Am. J. Hum. Genet. 63:347–359, 1998

Identification of *PEX10,* the Gene Defective in Complementation Group 7 of the Peroxisome-Biogenesis Disorders

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Summary

The peroxisome-biogenesis disorders (PBDs) are a group of genetically heterogeneous, lethal diseases that are characterized by neuronal, hepatic, and renal abnormalities; severe mental retardation; and, in their most severe form, death within the 1st year of life. Cells from all PBD patients exhibit decreased import of one or more classes of peroxisome matrix proteins, a phenotype shared by yeast *pex* mutants. We identified the human orthologue of yeast *PEX10* and observed that its expression rescues peroxisomal matrix-protein import in PBD patients' fibroblasts from complementation group 7 (CG7). In addition, we detected mutations on both copies of *PEX10* in two unrelated CG7 patients. A Zellweger syndrome patient, PBD100, was homozygous for a splice donor–site mutation that results in exon skipping and loss of 407 bp from the *PEX10* open reading frame. A more mildly affected neonatal adrenoleukodystrophy patient was a compound heterozygote for a missense mutation in the *PEX10* zinc-binding domain, H290Q, and for a nonsense mutation, R125ter. Although all three mutations attenuate *PEX10* activity, the two alleles detected in the mildly affected patient, PBD052, encode partially functional PEX10 proteins. *PEX10*-deficient PBD100 cells contain many peroxisomes and import peroxisomal membrane proteins but do not import peroxisomal matrix proteins, indicating that loss of *PEX10* has its most pronounced effect on peroxisomal matrix-protein import.

Received January 21, 1998; accepted for publication June 8, 1998; electronically published July 10, 1998.

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0002-9297/98/6302-0010$02.00

Introduction

Peroxisomes, which are present in almost all eukaryotic cells, are single-membrane–bound organelles that participate in numerous metabolic processes (Lazarow and Fujiki 1985). In humans, these processes include β-oxidation of fatty acids; synthesis of bile acids, cholesterol, and plasmalogens; and a variety of H_2O_2-producing oxidation reactions (van den Bosch et al. 1992). The enzymes responsible for these functions are encoded by nuclear genes, synthesized on cytoplasmic ribosomes, and posttranslationally imported into peroxisomes (Lazarow and Fujiki 1985). The import process, which is conserved from yeast to humans (Gould et al. 1990), is mediated by *cis*-acting peroxisome-targeting signals (PTS). For peroxisomal matrix proteins, import is most commonly specified by the type-1 PTS (PTS1), which consists of a C-terminal tripeptide of the sequence Ser-Lys-Leu_{COOH} (SKL_{COOH}) or a conservative variant (Gould et al. 1989; Subramani 1993). Less commonly used is the PTS2, a 9-amino-acid signal located near the amino terminus of proteins with a consensus sequence RLX_5H/QL (Subramani 1993; Swinkels et al. 1991). Although peroxisomal membrane proteins also contain *cis*-acting sequence elements that target them to peroxisomes (Dyer et al. 1996), a consensus sequence has yet to be established.

The identification of *trans*-acting components of the peroxisomal protein import machinery has relied heavily on genetic screens and selections in yeast. These studies have identified at least 16 different genes (*PEX*) and gene products (peroxins) that are required for peroxisome biogenesis and normal matrix-protein import (Distel et al. 1996; Albertini et al. 1997; Eitzen et al. 1997) (S.J.G., unpublished results). Information derived from studying yeast *pex* mutants, which have a defect in the import of one or more classes of peroxisome matrix proteins, has shaped our understanding of peroxisome protein import. Import is thought to begin with two predominantly cytoplasmic receptors, PEX5 and PEX7, that recognize newly synthesized proteins that contain a PTS (McCollum et al. 1993; Marzioch et al. 1994; Dodt et al. 1995; Dodt and Gould 1996; Elgersma et al. 1996;

Gould et al. 1996; Braverman et al. 1997). PEX5 binds PTS1-containing proteins, whereas PEX7 binds PTS2-containing proteins, and together these receptors direct newly synthesized matrix proteins to the peroxisome. The receptor-bound proteins are recognized by membrane-anchored docking factors on the cytoplasmic face of peroxisome membranes and subsequently are translocated into the peroxisome matrix, presumably through a membrane pore, although there is no evidence yet for such a structure.

The peroxisome-biogenesis disorders (PBDs) are a genetically heterogeneous group of lethal human diseases that are caused by an inability to import peroxisomal proteins (Lazarow and Moser 1995), the same phenotype displayed by yeast *pex* mutants. The PBDs include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD), and rhizomelic chondrodysplasia punctata (RCDP) (Lazarow and Moser 1995), and cell fusion studies have divided the PBDs into 10 complementation groups (CGs) (Moser et al. 1995). ZS, NALD, and IRD are characterized by similar symptoms, with ZS representing the most severe form and with NALD and IRD representing progressively less severe phenotypic variants (Lazarow and Moser 1995). Seven of the known CGs contain both mildly and severely affected patients, suggesting that the different phenotypes can result from mutations in the same gene (Moser et al. 1995). The phenotype of classical RCDP patients, which all belong to CG11, is somewhat distinct from the phenotypic spectrum of ZS, NALD, and IRD and is caused by defective import of PTS2-containing proteins (Braverman et al. 1997; Motley et al. 1997; Purdue et al. 1997).

Given the conserved nature of the peroxisomal protein-import process (Gould et al. 1990), it is not surprising that the PBDs are caused by mutations in human orthologues of yeast *PEX* genes. This was first demonstrated by the identification of human *PEX5,* the gene mutated in PBD CG2 (Dodt et al. 1995; Wiemer et al. 1995), and has been confirmed by the identification of human *PEX1, PEX6, PEX7,* and *PEX12* as the genes defective in CG1 (Portsteffen et al. 1997; Reuber et al. 1997), CG4 (Fukuda et al. 1996; Yahraus et al. 1996), CG11 (Braverman et al. 1997; Motley et al. 1997; Purdue et al. 1997), and CG3 (Chang et al. 1997; Okumoto and Fujiki 1997) of the PBDs, respectively. We report here the cloning of the human orthologue of yeast *PEX10* and demonstrate that it is the gene defective in CG7 of the PBDs.

Material and Methods

Cell Lines and Strains

Skin fibroblast cell lines were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies-BRL), as described elsewhere (Slawecki et al. 1995). All PBD cell lines are referred to by patient number and were obtained from Ann Moser (Kennedy Krieger Institute, Baltimore). All bacterial manipulations were performed with *Escherichia coli* strain DH10B.

Cloning of Human PEX10

PCR primers PEX10.1 (5′-AGG TTG AGC TGC TCT CAG ATG TGG-3′) and PEX10.2 (5′-GAT GGG TCC ACC GGA TGA TGC-3′) were designed from the sequence of EST H83562 and were used to amplify a 103-bp fragment of the putative *PEX10* cDNA from a human muscle cDNA library (Clontech). This fragment, which encodes the conserved TLGEEYV motif, was used to probe a λZAP human fetal brain cDNA library (Stratagene) at high stringency (Sambrook et al. 1989). Of the 16 independent library clones that hybridized to the *PEX10* probe, 6 were sequenced from both ends of the cDNA. The entire cDNA insert of one of the clones, 28-2.3, was sequenced and was found to contain an open reading frame (ORF) (GenBank accession number AF060502) similar in size to those of the yeast *PEX10* genes.

