Pancreatic agenesis attributable to a single nucleotide deletion in the human *IPF1* gene coding sequence

Pancreatic agenesis in humans is a rare disorder; only eight cases have been reported in the literature[8–14]. Recently, a female Caucasian infant was described in which the diagnosis of pancreatic agenesis was made shortly after birth and appropriate therapy was instituted[8]. The infant was underweight for gestational age and presented with neonatal diabetes mellitus at birth and, at age 18 days, with pancreatic exocrine insufficiency. Ultrasound examination revealed that the pancreas was absent. After replacement of insulin and pancreatic enzymes was begun, she developed normally and continues to do well at 5 years of age. There is a strong family history of non-insulin dependent diabetes mellitus (NIDDM). The patient's father was recently diagnosed with NIDDM and the mother had glucosuria during pregnancy. The paternal grandmother and great grandmother have NIDDM, as do a maternal uncle, grandfather and numerous siblings of the maternal grandfather.

To examine the *IPF1* gene for possible mutations in patients, the nucleotide sequence of the normal human gene was isolated and characterized. Two human genomic phage clones representing overlapping segments of the human *IPF1* gene (13q12.1)[15,16] were isolated, mapped and partially sequenced. The contig consists of 15 kb and encompasses the entire coding region, comprising two exons based on a comparison with the human cDNA sequence[15,16] (Fig. 1*a*). The coding sequences of the human and mouse genes exhibit 100% amino acid identity in the homeodomain and high amino acid homology in the flanking domains (86%).

Our karyotype analysis of the patient revealed a normal complement of chromosomes (46 XX) without macrodeletions or translocations (data not shown). Southern blot analysis of her genomic DNA with a probe spanning the coding exons and flanking regions showed no rearrangements within the *IPF1* gene (data not shown). The nucleotide sequence of both exons was determined by PCR amplification of the patient's genomic DNA followed by subcloning and sequencing. A deletion of a single cytosine was found in codon 63 of exon 1 resulting in a frame shift of translation that terminates 59 codons downstream (Fig. 1*b*; Fig. 2*a,b*). This cytosine deletion was observed in 100% of the PCR subclones of exon 1 (Table 1) suggesting homozygosity. Evidence for this deletion was obtained using four different sequencing primers which revealed the point deletion on both DNA strands of the PCR subclones. No mutations were detected in exon 2.

The patient has no siblings. Our analysis of the parents' genomic DNA, however, indicated that each parent was heterozygous for the same cytosine deletion in codon 63. Approximately half of the exon 1 PCR subclones from each parent possess the point deletion (Table 1). As no restriction enzyme site was gained or lost as a result of this point deletion, we optimized conditions for allele-specific hybridization to confirm the homozygous versus heterozygous presence of the mutation in the patient and her parents. A mutation-specific oligonucleotide hybridized to the same amount of parental exon 1 PCR product with an intensity intermediate between the patient (homozygote) and a normal control (Fig. 1*c*). Similarly, a wild-type sequence oligonucleotide hybridized to parental DNA with an intermediate intensity as well (Fig. 1*c*). Similar results were obtained when we employed the higher fidelity *Pfu* DNA polymerase in the PCR reactions. Collectively, these data indicate that the parents are heterozygous, and then proband, homozygous, for the cytosine deletion in codon 63. We did not detect the cytosine deletion in any of 92 normal control subjects tested, decreasing the likelihood that the point deletion is simply a DNA sequence polymorphism (data not shown).

Premature termination of translation is predicted to occur aminoproximal to the essential DNA-binding domain and give rise to a product of 13.2 kD (Fig. 2*a,b*). The transactivation domain is required for synergistic interaction with another transcription factor, E47, in the transcriptional regulation of the insulin gene[6]. The truncated protein is thus missing the homeodomain as well as the FPWMK motif required for the interaction with another homeoprotein, PBX (Fig. 2*b*)[17]. The mutant protein could potentially interfere with normal IPF1 function by competing for transcriptional coactivators. It is unlikely, however, that the truncated protein is transported to the nucleus, because the nuclear localization signal resides in the homeodomain[18].

