations that affect the POU3F4 protein. At least five POU domain genes are expressed in different parts of the developing inner ear (28), and defects of POU domain genes may play a major role in the etiology of nonsyndromic hearing impairment.

Fig. 3. Mutation analysis of POU3F4. (**A**) PCR-SSC analysis of the homeodomain of POU3F4 in 14 patients with X-linked deafness and a male control (46, XY) with the use of the primer set V (20). The observed SSC shifts are indicated by arrows. (**B**) PCR-SSC analysis of the DFN3 family of patient 5274 (indicated by an arrow) with the use of primer set IV. Obligate carrier females are marked as such in the pedigree (circles with dots). DFN3 patients are indicated with solid squares; symbols with lines designate deceased individuals. The asterisk indicates the presence of a weak wild-type (WT) SSC band in lane 12. Of the four females at risk of carrying the CAAA deletion, individual 10 carries the wild-type POU3F4 sequence, whereas individuals 7, 13, and 18 carry the mutation. (**C**) Nucleotide (nt) sequence of part of the POU homeodomain of patient 3055 and a control male.

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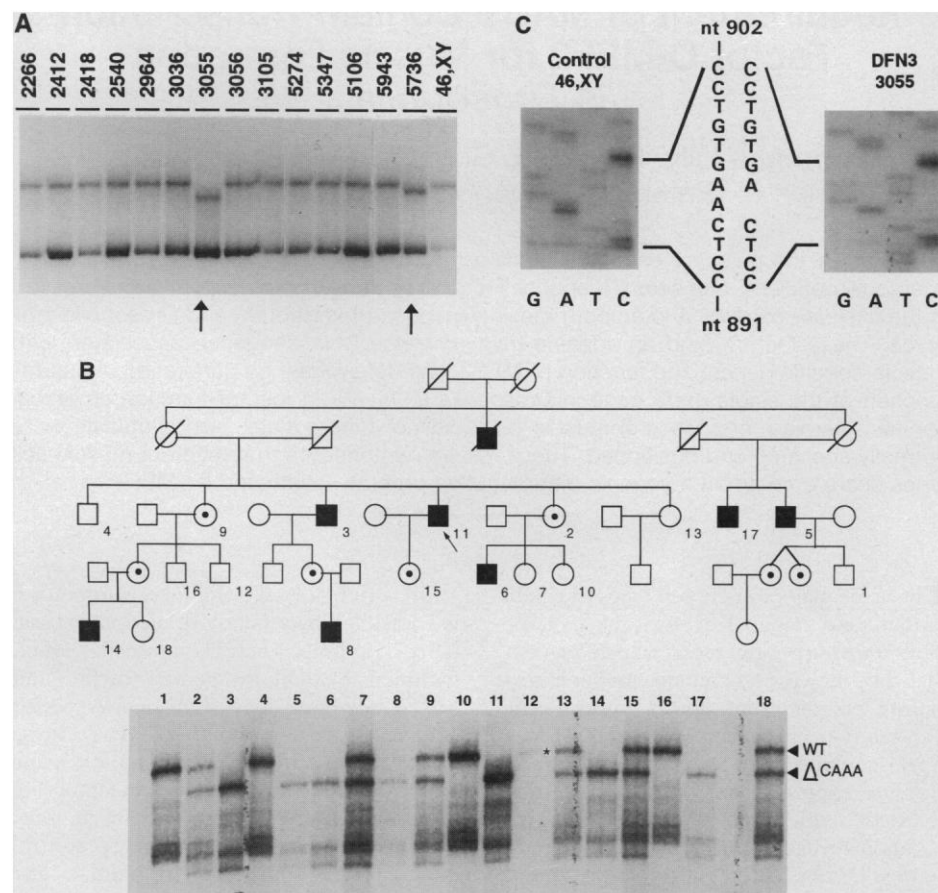14. P. J. Douville, S. Atanasoski, A. Tobler, A. Fontana,