Plasmids

To generate the *PEX10* cDNA expression plasmid (cDNA-*PEX10*) a 1.1-kb *Ngo*MI fragment, containing the entire *PEX10* ORF, was excised from cDNA clone 28-2.3, and the Klenow fragment of DNA polymerase (Boehringer Mannheim) was used to make the overhangs blunt. This fragment was ligated with pcDNA3 (Invitrogen) that had been digested with *Eco*RI and that had been made blunt by means of the Klenow fragment. The *PEX10myc* cDNA was created by amplification of the *PEX10* ORF (lacking its stop codon), with the primers PEX10myc5 (5′-CCC GGT ACC ATG GCC CCG GCC GCC GCC AG-3′) and PEX10myc3 (5′-CCC GGA TCC GCG GTA GTG CCG AAG GTA G-3′). The resulting fragment was cleaved with *Asp*718 and *Bam*HI and was cloned in-frame with the myc-epitope-tag–encoding sequence in pcDNA3*myc* (Yahraus et al. 1996). The pcDNA3-*PEX10myc* clone was sequenced, ensuring that no errors were incorporated during PCR amplification. *PEX10* cDNA was synthesized from PBD052 and PBD100 RNA by reverse transcription (RT)–PCR and was cloned into PCR 2.1 (Invitrogen), as described in the "Mutation Analysis" section below. The three mutant cDNAs identified in these patients were excised by *Eco*RI restriction-endonuclease digestion and were inserted into the *Eco*RI site of pcDNA3. The resulting plasmids, pcDNA3-*PEX10*/H290Q, pcDNA3-*PEX10*/R125ter, and pcDNA3-*PEX10*/PBD100, were used in

the relative rescue experiments. pcDNA3-*PEX10*/H290Q contains the entire *PEX10* coding region, with a single nucleotide change, a C→G transversion at nucleotide 870 of the ORF, that results in the H290Q substitution. pcDNA3-*PEX10*/R125ter also carries the entire *PEX10* coding region, although a single nucleotide change, a C→T transition at nucleotide 373 of the ORF, results in a premature-termination codon, R125ter. pcDNA3-*PEX10*/PBD100 carries a shortened form of the *PEX10* cDNA, which is missing 407 bp, nucleotides 194–600 of the ORF, from the middle of the coding region. This fragment corresponds to the major *PEX10* mRNA product of these cells. All PCR reactions were performed with a low–error-rate mixture of polymerases (Expand; Boehringer Mannheim).

Transfections, Indirect Immunofluorescence, Antibodies, and Fluorescence Microscopy

Transfection was performed either by lipofection (Lipofectamine; Life Technologies-BRL) or by electroporation, according to the protocol outlined by Chang et al. (1997). Two days after transfection, the cells were processed for immunofluorescence. The transfected cells were fixed for 20 min in 3% formaldehyde/1 × Dulbecco's PBS solution (DPBS). After fixation, the cells were washed 3 times in 1 × DPBS and were permeabilized by incubation in either 1% Triton X-100/1 × DPBS (standard conditions) or 25 μg/ml digitonin/1 × DPBS (for differential permeabilization experiments), for 5 min. To remove excess detergent, the cells were washed three times in 1 × DPBS, which was followed by a 30-min incubation with primary antibodies at the appropriate dilutions. The cells then were washed seven times in 1 × DPBS and were incubated with the appropriate secondary antibodies for 10 min. To remove excess secondary antibodies, the cells were washed seven times in 1 × DPBS and then were placed on glass slides in 10 μl of mounting solution (1 mg *p*-phenylenediamine/ml, 100 mM Tris pH 8.5, 90% glycerol). Anti-SKL_{COOH} antibodies have been described elsewhere (Gould et al. 1990); anti-myc antibodies were obtained from Boehringer Mannheim; anti-catalase antibodies were obtained from The Binding Site; secondary antibodies were obtained from Jackson Immunochemicals; and anti-peroxisomal membrane protein 70 (PMP70) antibodies were a generous gift from Dr. Suresh Subramani. Micrographs were captured on an Olympus fluorescence microscope.

Mutation Analysis

Genomic DNA was prepared by means of the TurboGen kit (Invitrogen). RNA isolation and RT-PCR was performed as described elsewhere (Michaud et al. 1992). The RT reactions were performed with Superscript II (Life Technologies-BRL) and the PEX10.12 primer (5′-CAG CCA GGG ACA GCT TTC-3′). The entire *PEX10* ORF was amplified from PBD100, PBD052, and control RNA samples by means of PEX10.5 (5′-CTG TGC AAG CAA GGT TAA TC-3′) and PEX10.7 (5′-GTG GCT GCT CGG GAC CAC-3′). The products were T/A cloned into PCR2.1 (Invitrogen), and multiple clones were sequenced. The mutations discovered in cDNA clones were examined also by restriction-endonuclease digestion of PCR-amplified genomic DNA fragments of the *PEX10* gene. Genomic DNA from PBD052 and an unaffected individual was amplified by means of the primers PEX10.15 (5′-CGC TGC ATG CCG TCC TGC CCT ACC-3′) and PEX10.22 (5′-GCC CCT GAG CAG CCA CGC CCA CCT G-3′), and this was followed by restriction digestion with *Bst*EII, a restriction-enzyme site introduced by the R125ter mutation (allele A). Genomic DNA from PBD052 and an unaffected individual also was amplified, by means of the primers PEX10.18 (5′-CGT TTC CAG AAA CCC CCT GTG CAC C-3′) and PEX10.24 (5′-CAC CAC GCG GTG ATG CAC TCC CAG C-3′), and this was followed by restriction-endonuclease digestion with *Pvu*II, a restriction-enzyme site introduced by the H290Q mutation (allele B). The 407-bp deletion in *PEX10* cDNAs from PBD100 suggested an RNA processing defect. We therefore performed a limited analysis of *PEX10* gene structure, and we found that the region deleted from PBD100 *PEX10* cDNAs corresponds to an exon of the *PEX10* gene. Sequence analysis of this exon and flanking intronic sequences revealed the presence of a G→A mutation at the splice-donor site of the downstream intron. Genomic DNA from PBD100 and an unaffected individual was amplified by means of the primers PEX10.16 (5′-CCT GGA GCA GGA GCT GCA GGC TGA C-3′) and PEX10.32 (5′-CCT TGA CAC AGA TGC TGG ATG TAG AAC C-3′), which was followed by restriction digestion with *Sna*BI, a restriction-enzyme site destroyed by the G→A mutation.