To directly determine whether the proposed translational reading frame imposed by the cytosine deletion in codon 63 results in termination after 59 additional codons, we prepared expression plasmids containing the mutation, transfected them into Cos-1 cells, and analysed expressed protein products by western blot analysis and by immunoprecipitation of [^{35}S]-labelled proteins using antisera specific for the detection of either N- or C-terminal sequences of IPF1 (Fig. 2*c,d*). The N-terminal specific anti-

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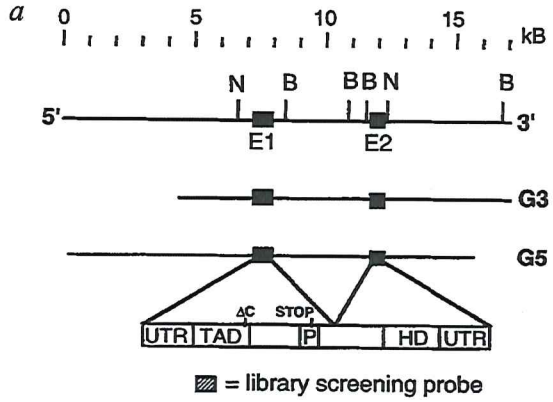

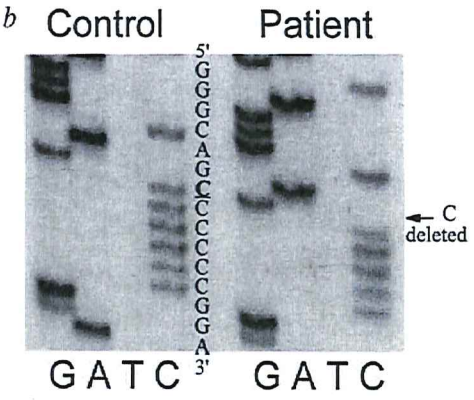

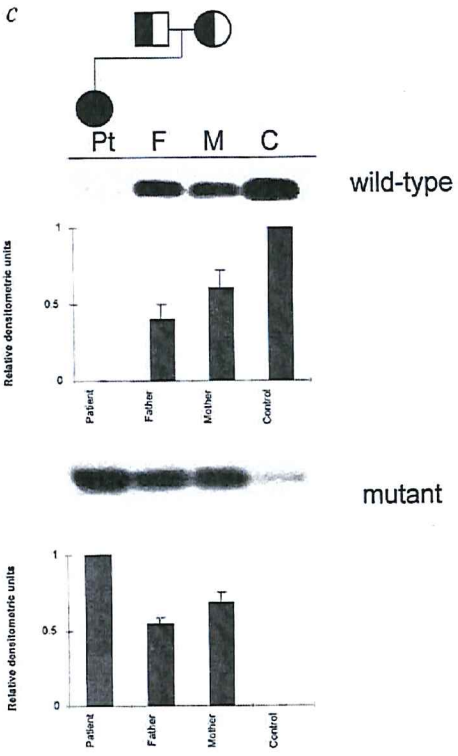

Fig. 1 Organization of the human *IPF1* gene; a single nucleotide deletion in the protein-coding sequence of the *IPF1* gene in the patient with pancreatic agenesis. ***a***, Restriction map of overlapping genomic phage inserts, G3 and G5. Restriction sites are: N, *Not*I ; B, *Bam*HI. The library screening probe is indicated by a hatched rectangle. The putative positions of the exons (E1, E2) indicated are based on Southern blot analysis (not shown) of phage DNA with the library-screening probe (hatched bar) and with a [^{32}P]-end-labelled oligonucleotide complementary to a highly conserved portion of the homeodomain. The structure of the mRNA is represented by the open box, where: UTR, untranslated region; TAD, transactivation domain; P, pentapeptide PBX interaction motif; HD, homeodomain. ***b***, DNA sequence analysis of an exon 1 PCR subclone from a control and from the affected patient. The DNA sequence is shown between the two autoradiograms. The deletion mutation in DNA is depicted by an arrow and is indicated in bold underline in the sequence. ***c***, Allele-specific hybridization of Southern blots of amplified genomic DNA for exon 1 was performed to confirm the mutation status in the patient (Pt) and both parents (F=father, M=mother) as well as a normal control (C). Equal amounts of DNA were loaded in each lane, verified by quantitation of ethidium bromide fluorescence of the agarose gel prior to Southern transfer. Both parents exhibited an intermediate intensity of hybridization to the wild-type sequence and mutation-specific oligonucleotides, supporting heterozygote status. The affected patient exhibited no hybridization with the wild-type oligonucleotide, while the normal control subject had no hybridization with the mutation-specific oligonucleotide. The pedigree is depicted above the results. The graphs below each blot represent densitometry of autoradiograms and/or quantitation of phosphorimager scans. In the case of the wild-type oligonucleotide probe (n=2), the patient's density of hybridization was set to 0.0 and the normal to 1.0 (based on sequencing of PCR subclones; see Table 1) after an initial background correction. Conversely, for the mutation-specific oligonucleotide (n=4), the patient's density of hybridization was set to 1.0 and that of the normal to 0.0.

