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Loss-of-Function Mutations in a Human Gene Related to *Chlamydomonas reinhardtii* Dynein IC78 Result in Primary Ciliary Dyskinesia

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Summary

Primary ciliary dyskinesia (PCD) is a group of heterogeneous disorders of unknown origin, usually inherited as an autosomal recessive trait. Its phenotype is characterized by axonemal abnormalities of respiratory cilia and sperm tails leading to bronchiectasis and sinusitis, which are sometimes associated with situs inversus (Kartagener syndrome) and male sterility. The main ciliary defect in PCD is an absence of dynein arms. We have isolated the first gene involved in PCD, using a candidate-gene approach developed on the basis of documented abnormalities of immotile strains of *Chlamydomonas reinhardtii*, which carry axonemal ultrastructural defects reminiscent of PCD. Taking advantage of the evolutionary conservation of genes encoding axonemal proteins, we have isolated a human sequence (*DNAI1*) related to IC78, a *C. reinhardtii* gene encoding a dynein intermediate chain in which mutations are associated with the absence of outer dynein arms. *DNAI1* is highly expressed in trachea and testis and is composed of 20 exons located at 9p13-p21. Two loss-of-function mutations of *DNAI1* have been identified in a patient with PCD characterized by immotile respiratory cilia lacking outer dynein arms. In addition, we excluded linkage between this gene and similar PCD phenotypes in five other affected families, providing a clear demonstration of locus heterogeneity. These data reveal the critical role of *DNAI1* in the development of human axonemal structures and open up new means for identification of additional genes involved in related developmental defects.

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Introduction

Functional and ultrastructural abnormalities of respiratory cilia have been described in patients with a congenital respiratory disease known as primary ciliary dyskinesia (PCD) (Afzelius 1976; Rossman et al. 1980). PCD, previously described as "immotile cilia syndrome" (MIM 242650), represents a heterogeneous group of genetic disorders affecting 1/16,000 individuals; the PCD phenotype is characterized by impaired mucociliary clearance resulting from a lack of ciliary movements believed to be responsible for chronic lung, sinus, and middle ear diseases. Approximately 50% of patients with PCD display situs inversus, which defines the Kartagener syndrome (MIM 244400). Most male patients are infertile as a result of nonmotile spermatozoa in relation to functional and ultrastructural abnormalities of sperm flagella (Afzelius 1985). Studies to date, however, have failed to decipher the molecular basis of this disease. PCD is usually transmitted as an autosomal recessive trait. Indeed, most familial cases have been confined to sibs; in several instances, patients were born to consanguineous unions; an absence of respiratory symptoms is usually reported in the parents (Sturgess et al. 1986). However, other inheritance patterns (i.e., autosomal dominant and X-linked transmission of the disease phenotype) have seldom been reported (Narayan et al. 1994), thereby suggesting genetic heterogeneity in this condition. This hypothesis is further supported by the various functional and ultrastructural abnormalities of respiratory cilia documented in patients with PCD (Chao et al. 1982).

The main ciliary defect found in PCD is an absence of dynein arms, affecting almost all cilia (Afzelius 1985). Dyneins consist of a large family of proteins involved in many types of microtubule-dependent cell motility in both lower and higher eukaryotes. Two major classes of dyneins have been described: cytoplasmic and axonemal dyneins. Cytoplasmic dyneins are involved in a wide range of intracellular functions, including chromosome movements on the mitotic spindle and the transport of

membranous organelles toward the minus ends of microtubules (Holzbaur and Vallee 1994). Axonemal dyneins are found in ciliary and flagellar axonemes. The axonemal ultrastructure, which is highly conserved through evolution, is composed of nine outer-doublet microtubules that surround a central pair of singlet microtubules. Two dynein arms—outer and inner—are bound to each peripheral microtubule doublet; these dynein arms are essential for ciliary and flagellar beating, which they generate through ATP-dependent cycles of attachment/detachment to the adjacent microtubule doublet (Witman 1992; Shingyoji et al. 1998).