Relative Rescue Experiments

For analysis of *PEX10myc* activity, PBD100 cells were transfected with pcDNA3, pcDNA3-*PEX10,* and pcDNA3-*PEX10myc.* Two days later, each cell population was examined by indirect immunofluorescence, to determine the percentage of cells importing anti-SKL–reactive proteins into peroxisomes. To assess the effect of the mutations in PBD052 and PBD100, pcDNA3-*PEX10*/H290Q, pcDNA3-*PEX10*/R125ter, pcDNA3-*PEX10*/PBD100, pcDNA3-*PEX10,* and pcDNA3 each were cotransfected with pcDNA3 *PAHXmyc,* into PBD100 cells. Double indirect immunofluorescence was performed 2 d later with anti-myc and anti-catalase antibodies. The number of cells dis-

playing peroxisomal catalase staining was recorded for each transfection, as were both the number of cells displaying peroxisomal PAHXmyc staining and the total number of cells that expressed PAHXmyc.

Results

Identification of Human PEX10

Pichia pastoris Pex10p (PpPex10p) is essential for peroxisomal matrix-protein import (Kalish et al. 1995) and contains a C-terminal C_3HC_4 zinc-binding domain necessary for PpPex10p function. To identify the human *PEX10* gene, we searched for human ESTs capable of encoding proteins with significant similarity to PpPex10p. However, these attempts were unsuccessful, primarily because the only similarities detected were in the C_3HC_4 domain, and we were unable to discern the human PEX10 orthologue from the large number of other C_3HC_4 proteins. Therefore, we modified our strategy and instead searched for *PEX10* orthologues in other species. A screen of the nonredundant protein databases led to the identification of putative PEX10 homologues from *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (BLAST *P* values of 1.9^{-74} and 1.0^{-17}, respectively). We rescreened dbEST with the sequence of PEX10 from *C. elegans,* an evolutionary relative closer to humans, and identified one candidate human *PEX10* cDNA (GenBank accession number H83562; clone 249368) that encoded a 7-amino-acid segment, TLGEEYV. This sequence is present in the *P. pastoris* (Kalish et al. 1995), *Hansenula polymorpha* (Tan et al. 1995), *S. cerevisiae,* and *C. elegans PEX10* gene products but not in any other known protein. Unfortunately, the sequence of clone 249368 did not correspond to the sequence of EST H83562 deposited in GenBank. Thus, we used primers based on the EST sequence to amplify a 103-bp fragment of the candidate *PEX10* cDNA from a human muscle cDNA library. This fragment contains the TLGEEYV motif and was used to probe a human fetal brain cDNA library. The fragment was amplified and was used to probe a human fetal brain cDNA library. Multiple *PEX10* cDNA clones were identified and sequenced, and one contained an ORF that encodes a protein that is similar in size to the previously identified yeast PEX10 proteins. An alignment between the predicted human PEX10 sequence and those of the *P. pastoris, S. cerevisiae,* and *C. elegans PEX10* gene products shows that the TLGEEYV region and the zinc-binding domain are the most highly conserved regions of the protein (fig. 1). Human PEX10 is predicted to have two membrane-spanning domains, amino acids 51–67 and 219–239.

Human PEX10: A Peroxisomal Protein

To determine whether human PEX10 shared the peroxisomal membrane localization reported for yeast PEX10 proteins (Kalish et al. 1995; Tan et al. 1995), we created a modified version of the *PEX10* cDNA encoding the 10-amino-acid c-myc epitope at the 3′ end of the ORF (*PEX10myc*). *PEX10myc* was expressed in normal human fibroblasts and was found to colocalize with PMP70, a known integral peroxisome membrane protein (Kamijo et al. 1990), demonstrating that human PEX10 is a peroxisomal protein (fig. 2*A* and *B*).

Differential permeabilization experiments were used to assess the topology of PEX10 in the peroxisome. Normal human fibroblasts were transfected with pcDNA3-*PEX10myc* and, 2 d later, were processed for indirect immunofluorescence by means of a technique in which the cells were permeabilized with limiting amounts of digitonin instead of with 1% Triton X-100. This treatment permeabilizes the cell membrane while leaving peroxisome membranes intact, thus allowing antibodies to bind cytoplasmic antigens but not intraperoxisomal antigens. Double-labeling experiments with anti-myc (fig. 2*C*) and anti-SKL (fig. 2*D*) antibodies revealed that the C-terminus of PEX10myc is exposed to the cytosol under conditions that prevent detection of intraperoxisomal SKL-containing proteins. Double-labeling experiments with anti-myc (fig. 2*E*) and anti-PMP70 antibodies (fig. 2*F*), which bind the cytoplasmic tail of PMP70, demonstrated colocalization of PEX10myc and PMP70 under these conditions, confirming that the punctate distribution of PEX10myc staining (as seen in fig. 2*C*) corresponds to peroxisomes and that peroxisomes remain intact during treatment with digitonin. These results indicate that PEX10 is a peroxisomal membrane protein with its C-terminus oriented toward the cytoplasm.

Accordingly, PEX10 should localize to peroxisomes in cell lines that do not import matrix proteins but that do import peroxisomal membrane proteins. One such cell line is PBD062, which lacks functional *PEX2* (Shimozawa et al. 1992; Chang et al. 1997). PBD062 cells were transfected with pcDNA3-*PEX10myc* and were processed for double indirect immunofluorescence with anti-myc and anti-PMP70 antibodies. The colocalization of PEX10myc (fig. 2*G*) with PMP70 (fig. 2*H*) in these cells demonstrated that the targeting of human PEX10 to peroxisomes is independent of the matrix-protein–import machinery and provides additional evidence that PEX10 is a peroxisomal membrane protein.

Expression of PEX10: *Restoration of Import in CG7 Cell Lines*

Given that PpPex10p is required for peroxisome biogenesis, it is likely that mutations in human *PEX10*