serum detected proteins of 42–43 kD by western blot analysis (Fig. 2*c*, lanes 3,5) and by immunoprecipitation (Fig. 2*d*, lanes 4,6) in Cos-1 cells transfected with wild-type IPF1 plasmids. These proteins are also detected by a C-terminal specific antiserum (Fig. 2*c*, lanes 10,11; Fig. 2*d*, lanes 10,11) and are present in nuclear extracts (Fig. 2*c*, lanes 4,6). In contrast, Cos cells transfected with the mutant IPF1 plasmid expressed only a 16-kD protein (Fig. 2*c*, lane 7; Fig. 2*d*, lane 8). This truncated protein was not detected in nuclear extracts (Fig. 2*c*, lane 8) and it could not be visualized with a C-terminal specific antiserum (Fig. 2*c*, lane 12; Fig. 2*d*, lane 12). We confirmed its predicted cytoplasmic localization by immunocytochemistry of transfected Cos-1 cells using the N-terminal antiserum (Fig. 2*e*). By electrophoretic mobility shift assays, we showed

Table 1 • *IPF1* point deletion analysis

Subject	PCR subclones analysed	Clones with ΔC in codon 63 (%)
Patient	16	16 (100)
Father	13	8 (61)
Mother	12	6 (50)
Normal Control	14	0 (0)

The number and % of exon 1 PCR subclones analysed possessing the point deletion for the patient, both parents and a normal control are shown.

the expected binding of transfected cell nuclear extracts to known IPF1 binding sites for wild-type IPF1 proteins but failed to show specific binding in nuclear extracts from mutant IPF1 transfected cells (data not shown). These data indicate that a truncated protein lacking the homeodomain (and nuclear localization signal) is produced as a result of the mutation.

The homozygous disruption of *IPF1* in a human case of pancreatic agenesis further underscores the critical role that IPF1 plays in normal pancreatic development. Previously, pancreatic agenesis was demonstrated in homozygous mice harbouring a targeted disruption of exon 2 of the *Ipf1* gene[4,7]. Offield *et al.*[7], however, found that the pancreatic buds do form in the homozygous null mutants, but that they undergo only limited ductal outgrowth and branching, with a blockage of both pancreatic endocrine and exocrine differentiation. The proximal duodenum showed a local absence of the normal columnar epithelial lining, villi, and Brun-