Detailed structural and biochemical studies of dynein arms have been performed in various species, including sea urchins and *Chlamydomonas reinhardtii,* a unicellular alga with two flagella containing an axonemal structure similar to that of human respiratory cilia and sperm tails (Blair and Dutcher 1992; Vallee 1993; Porter 1996). Such studies have revealed that, depending on the species, the outer dynein arms are composed of either two or three heavy chains (Mr >400,000), two to four intermediate chains (Mr 45,000–110,000), and several light chains (Mr <30,000) (Porter 1996). Numerous genes encoding dynein heavy and light chains have been identified in various species (Gibbons et al. 1991; Mitchell and Kang 1991; Ogawa 1991; Asai et al. 1994; Gibbons et al. 1994; Rasmusson et al. 1994; Wilkerson et al. 1994; LeDizet and Piperno 1995*b;* Tanaka et al. 1995; Andrews et al. 1996; Porter et al. 1996; Vaughan et al. 1996; Neesen et al. 1997), including humans (Milisav et al. 1996; Vaughan et al. 1996; Chapelin et al. 1997*b;* Kastury et al. 1997; Neesen et al. 1997). However, to date, genes encoding intermediate chains belonging to the outer arms have been identified only in *Chlamydomonas* (IC69 [GenBank accession number X55382] and IC78 [GenBank accession number U19120]) (Mitchell and Kang 1991; Wilkerson et al. 1995) and in sea urchins (IC1 [GenBank accession number D63884], IC2 [GenBank accession number D38538], and IC3 [GenBank accession number D28863]) (Ogawa et al. 1995, 1996) but not in humans.

Given the high complexity of the structures involved in ciliary movements, the genetic heterogeneity underlying PCD is expected to be extremely important. Such a complexity may account for the failure to establish a reproducible linkage between the PCD phenotype and a genetic region (Chapelin et al. 1997*a*). Strikingly, several immotile strains of *Chlamydomonas* have been found to carry axonemal ultrastructural defects that are reminiscent of those reported in patients with PCD (Mitchell and Kang 1991; Sakakibara et al. 1991, 1993; Porter et al. 1994; LeDizet and Piperno 1995*a;* Rupp et al. 1996; Myster et al. 1997; Perrone et al. 1998). Among them, the *Chlamydomonas* flagellar mutants carrying a defect in IC78, a gene of relatively small size compared with dynein heavy-chain genes, have been the subject of detailed functional, ultrastructural, and molecular studies (Wilkerson et al. 1995). Of particular interest, the flagellar ultrastructural phenotype of these latter mutants is similar to the axonemal ultrastructural abnormality (i.e., absence of outer dynein arms) observed in several patients with PCD; we therefore designed a strategy, on the basis of evolutionary conservation of both the axonemal ultrastructure and the genes encoding axonemal proteins, to isolate a human gene related to IC78 and to test its involvement in PCD.

Subjects and Methods

Subjects

A 9-year-old boy (patient II-1), who was born to unrelated parents (family 1), presented in early childhood with chronic respiratory symptoms characterized by chronic sinusitis, serous otitis, and recurrent episodes of bronchitis associated with severe segmental atelectasis that had led to partial lobectomy. Chest radiography showed normal cardiac and visceral situs. Neither his parents nor his other relatives have a history of respiratory disease. At the time of bronchoscopy, samples of trachea mucosa were obtained and were processed for ciliary studies, as described elsewhere (Escudier et al. 1990). No ciliary beating was observed, and transmission electron microscopy showed the absence of outer dynein arms in all cilia, thereby confirming the diagnosis of PCD. Five other unrelated consanguineous families with PCD were also investigated (families 2–6). In two independent families (families 2 and 3), the ultrastructural phenotype was identical to that documented in patient II-1 from family 1; however, in one case (family 3), the patients with PCD also displayed situs inversus. In the three remaining families (families 4–6), both outer and inner dynein arms were absent in the patients' cilia; in one case (family 6), the disease phenotype was associated with situs inversus. In all these individuals, DNA samples were isolated from peripheral-blood samples, according to standard techniques.

Cloning and Sequencing of Human DNAI1 *cDNA and Genomic DNA*

To isolate a human gene homologous to the *Chlamydomonas* IC78 gene and the sea urchin IC2 gene, we performed an alignment of the amino acid sequences of IC2 and IC78, using the Clustal W1.7 program, and we designed three degenerated primers (P1–P3) for PCR in the most conserved regions of these two chains. The

primers were used in reverse transcriptase–PCR (RT-PCR) assays, with total RNA from human adult testis and trachea used as templates (Clontech). PCR performed with primers P1 (antisense) and P2 (sense) led to the characterization of a 975-bp fragment of the human dynein axonemal intermediate chain–1 (*DNAI1*) coding sequence. A specific primer (P10, sense) designed in the new sequence was used with primer P3 (antisense) in a PCR assay that gave rise to an additional *DNAI1* cDNA fragment. On the basis of the latter sequence, both the 5′ and 3′ portions of the human *DNAI1* cDNA sequence were obtained, by rapid amplification of cDNA ends (RACE), with the use of Marathon-ready cDNA from human testis (Clontech). The full-length coding sequence of *DNAI1* was subsequently amplified on total trachea and testis RNAs, by means of RT-PCR with primers P4 (forward) and P5 (reverse).