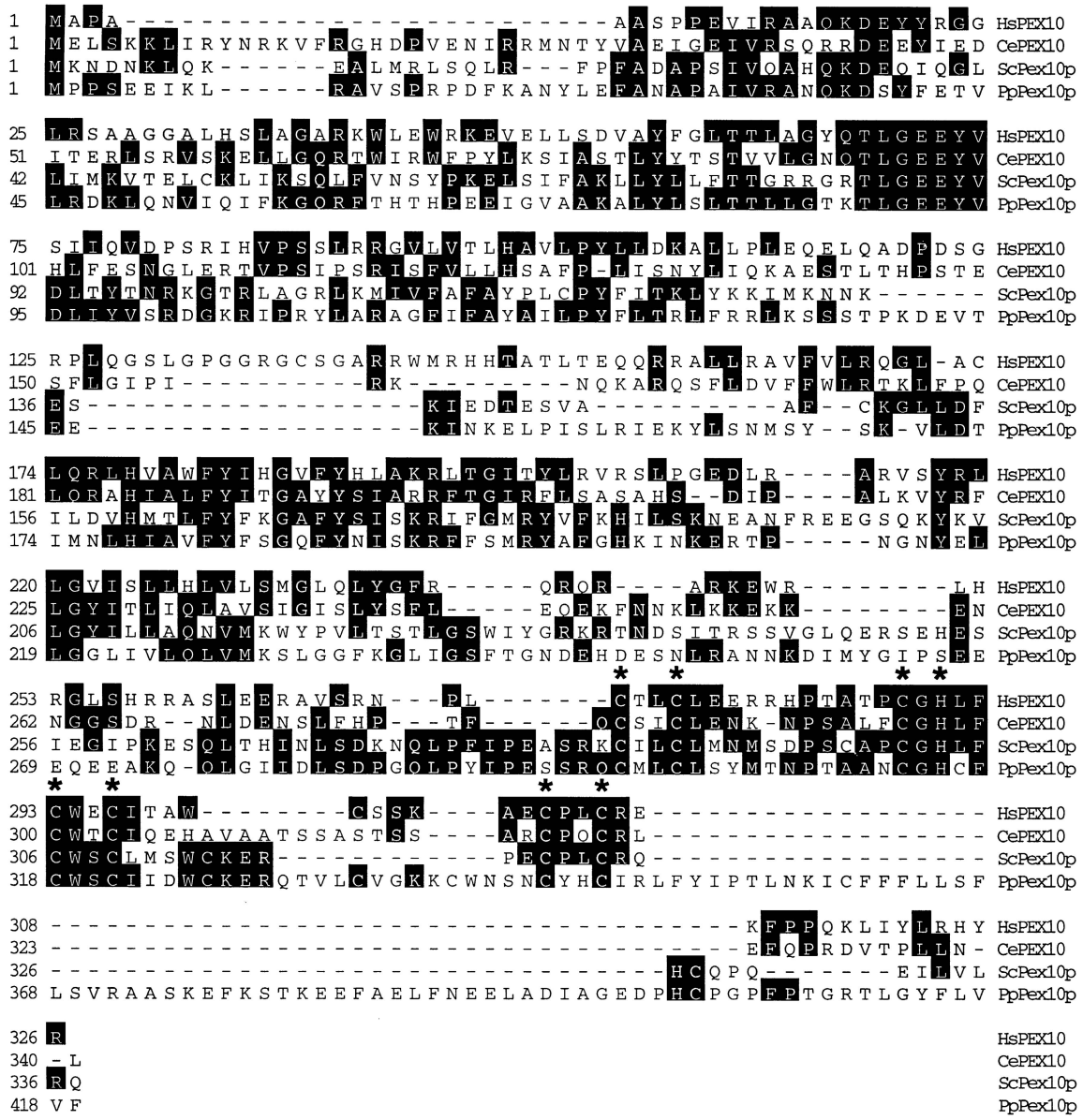

Figure 1 Alignment of deduced *Homo sapiens, C. elegans, S. cerevisiae,* and *P. pastoris PEX10* gene products. Sequence alignment was performed by means of MegAlign/DNASTAR. Blackened letters denote identical amino acids present in at least two of the four proteins; and asterisks (*) indicate the zinc-binding residues of the C_3HC_4 domain. The putative *S. cerevisiae PEX10* gene (YDR265W) is more similar to *PpPEX10* than is any other gene of this species, and *C. elegans PEX10* (GenBank accession number U10402) is the only gene in this species with significant similarity to *PpPEX10*.

would result in ZS or a related PBD phenotype. To test this possibility, the human *PEX10* cDNA was expressed in skin fibroblasts from patients representing CG7, CG9, and CG12 of the PBDs, each of unknown genetic etiology. *PEX10* expression restored peroxisomal matrix-protein import in fibroblasts from CG7 but not in cells from the other CGs. Prior studies of three CG7 cell lines, all from NALD patients, revealed mild cellular defects associated with significant import of PTS1 and PTS2 proteins and even import of PEX5, the PTS1 receptor (Slawecki et al. 1995; Dodt and Gould 1996). However, we have since identified multiple ZS patients who belong to CG7. Cells from one such patient, PBD100, fail to import PTS1 or PTS2 matrix proteins but contain peroxisomes that are capable of importing peroxisomal membrane proteins (fig. 3*A–C*). Expression of *PEX10* in PBD100 cells restores import of both PTS1 and PTS2 proteins into peroxisomes (fig. 3*D* and *E*). Colocaliza-