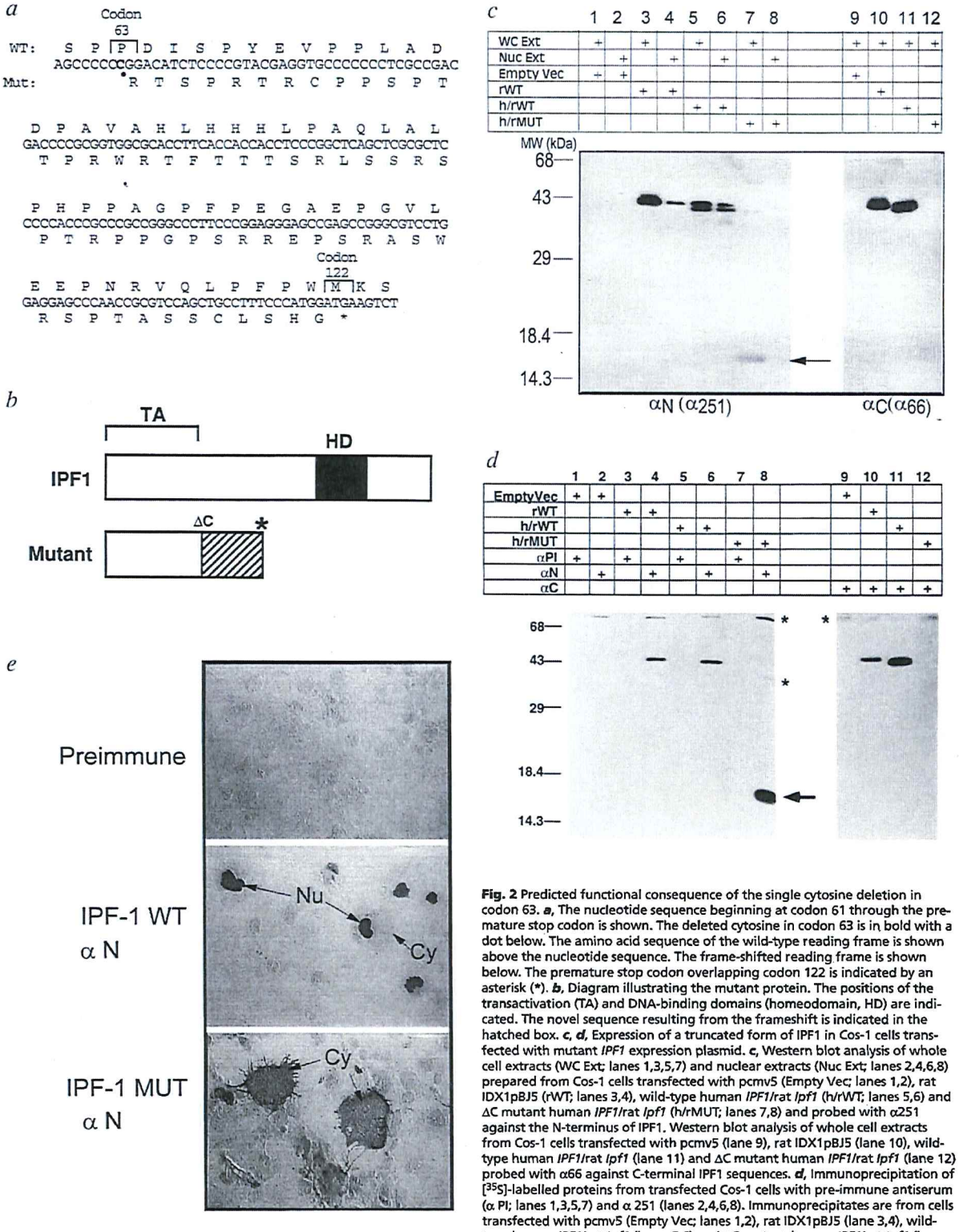

Fig. 2 Predicted functional consequence of the single cytosine deletion in codon 63. ***a***, The nucleotide sequence beginning at codon 61 through the premature stop codon is shown. The deleted cytosine in codon 63 is in bold with a dot below. The amino acid sequence of the wild-type reading frame is shown above the nucleotide sequence. The frame-shifted reading frame is shown below. The premature stop codon overlapping codon 122 is indicated by an asterisk (*). ***b***, Diagram illustrating the mutant protein. The positions of the transactivation (TA) and DNA-binding domains (homeodomain, HD) are indicated. The novel sequence resulting from the frameshift is indicated in the hatched box. ***c***, ***d***, Expression of a truncated form of IPF1 in Cos-1 cells transfected with mutant *IPF1* expression plasmid. ***c***, Western blot analysis of whole cell extracts (WC Ext; lanes 1,3,5,7) and nuclear extracts (Nuc Ext; lanes 2,4,6,8) prepared from Cos-1 cells transfected with pcmv5 (Empty Vec; lanes 1,2), rat IDX1pBJ5 (rWT; lanes 3,4), wild-type human *IPF1*/rat *Ipf1* (h/rWT; lanes 5,6) and ΔC mutant human *IPF1*/rat *Ipf1* (h/rMUT; lanes 7,8) and probed with α251 against the N-terminus of IPF1. Western blot analysis of whole cell extracts from Cos-1 cells transfected with pcmv5 (lane 9), rat IDX1pBJ5 (lane 10), wild-type human *IPF1*/rat *Ipf1* (lane 11) and ΔC mutant human *IPF1*/rat *Ipf1* (lane 12) probed with α66 against C-terminal IPF1 sequences. ***d***, Immunoprecipitation of [^{35}S]-labelled proteins from transfected Cos-1 cells with pre-immune antiserum (α PI; lanes 1,3,5,7) and α 251 (lanes 2,4,6,8). Immunoprecipitates are from cells transfected with pcmv5 (Empty Vec; lanes 1,2), rat IDX1pBJ5 (lane 