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16. Genomic DNAs (10 µg of each) were digested with Eco RI. The fragments were separated by gel electrophoresis and blotted on GeneScreenPlus membranes (NEN) as recommended by the manufacturer. The mouse *Pou3f4* genomic segment was amplified from 500 ng of mouse genomic DNA, 250 ng of the primers 977 [nucleotides 119 to 140 of the *POU3F4* open reading frame (ORF) (*19*); GTGAC-TACTTGCAGGGAGTTCC] and 978 (nucleotides 521 to 540 of the ORF; GCAGTGGTGCGAGC-CCAGCT; Isogen Biosciences), 10 mM tris-HCl (pH 8.0), 50 mM KCl, 3 mM $MgCl_2$, and 0.01% gelatin. After initial denaturation for 5 min at 94°C, amplification was done in 30 cycles of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C, with a final elongation step for 6 min at 72°C. The PCR product was purified from low melting temperature (LMT) agarose and radiolabeled with the use of random hexamer priming. The blot in Fig. 1A was hybridized in 0.5 M NaH_2PO_4 (pH 6.8), 7% (w/v) SDS, 1 mM EDTA, and 50 µg/ml of sonicated, denatured herring sperm DNA at 55°C for 18 hours. Washing was done in 40 mM NaH_2PO_4 and 1% (w/v) SDS at 60°C for 1 hour. The mouse *Alas2* cDNA probe pMS20 [D. S. Schoenhaut and P. J. Curtis, *Gene* **48**, 55 (1986)] was hybridized to the blot shown in Fig. 1A, as described for the mouse *Pou3f4* probe. The gene encoding human ALAS2 is located in Xp11.2 [P. D. Cotter, H. F. Willard, J. L. Gorski, D. F. Bishop, *Genomics* **13**, 211 (1992)].
17. We used the mouse *Pou3f4* primers 977 and 978 (*16*) to amplify a 420-bp segment of the human *POU3F4* gene using 10 ng of cosmid IC3. The PCR product was purified in an LMT agarose gel and labeled as described (*16*). Phage recombinants (10⁶) of a fetal brain cDNA library (Stratagene) were screened with this DNA fragment, as described [F. P. M. Cremers, D. J. R. van de Pol, L. P. M. van Kerkhoff, B. Wieringa, H.-H. Ropers, *Nature* **347**, 674 (1990)]. Six cDNA clones were isolated, four of which were analyzed further. The ORF of the human *POU3F4* gene was sequenced on both strands with the use of mouse and human *POU3F4* oligonucleotides that amplified DNA from cosmid IC3 and DNA from the four cDNA clones. Sequences from the 5′ and 3′ untranslated regions were obtained from the cDNA clones. Sequence analysis was performed with the dye terminator sequencing kit on an Applied Biosystems automated sequencer. The human *POU3F4* cDNA sequence has been deposited in the European Molecular Biology Laboratory database with accession number X82324.
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20. PCR-SSC analysis [M. Orita, Y. Suzuki, T. Sekiya, K. Hayashi, *Genomics* **5**, 874 (1989)] was performed with five partially overlapping PCR products that span the ORF of *POU3F4*. We added 250 ng of each primer from the five sets of primers [Ia (ACTAG-TAGGGGATCCTCACCG) and Ib (CCGTCGCTCAG-ACTGGTCAC); IIa (GTGACTACTTGCAGGGAGTT-CC) and IIb (CCAGCATACCGCTCACCGTG); IIIa (ACGTGTACTCGCAGCCTGGC) and IIIb (CGGTCT-GCGTGAAGCCCAAC); IVa (GATGAGTTGGAACA-GTTCGCCAA) and IVb (TGACACTCACCTCGATG-GAGG); and Va (CATTGACAAGATCGCTGCAC) and Vb (GCCTCCTCGCTTCCTCCA)] to buffers containing either 10 mM tris-HCl (pH 8.0), 50 mM KCl, 3 mM $MgCl_2$, and 0.01% gelatin (primer set I), 10 mM tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol (primer sets II, IV, and V), or 30 mM tricine-HCl (pH 8.4), 2 mM $MgCl_2$, 0.01% gelatin, and 5 mM β-mercaptoethanol (primer set III), supplemented with 500 ng of genomic DNA, 4 mM deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate (dTTP), 1 mM deoxycytidine triphosphate (dCTP), 1 µCi of [α-³²P]dCTP, and 1 unit of Taq DNA polymerase (Boehringer). After initial denaturation for 5 min at 94°C, amplification was done in 30 cycles of 1 min at 94°C, 1 min at 64°C (primer sets I through IV), or 1 min at 58°C (primer set V) and 1 min at 72°C

with a final elongation step for 6 min at 72°C. The PCR products were separated on a 6% polyacrylamide gel, with or without 10% (w/v) glycerol, for 4 hours at 35 W at 4°C. After vacuum drying, the gels were exposed to Xomat films (Kodak) for 18 hours at room temperature. The sequence of PCR products was established for both strands as described (*17*). Primers IIa, IIb, and IIIb were deduced from mouse *Pou3f4* sequences. The nucleotides that do not match the human *POU3F4* sequence are underlined above. The sequence shown in Fig. 3B was established with the use of ³²P end-labeled primer Va with the cycle sequencing kit (Boehringer).
21. Mutations are indicated with the single-letter amino acid abbreviations; thus, Leu³¹⁷ → Trp is indicated by L317W. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Requirement of MADS Domain Transcription Factor D-MEF2 for Muscle Formation in *Drosophila*

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Members of the myocyte enhancer binding factor–2 (MEF2) family of MADS (MCM1, agamous, deficiens, and serum response factor) box transcription factors are expressed in the skeletal, cardiac, and smooth muscle lineages of vertebrate and *Drosophila* embryos. These factors bind an adenine-thymidine–rich DNA sequence associated with muscle-specific genes. The function of MEF2 was determined by generating a loss-of-function of the single *mef2* gene in *Drosophila* (*D-mef2*). In loss-of-function embryos, somatic, cardiac, and visceral muscle cells did not differentiate, but myoblasts were normally specified and positioned. These results demonstrate that different muscle cell types share a common myogenic differentiation program controlled by MEF2.

The three major muscle cell types (skeletal, cardiac, and smooth) express many of the same muscle-specific genes, which suggests that they may use a common myogenic regulatory program that directs muscle gene transcription. However, each muscle cell type is unique with respect to the muscle proteins expressed, contractile properties, ability to divide, and morphology. Thus, if a common myogenic program exists, it must be modified by additional regulatory factors to generate muscle cell diversity.

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In vertebrates, skeletal muscle formation is controlled by a family of myogenic basic helix-loop-helix (bHLH) proteins, which includes MyoD, myogenin, myf5, and MRF4 [reviewed in (*1*)]. When expressed ectopically in nonmuscle cell types, these factors can activate skeletal muscle gene expression. The myogenic bHLH factors are exclusively expressed in skeletal muscle. Therefore, other regulators must control muscle gene expression in cardiac and smooth muscle. MEF2, which recognizes an A-T–rich DNA sequence associated with skeletal, cardiac, and smooth muscle genes, may be such a factor (*2*). Four *mef2* genes, designated *mef2a*, *-b*, *-c*, and *-d*, have been cloned from several vertebrate species (*3, 4*). MEF2 factors, also known as related to serum response factors (RSRFs) (*4*), belong to the MADS family of transcription factors