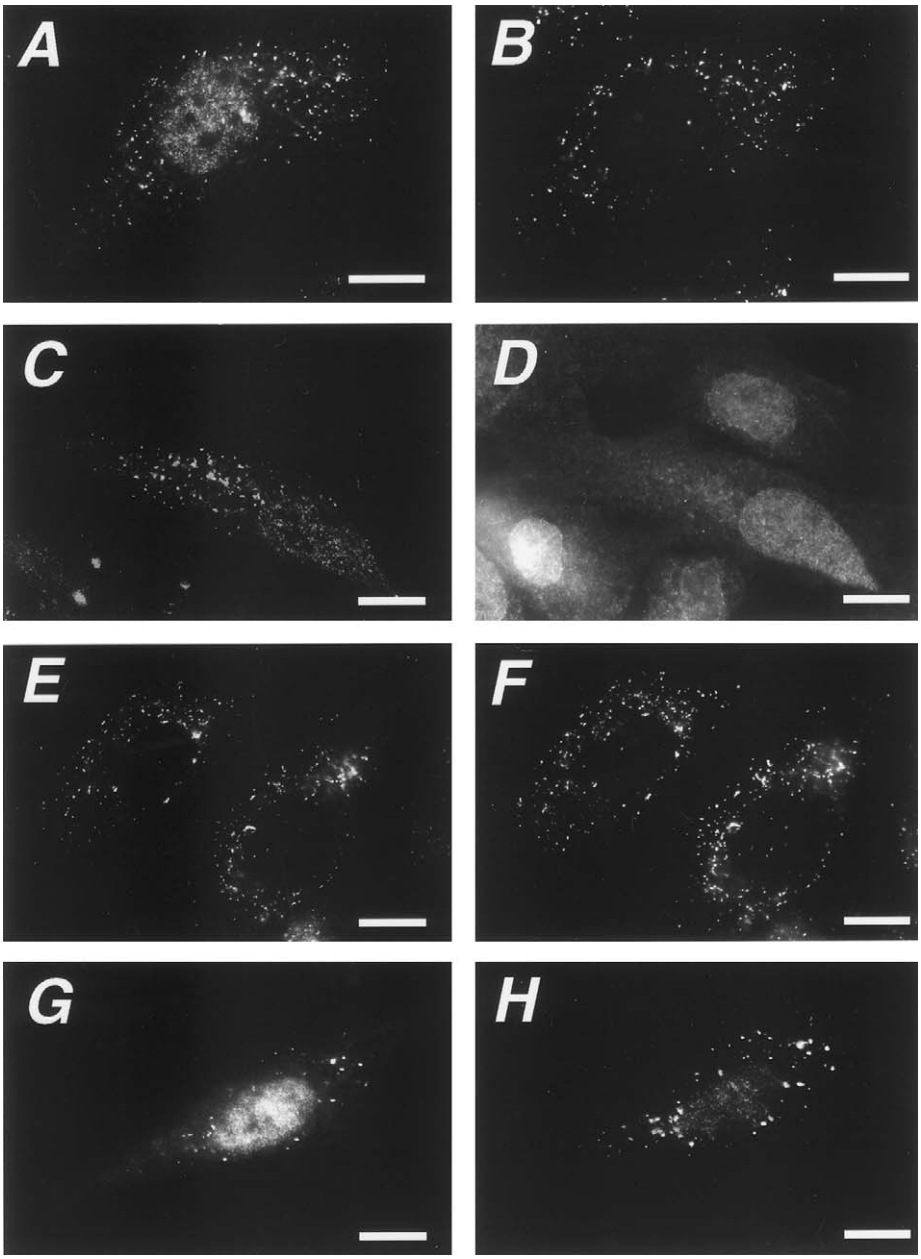

Figure 2 PEX10, a peroxisomal protein with its C-terminus exposed to the cytoplasm. Normal human skin fibroblasts (5756-T) were transfected with pcDNA3-*PEX10*myc. The cells were fixed, permeabilized with 1% Triton X-100, and processed for double indirect immunofluorescence with use of both a monoclonal antibody specific for the 10-amino-acid myc epitope tag and rabbit antibodies specific for PMP70, and this was followed by incubation with fluorescein-labeled goat anti-mouse and Texas red–labeled goat anti-rabbit secondary antibodies. Colocalization of PEX10myc (*A*) and PMP70 (*B*) in cells that were fixed and permeabilized with Triton X-100 shows that PEX10 is a peroxisomal protein. To assess PEX10 topology, 5756-T cells were transfected with pcDNA3-*PEX10*myc, were fixed, and were permeabilized with 25 μg digitonin/ml, which permeabilizes the cell membrane but leaves the peroxisome membrane intact. One set of these cells was processed for double indirect immunofluorescence with use of anti-myc (*C*) and anti-SKL (*D*) antibodies, and this was followed by incubation with fluorescein-labeled goat anti-mouse and Texas red–labeled goat anti-rabbit secondary antibodies. Another set of these cells was processed for double indirect immunofluorescence with use of anti-myc (*E*) and anti-PMP70 (*F*) antibodies, and this was followed by incubation with fluorescein-labeled goat anti-mouse and Texas red–labeled goat anti-rabbit secondary antibodies. PBD062 cells also were transfected with pcDNA3-*PEX10myc* and were processed for double indirect immunofluorescence under standard permeabilization conditions (Triton X-100), with use of anti-myc (*G*) and anti-PMP70 (*H*) antibodies, and this was followed by incubation with fluorescein-labeled goat anti-mouse and Texas red–labeled goat anti-rabbit secondary antibodies.

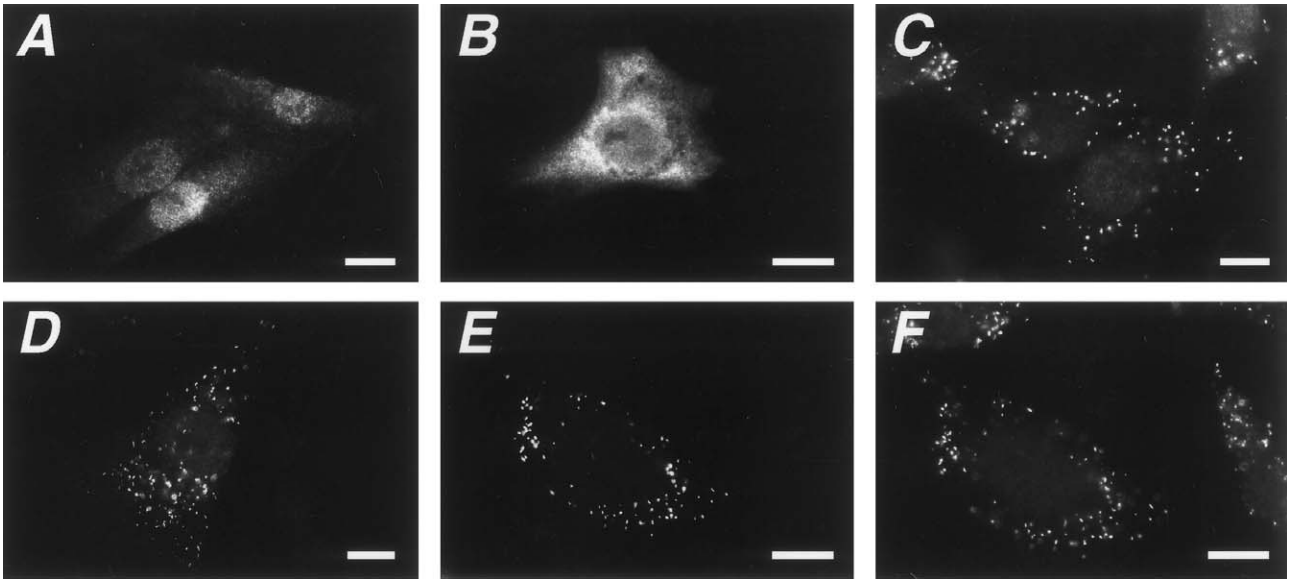

Figure 3 Functional complementation in PBD100 cells. To assess PTS1 import, PBD100 cells were transfected with pcDNA3 (*A*) or pcDNA3-*PEX10* (*D*) and were processed for indirect immunofluorescence with use of rabbit antibodies specific for SKL-containing proteins and fluorescein-labeled goat anti-rabbit secondary antibodies. To assess PTS2 import, PBD100 cells were cotransfected with both pcDNA3-*PAHXmyc*, a plasmid designed to express a c-myc–tagged version of the PTS2 protein phytanoyl-CoA α-hydroxylase (Mihalik et al. 1997), and either pcDNA3 (*B*) or pcDNA3-*PEX10* (*E* and *F*). Cells transfected with pcDNA3 and pcDNA3-*PAHXmyc* were processed for indirect immunofluorescence with use of a monoclonal antibody directed against the 10-amino-acid myc epitope tag and fluorescein-labeled goat anti-mouse secondary antibodies. Cells transfected with pcDNA3-*PEX10* and pcDNA3-*PAHXmyc* were processed for double indirect immunofluorescence with use of antibodies specific for the myc epitope tag (*E*) and PMP70 (*F*), and this was followed by incubation with fluorescein-labeled goat anti-mouse and Texas red–labeled goat anti-rabbit secondary antibodies. *C*, PBD100 cells transfected with pcDNA3, processed for indirect immunofluorescence with use of rabbit antibodies specific for PMP70 and Texas red–labeled goat anti-rabbit secondary antibodies, demonstrating the presence of numerous PMP-containing peroxisomes in the absence of PEX10.

tion (fig. 3*E* and *F*) and selective permeabilization (data not shown) experiments confirmed that the punctate distribution of peroxisomal matrix proteins in transfected cells represents import into the peroxisome lumen.

In contrast to PBD100 cells, the mildly affected CG7 cell line PBD052 imports significant quantities of PTS1- and PTS2-containing peroxisomal matrix proteins, as has been described elsewhere (Slawecki et al. 1995). In fact, peroxisomal matrix-protein import in these cells is so high that it was difficult to assess rescue of this cell line by following the distribution of SKL-containing PTS1 proteins or the PTS2 marker-protein PAHX-myc (fig. 4*A* and *B*). However, peroxisomal catalase, which carries an unusual form of PTS1 (Purdue and Lazarow 1996) and is the most inefficiently imported mammalian protein known ($\frac{1}{2}$ time of import = 15 min) (Lazarow et al. 1982), was found to be cytosolic in these cells. Transfection of PBD052 cells with the *PEX10* expression vector rescued catalase import in these cells (fig. 4*C* and *D*), suggesting that this cell line is also defective in *PEX10*.