3,4), wild-type human *IPF1*/rat *Ipf1* (lanes 5,6) and ΔC mutant human *IPF1*/rat *Ipf1* (lanes 7,8) using antiserum 253. Immunoprecipitation of ^{35}S-labelled proteins from whole cell extracts of transfected Cos-1 cells using antiserum 251: pcmv5 (lane 9, rat IDX1pBJ5 (lane 10), wild type human *IPF1*/rat *Ipf1* (lane 11), ΔC mutant human *IPF1*/rat *Ipf1* (lane 12). The truncated protein is indicated by an arrow. Asterisks (*) denote non-specific cross-reacting proteins endogenous to Cos-1 cells. ***e***, Immunocytochemistry of transfected Cos-1 cells stained with pre-immune serum (top), α251 (N-terminal) (wild-type IPF1, centre) or mutant IPF1 (bottom). Examples of nuclei (Nu) and cytoplasm (Cy) are indicated (arrows).

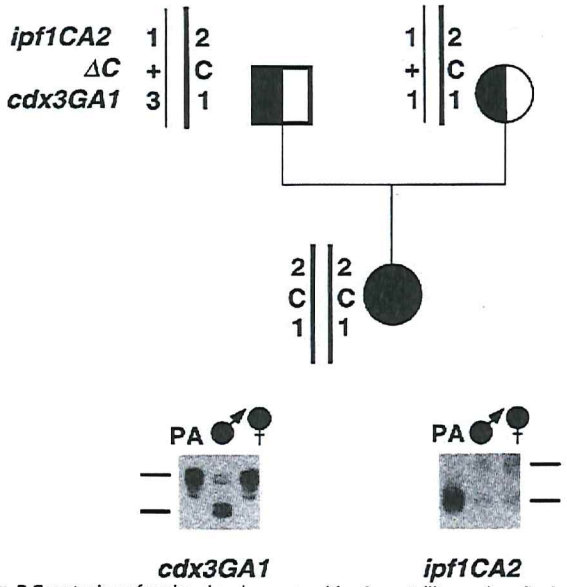

Fig. 3 Genotyping of proband and parents with microsatellite markers flanking the *IPF1* gene. Nearby markers, *Ipf1CA2* and *cdx3GA1*, were used to genotype genomic DNA from the proband and her parents as well as several unrelated controls. Representation of scored allelles are shown above. Below, autoradiogram of end-labelled PCR products resolved on a 5% denaturing sequencing gel (PA, proband; +, wild-type *IPF1* allele; C, allele possessing the cytosine deletion in codon 63).

ner's glands, which were replaced by GLUT2-positive cuboidal epithelium[7]. We have observed strong expression of a β-galactosidase transcriptional reporter gene driven by the *IPF1* promoter in the gall bladder and common bile duct of transgenic mice as well as in the pancreas, duodenum, Brunner's glands, and distal stomach (our unpublished observations). It would be interesting to know whether abnormalities in the development of the duodenum, distal stomach, common bile duct and gall bladder also exist in our patient.