Having established that expression of an unmodified *PEX10* cDNA restored peroxisome matrix-protein import in CG7 cells, we were able to test the effect of the C-terminal myc tag on *PEX10* function. PBD100 cells were transfected with either pcDNA3, pcDNA3-*PEX10*, or pcDNA3-*PEX10myc* and were processed for indirect immunofluorescence, with use of rabbit antibodies specific for SKL_{COOH}-containing proteins. *PEX10myc* expression restored peroxisomal protein import in PBD100 cells (54%) as efficiently as the wild-type *PEX10* cDNA (56%) (table 1), demonstrating that *PEX10myc* is a functional form of *PEX10* (note that rescue activities are limited by transfection efficiency).

CG7 Patients' Mutations in PEX10

PEX10 cDNAs containing the entire *PEX10* ORF were synthesized from PBD100 and PBD052 RNA samples by RT-PCR. The *PEX10* cDNA from ZS patient PBD100 was ~400 bp shorter than normal. Analysis of genomic DNA clones from PBD100 and controls revealed that this patient has a G→A transition mutation at the splice-donor site (GT→AT) in an intron that lies downstream from a 407-bp exon. The sequence of this exon corresponds to the 407 bp that are missing from PBD100 *PEX10* cDNAs, suggesting that the mutation causes aberrant mRNA processing and leads to exon

skipping. It also should be noted that this mutation corresponds to a C→T transition at a CpG dinucleotide on the antisense strand. The homozygous nature of this allele, which destroys a *Sna*BI site, was confirmed by restriction-endonuclease digestion of an amplified genomic DNA fragment spanning this mutation (fig. 5*B*).

In contrast to PBD100, cells from NALD patient PBD052 express full-length *PEX10* mRNA. Sequence analysis of *PEX10* cDNA clones from this patient revealed two distinct alleles, indicating that this patient is a compound heterozygote. One *PEX10* mutation, a C→T transition at a CpG dinucleotide (fig. 5*C*), converts the Arg125 codon to a stop codon (R125ter). The other *PEX10* mutation, a C→G transversion (fig. 5*D*), results in a substitution of glutamine for the zinc-coordinating histidine (H290) of the C_3HC_4 zinc ring domain and was found on >90% (54/59) of the *PEX10* cDNA clones generated from this patient. This histidine is conserved in PEX10 from all species that have been examined. The low number of PBD052 *PEX10* cDNA clones that carry the R125ter mutation is consistent with a decreased abundance of the corresponding mRNA, a decrease that may be caused by nonsense-mediated RNA decay (Maquat 1995). Both mutations create new restriction-enzyme sites, and the presence of both mutations was confirmed at the genomic level by restriction-endonuclease digestion of amplified genomic DNA fragments of the *PEX10* gene (fig. 5*C* and *D*).

Table 1

Expression of *PEX10myc:* Restoration of PTS1 Protein Import in PBD100 Cells

Transfected Plasmid	Proportion (%) of PBD100 Cells Importing PTS1 Proteins
pcDNA3	0/210
pcDNA3 PEX10	122/217 (56)
pcDNA3 PEX10myc	166/306 (54)

Relative Activity of PEX10 *Alleles from PBD052 and PBD100*

Having established that expression of the normal *PEX10* cDNA restores PTS1 and PTS2 import in PBD100 cells, we wanted to determine the relative in vivo activity of the mutant *PEX10* cDNAs identified in PBD100 and PBD052. The corresponding expression vectors—pcDNA3-*PEX10*/H290Q, pcDNA3-*PEX10*/R125ter, and pcDNA3-*PEX10*/PBD100—were cotransfected with pcDNA3-*PAHXmyc,* which encodes a myc-tagged PTS2 protein, into PBD100 cells. pcDNA3-

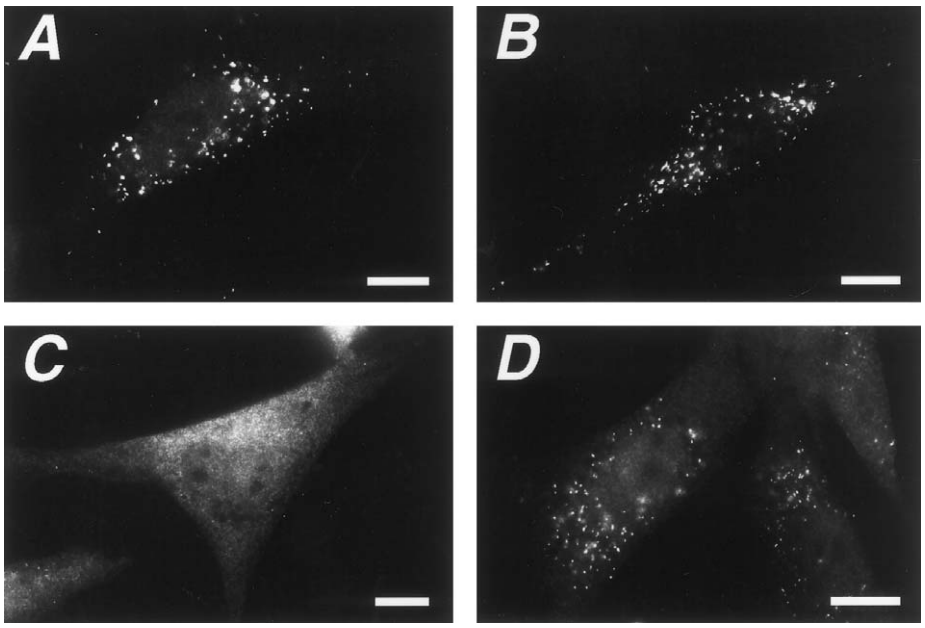

Figure 4 Functional complementation in PBD052 cells. PBD052 cells were transfected with pcDNA3, processed for indirect immunofluorescence with use of rabbit antibodies specific for SKL-containing proteins and fluorescein-labeled goat anti-rabbit secondary antibodies (*A*). Note the significant import of SKL-reactive proteins into punctate structures. PBD052 cells were cotransfected with pJM282, the *PAHXmyc* expression vector, and pcDNA3, processed for indirect immunofluorescence with use of a monoclonal antibody directed against the 10-amino-acid myc epitope tag and fluorescein-labeled goat anti-mouse secondary antibodies, revealing significant import of PAHXmyc, the PTS2 marker protein (*B*). PBD052 cells transfected with pcDNA3 (*C*) or pcDNA3-*PEX10* (*D*) were processed for indirect immunofluorescence with use of sheep antibodies specific for peroxisomal catalase and rhodamine-labeled donkey anti-sheep secondary antibodies, demonstrating rescue of this mildly affected line by expression of *PEX10*.