Given the rarity of pancreatic agenesis, an extended haplotype analysis using two closely linked microsatellite markers (*ipf1CA2* and *cdx3GA1*)[16] was performed in the parents and proband. This analysis was consistent with homozygosity by descent of the chromosomal segment containing *IPF1*ΔC (Fig. 3). Thus, as expected for a rare allele ocurring in the homozygous state, both of the proband's *IPF1*ΔC alleles are likely to have been derived from a single common ancestor. There was no clear history of consanguinity in our patient.

In addition to pancreatic agenesis, three cases of severe pancreatic hypoplasia and one of complete absence of the islets of Langerhans in the context of a normal-sized pancreas have been reported[19–21]. It is tempting to speculate that the phenotypes of pancreatic hypoplasia and selective agenesis of the endocrine pancreas (islets) might represent a spectrum of less severe *IPF1* mutations which may impair, but not abolish, IPF1 functions. Alternatively, these disorders may be a consequence of mutations in other factors, transcriptional or otherwise, that are essential for full development of the pancreas.

Abnormal IPF1 function may also be a candidate factor in the development of adult onset diabetes mellitus. Chronic exposure of β-cell lines to high glucose results in decreased insulin gene transcription, accompanied by diminished binding of IPF1 to the glucose-responsive FLAT element of the insulin promoter[22–23] (our unpublished observations). Because IPF1 is a transactivator of insulin gene transcription, physiological defects in insulin synthesis under certain conditions may exist in heterozygous *IPF1* +/– animals. Such abnormalities have not yet been examined. This is of particular interest given the heterozygous status of both parents of the affected patient and the strong history of diabetes mellitus on both sides of the family. Whether this reflects abnormalities in glucose homeostasis which relate to carrier status of the *IPF1* mutation is unknown and will require further investigation.

Methods

Nomenclature. The human genome nomenclature committee has designated *IPF1* as the gene name for the homeodomain protein also known as *IDX-1*, *STF-1* and *PDX-1*. *IDX-1* is the gene name used in our laboratory. Consequently plasmid names and other related reagents carry that designation.

Genomic cloning of human *IPF1*. Recombinant phage (50,000) from a human genomic library (HUVEC, l DASH II, Stratagene) were screened using a [^{32}P]-labelled PCR-generated probe corresponding to the 5' end of the open reading frame in the mouse *Ipf1* cDNA (Fig. 1; hatched box) using standard methods[24]. This probe was chosen because of the high degree of sequence similarity between rat and mouse *Ipf1* cDNAs in this region and to avoid the homeodomain which is highly conserved among all members of the homeodomain family. Human genomic DNA revealed a single band (not shown)when southern blotted with this probe, indicating the likelihood that a single human gene contains sequences homologous to the probe. Four rounds of screening resulted in two plaque-pure clones, G3 and G5, from which phage DNA was prepared. Phage inserts were mapped by Southern blot hybridization of partial restriction enzyme digests with [^{32}P]-labelled oligonucleotide probes complementary to phage sequences adjacent to the cloning site of λ DASH II (Fig. 1).

Analysis of patient DNA. Genomic DNA was isolated from peripheral blood samples of the patient, parents and normal control subjects by standard methods[24]. Karyotype analysis was performed by the Cytogenetics Laboratory at Massachusetts General Hospital. Southern blot analysis was performed according to standard procedures. PCR amplification of exon 1 was performed in 2 sequential rounds using nested primers. Round 1 amplimers were: PCR4, 5'–GGAATTCGGCTGTGGTTCCCTCT–3' and S16, 5'–CAGAGAGAAGGCTCCTG–3'. Round 2 amplimers were: S17b, 5'–AGCGAGCAGGGGTGGCG–3' and S18, 5'–GGGACGCTTGGAGGTAA–3'. Each round consisted of 26 cycles with denaturation at 97.5 °C for 15 s, annealing at 60 °C for 90 s, and extension at 72 °C for 2 min. Where indicated, the resultant fragment (610 bp) was subcloned (TA cloning vector PCRII; Invitrogen) and sequenced (Sequenase 2.0; United States Biochemicals). Exon 2 screening was performed in 3 overlapping PCR fragments. Amplimers were: (A) PCR2, 5'–CGGGATCCGCCGAG-CTTCTTGTC–3' and PCR3, 5'–GGATTCTGGGGCTTGGTGGCTC–3' (annealing temperature = 60 °C); (B) S1, 5'–CGCCTACGCTGCGGAGC–3' and S14, 5'–AGAAGCTCCTCGCCGGAG–3' (annealing temperature = 37 °C); (C) S12, 5'–AGGAGGAGGACAAGAAGC–3' and PCR7, 5'–CGGATCCTAGGGCCTCTGCTCC–3' (annealing temperature = 37 °C).