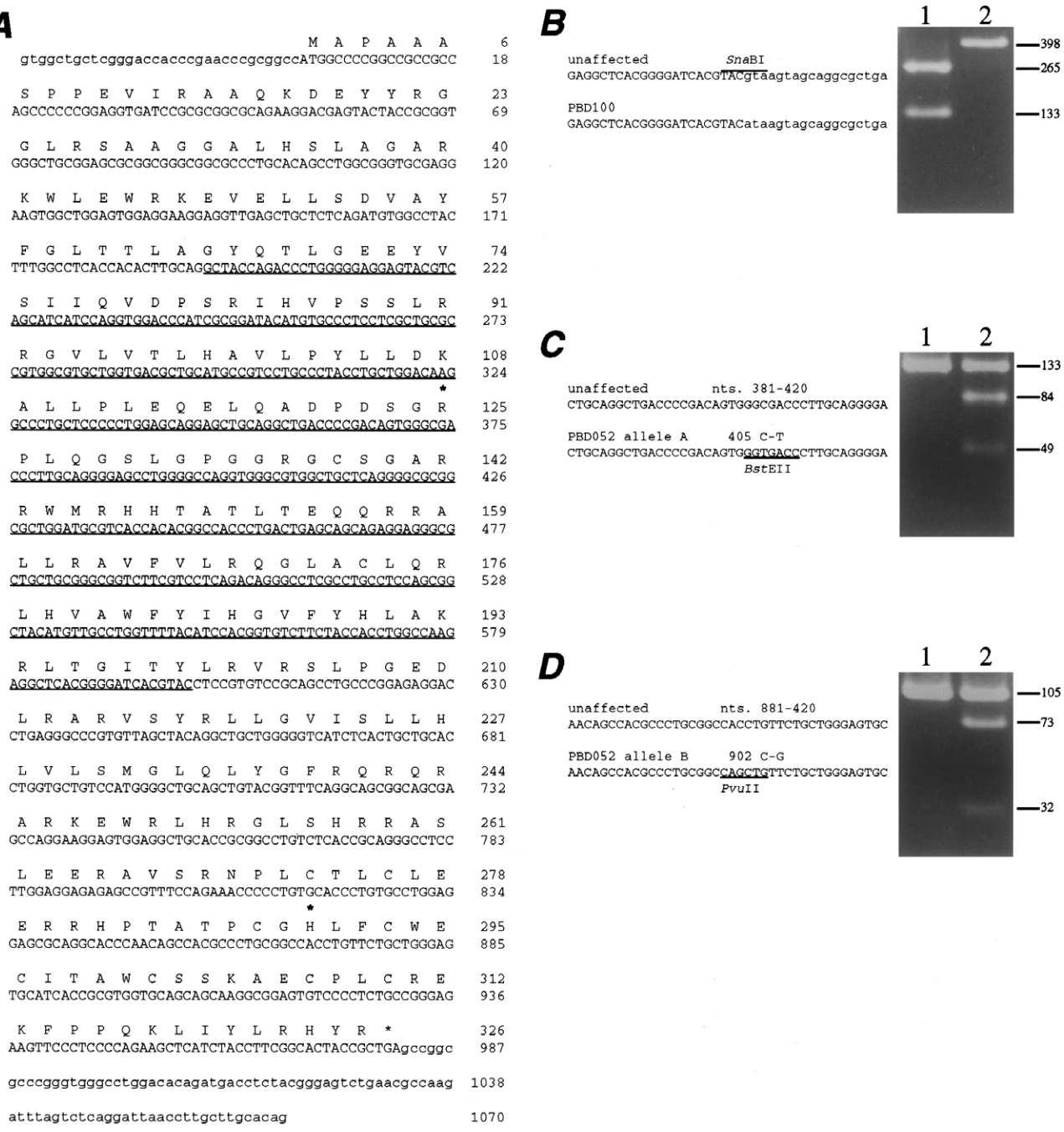

Figure 5 *PEX10* cDNA sequence and mutations in PBD100 and PBD052. *A,* Nucleotide and amino acid sequence of the *PEX10* gene. Nucleotides of the ORF are capitalized and numbered from the first nucleotide of the ORF; untranslated sequences are in lowercase; the segment lacking in *PEX10* cDNAs from PBD100 is underlined; and asterisks (*) indicate the sites of the H290Q and the R125ter mutations in PBD052. *B,* Mutation in PBD100, a G→A transition at a splice-donor site, which destroys a *Sna*BI site. The sequence to the left shows the exon/intron boundary at the site of the mutation in control and PBD100 genomic DNA samples. Intronic sequences are in lowercase. A 398-bp fragment of the *PEX10* gene flanking this site was amplified from the genomic DNA of an unaffected individual and from PBD100, was digested with *Sna*BI, and was separated on a 3% agarose gel. DNA amplified from an unaffected individual (lane 1) was digested completely with *Sna*BI, whereas the DNA amplified from PBD100 (lane 2) was resistant to digestion, indicating that PBD100 is homozygous for this mutation. Note that this point mutation causes a 407-bp deletion in the *PEX10*cDNA. *C,* PBD052 allele A (R125ter), a C→T transition at nucleotide 373 of the ORF, which creates a *Bst*EII site. A 133-bp fragment spanning the site of this mutation was amplified from the genomic DNA of an unaffected individual and from PBD052, was digested with *Bst*EII, and was separated on a 4% agarose gel. DNA amplified from a normal individual (lane 1) was resistant to digestion with *Bst*EII, whereas ~50% of the DNA amplified from PBD052 (lane 2) is cleaved into two smaller fragments, suggesting that PBD052 is not homozygous for this mutation. *D,* PBD052 allele B (H290Q), a C→G transversion at nucleotide 870 of the ORF, which creates a *Pvu*II site. A 105-bp fragment spanning the site of this mutation was amplified from genomic DNA of an unaffected individual and from PBD052, was digested with *Pvu*II, and was separated on a 4% agarose gel. The fragment from the unaffected individual was resistant to digestion with *Pvu*II (lane 1), whereas ~50% of the DNA amplified from PBD052 (lane 2) is cleaved into two smaller fragments, indicating that PBD052 is a compound heterozygote.

PEX10 and pcDNA3 also were cotransfected with pcDNA3-*PAHXmyc,* as positive and negative controls, respectively. Each transfected cell population was processed for double indirect immunofluorescence with anti-myc and anti-catalase antibodies, and the number of cells importing PTS1 and PTS2 proteins was recorded. These experiments were repeated three times, and similar results were obtained in each instance. The data from a representative trial are shown in table 2. We found that cells transfected with pcDNA3-*PEX10*/PBD100 do not import PTS1 or PTS2 proteins above background levels, consistent with the severe clinical and cellular defects of PBD100. In contrast, pcDNA3-*PEX10*/H290Q and pcDNA3-*PEX10*/R125ter rescued PTS2 protein import as efficiently as did pcDNA3-*PEX10* and restored import of catalase, a PTS1 protein, to almost 50% of the level observed for pcDNA3-*PEX10.* These results indicate that both PBD052 alleles, when overexpressed, have significant *PEX10* activity. However, it should be noted that the consistent underrepresentation of the R125ter allele in RT-PCR reactions (data not shown) indicates that this mutation may affect mRNA abundance.