For allele-specific hybridization, wild-type sequence (5'–ATGTCCGGGGGGCTGCC–3') and mutation-specific (5'–CAGGGCAGCCCCCGGAC–3') oligonucleotides were end-labelled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. Hybridizations were performed in 6× SSC, 5× Denhardt's, 1% SDS, 50mM sodium phosphate, pH 6.8 and 100 μg/ml denatured salmon sperm DNA at 37 °C for 6–15 h. Washes were: 4 × 10 min in 5× SSC, 0.1% SDS at room temperature, followed by 1 × 10 min in 5× SSC, 0.1% SDS at 37 °C, 2 × 30 min in 3M tetramethylammonium chloride, 50 mM Tris, pH 8.0, 0.2% SDS at 52 °C and a brief rinse in 2× SSC, 0.1% SDS at room temperature[25].

Expression studies in Cos-1 cells. As a full-length human *IPF1* cDNA was not available, a fusion *IPF1* expression construct was created in which the unique conserved *Afl*III site was used to fuse exon 1 from human *IPF1* in frame to the remainder of exon 1 and exon 2 of rat *Ipf1* cDNA[1]. The wild-type human *IPF1* portion was the 0.5-kb *Eco*RI-*Afl*III fragment from a fully sequenced TA subclone of a nonaffected individual, while the mutation-containing exon 1 fragment was taken from a sequenced TA subclone from the proband. Thus, the 5' end of the open reading frame

including the mutation, ensuing 59 codons and the premature stop codon are all derived from human *IPF1*. The rat-derived portion was the 0.9-kb *Afl*III-*Bam*HI fragment of rat *Ipf1* (plasmid: idx1pBJ5)[18]. These fragments were ligated into the *Eco*RI and *Bam*HI double-digested eukaryotic expression vector pcmv5 (ref. 26). The resulting wild-type human rat fusion plasmid results in an IPF1 protein with similar MW (43 kD)(Fig. 2 *c,d*) and DNA-binding properties in EMSAs as rat IPF1 (data not shown).

The N-terminal IPF1 antiserum (α251) was a rabbit polyclonal antiserum raised against the the first 12 amino acids of rat IPF1 (100% conserved between rat and human). Two C-terminal antisera were utilized, Hm66 (ref. 1) directed against GST IDX-1(164–283) which recognizes predominantly homeodomain sequences and α253, directed against the C-terminal 12 amino acids of IPF1.

Transfections into Cos-1 cells were performed using diethylaminoethyl-dextran and a brief dimethylsulfoxide shock[27]. Western blot analysis of whole cell[1] or nuclear extracts[28] was performed as described. For immunoprecipitations, cells were labelled with [^{35}S] methionine/cysteine (Trans [^{35}S]-label ICN) for 1 h prior to collection. Immunoprecipitation of whole cell lysates was carried out as described[29] using the N- and C-terminal antisera described above. Transfected Cos-1 cells were also examined by immunocytochemistry using the N-terminal antiserum (α251) as described[1].

Microsatellite marker analysis. Amplimers and PCR conditions for simple sequence repeat polymorphisms, *ipf1CA2* and *cdx3GA1*, were as described[16]. For *ipf1CA2*, 3 distinct alleles were resolved in the proband, parents and four normal controls. For *cdx3GA1*, 5 alleles were observed.

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