Table 2

Relative Rescue Activities of Control and Mutant *PEX10* cDNAs in PBD100 Cells

Transfected Plasmid	Proportion (%) of Cells Importing	
	PTS1 Proteins	PTS2 Proteins
pcDNA3	0/1,000	37/507 (7.3)
pcDNA3-*PEX10*/PBD100	0/1,000	54/505 (10.7)
pcDNA3-*PEX10*/H290Q	273/1,000 (27.3)	444/506 (87.7)
pcDNA3-*PEX10*/R125ter	291/1,000 (29.1)	450/501 (89.8)
pcDNA3-*PEX10*	631/1,000 (63.1)	449/507 (88.6)

Discussion

Defects in peroxisomal protein import have long been implicated in the PBDs, but for many years there was little progress in the elucidation of the molecular and genetic basis of these diseases. However, the advent of the EST projects, the identification of many yeast proteins required for peroxisome biogenesis, and the conserved nature of peroxisomal protein import have aided the identification of the genes defective in the PBDs. Prior to this report, the genes defective in five of the known CGs of the PBDs, comprising >80% of all PBD patients (Reuber et al. 1997), had been identified. Here we extend our knowledge of the genetic etiology of these diseases, with the identification of human *PEX10* as the gene mutated in patients from CG7 of the PBDs.

PEX10 expression rescues the peroxisomal protein import defects of cells from CG7 patients. We have found mutations on both copies of *PEX10* from two of these patients, PBD052 and PBD100, and currently are searching for *PEX10* mutations in the remaining CG7 patients. PBD100, a severely affected ZS patient and the progeny of a consanguineous marriage, is homozygous for a splice donor–site mutation in one of the introns of the *PEX10* gene and produces truncated *PEX10* mRNAs, most of which lack all of a 407-bp coding exon. We were unable to detect significant matrix-protein import in PBD100 cells, and the *PEX10* cDNA generated from PBD100 RNA lacked activity, even when overexpressed at high levels. This suggests that the splice-site mutation in PBD100 creates a null allele of *PEX10.*

PBD052, the second CG7 patient whom we have characterized at the molecular level, is a compound heterozygote for *PEX10* mutations. PBD052 displays mild clinical and cellular phenotypes, suggesting that one of the corresponding alleles would express at least some *PEX10* activity. Given that the two mutations are a missense mutation (H290Q) at a zinc-coordinating histidine residue of the zinc-binding domain and a nonsense mutation (R125ter) predicted to terminate translation after synthesis of only the N-terminal third of PEX10, we assumed that the residual activity would be derived from the allele with the H290Q mutation. However, we observed that *PEX10* cDNAs containing either the H290Q mutation or the R125ter mutation displayed extremely high levels of activity in an in vivo functional complementation assay.

A prior study of yeast *PEX10* had demonstrated that mutations at any of three zinc-binding residues of the C_3HC_4 domain, including the sole zinc-binding histidine H315, inactivated this gene in a functional complementation assay (Kalish et al. 1995). The fact that substitutions for H315 of yeast Pex10p (H315W), as well as for the analogous residue of human PEX10, H290 (H290Q), have phenotypic consequences lends support to the hypothesis that the zinc-binding domain is important for *PEX10* function. However, the consequences of the H315W and H290Q mutations appeared to differ, with the H315W mutation inactivating the yeast *PEX10* gene (Kalish et al. 1995) whereas the H290Q mutation results in only an attenuation of *PEX10* activity. Although it is possible that the threshold for activity in the yeast and human functional complementation assays is different, it is also possible that the nature of the substitution is responsible for the apparently severe consequences of the H315W substitution and for the mild consequences of the H290Q mutation. One possible difference is that glutamine may participate in coordination of metal ions indirectly through a water molecule, an activity that would not be expected for tryptophan.

Although the consequence of the H290Q mutation was unexpectedly mild, the high activity of the *PEX10*/R125ter cDNA was even more surprising. The deduced product of this allele includes only the N-terminal 124 amino acids of PEX10, a region that contains both the

first putative transmembrane domain of the protein and the TLGEEYV motif. If this is indeed the only product of the R125ter allele, it would mean that the second transmembrane domain and the zinc-binding domain can be removed without elimination of *PEX10* activity, a result that again conflicts with the inactivating effect of missense mutations in the zinc-binding domain of yeast *PEX10*. However, it is also possible that additional PEX10 products are synthesized from the R125ter cDNA, which may contribute to the *PEX10* activity of pcDNA-*PEX10*/R125ter. Although elucidation of the mechanism responsible for *PEX10*/R125ter activity is important for an understanding of the role of *PEX10* in peroxisome biogenesis, we doubt that the R125ter allele contributes significant *PEX10* activity to PBD052 cells. The R125ter mutation was present in <10% of PBD052 *PEX10* cDNAs, suggesting that this mRNA exists at reduced abundance in these cells. The R125ter mutation lies within the fourth-to-last coding exon of the *PEX10* gene (data not shown), and nonsense-mediated mRNA decay has been shown to affect steady-state mRNA levels of genes carrying frameshift or nonsense mutations in exons upstream from the last two coding exons (Maquat 1995).

Peroxisomal matrix-protein import is a multistep process that requires at least 16 different proteins. For PTS1 proteins, import appears to begin with recognition by the PTS1 receptor (PEX5) in the cytoplasm and subsequent transport of the receptor-ligand complex to the peroxisome surface (Dodt and Gould 1996). There the receptor binds via membrane-bound docking factors (Elgersma et al. 1996; Erdmann and Blobel 1996; Gould et al. 1996; Albertini et al. 1997), followed by translocation of matrix proteins and recycling of the receptor to the cytoplasm (Dodt and Gould 1996). van der Klei et al. (1995) have reported that *H. polymorpha* PEX5 is present in the cytoplasm, peroxisome membrane, and peroxisome matrix, suggesting that PEX5 may be imported along with its cargo of newly synthesized peroxisomal proteins and may be exported to the cytosol after dissociation from its ligands. Previous studies on human fibroblasts have demonstrated that PEX5 is also imported into the peroxisome lumen in certain mildly affected CG7 cell lines, including PBD052, which display relatively high levels of matrix-protein import (Dodt and Gould 1996). Although these earlier observations were consistent with a role for the CG7 gene in PEX5 export, we have found that PBD052 cells express alleles of *PEX10* that retain significant activity. Also, we now know that other CG7 cells with severe mutations in *PEX10,* such as PBD100, are associated with both a complete block in matrix-protein import and accumulation of PEX5 on the outer surface of peroxisomes (data not shown). The observation that PEX5 import occurs in cells with partial *PEX10* function (PBD052) and not in cells that completely lack *PEX10* function (PBD100) argues against a role for this protein in receptor export. Instead, our data are consistent with a role for *PEX10* in either the translocation of matrix proteins across the peroxisome membrane or in receptor-ligand dissociation prior to the translocation event. As such, it would appear that one or the other PBD052 alleles disrupts the specificity and/or efficiency of the translocation process.

Acknowledgments

We thank C. Dunbar for advice and technical assistance, S. Subramani for the anti-PMP70 antibodies, A. Moser and D. Gordon for providing the PBD cell lines, and C. Obie for providing RNA and genomic DNA samples. D.S.W. was supported by The Predoctoral Training Program in Human Genetics at The Johns Hopkins University; and D.V. is an Investigator of the Howard Hughes Medical Institute. This work was supported by National Institutes of Health grants DK45787 and HD10891.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (accession numbers AF060502, H83562, and U10402)

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