To characterize the intron-exon organization of the *DNAI1* gene, long-range PCRs were performed on human genomic DNA, by use of the Expand Long Template PCR System kit (Boehringer Mannheim) and various sets of exonic primers designed in the human *DNAI1* cDNA, according to the manufacturer's instructions. PCR products were purified with the use of the PCR purification kit (Boehringer Mannheim) prior to sequencing. All sequences were determined on both strands.

Sequence Analysis, Alignment, and Construction of Phylogenetic Tree

DNA homology searches were performed by use of BLAST. Deduced amino acid sequences of *Chlamydomonas* IC78, sea urchin IC2, and the newly identified human sequence, *DNAI1,* were multiply aligned with the use of the ClustalW program, version 1.8 (Thompson et al. 1994). The GCG PILEUP program was used to generate relationship trees, on the basis of the neighbor-joining method (Saitou and Nei 1987).

Northern Blot Analysis

For expression pattern analysis, two human multiple-tissue northern blot membranes were purchased (Clontech). Four probes were obtained by PCR. These probes recognize different cDNA regions defined by positions −77 to +2241, −47 to +490, 848–2204, and 2035–2241, respectively. Membranes were hybridized with these probes, according to the manufacturer's recommended protocol.

Mapping of the DNAI1 *Gene*

The chromosomal localization of the human *DNAI1* gene was first determined by the screening of a panel of 24 hybrid somatic cell lines (human/rodent), by use of PCR with *DNAI1*-specific primers (Quantum). PCR was performed, as described above, with primers P12 (intron 17, sense) and P13 (intron 18, antisense). Further localization of the genomic *DNAI1* probe was performed on human metaphase chromosomes, by use of FISH combined with R-banding, as described elsewhere (Lemieux et al. 1992). The probe consisted of four genomic DNA products amplified by PCR and covering 33.5 kb of the *DNAI1* gene. The probe labeled with biotin by means of nick-translation (Pinkel et al. 1986) was visualized with fluorescein isothiocyanate (FITC)–avidin, and the chromosomes were counterstained with 4,6-diamidino-2-phenylindole. The specific signal intensity and its sublocalization along the chromosome axis were analyzed with use of the Cytogen Fluoquant program (Imstar).

SSCP Mutation Analysis

DNAI1 exons were amplified with intronic primers (available on request). PCR products were electrophoresed inmutation-detection–enhancement gel (Bioprobe), with the use of 0.6 × Tris-borate EDTA (TBE) buffer at 4–10 W (depending on the size of each fragment), for 16 h at room temperature. When bandshifts were identified, the corresponding PCR products were sequenced.

Characterization of DNAI1 *Mutations*

We used the following primers to amplify the genomic DNA spanning the two mutated sites described in family 1: primers P6 (exon 1, sense) and P11 (intron 1, antisense), which bracket the paternal mutation, were used to amplify a 159-bp product in control subjects; primers P8 (intron 4, sense) and P9 (intron 5, antisense), which bracket the maternal mutation, were used to amplify a 276-bp product in control subjects. To determine the consequences of the paternal mutation on the processing of *DNAI1* primary transcript, total RNA obtained from both the patient and a control were prepared from nasal brushing, according to standard procedures (Escudier et al. 1990); RT-PCR was performed with exonic primers P6 and P7 (exon 4, antisense).

Results

Isolation of the Human cDNA and Gene Encoding DNAI1, a Protein Related to Chlamydomonas reinhardtii *Dynein IC78*

To isolate the human cDNA sequence encoding DNAI1 (dynein axonemal intermediate chain–1), we used testis cDNAs as templates in cross-species PCR assays, with degenerated primers (P1–P3; see the Subjects and Methods section, above) chosen from IC78 and IC2, two orthologous cDNA sequences encoding an axonemal dynein intermediate chain in *Chlamydomonas rein-*

hardtii and sea urchins, respectively. This gave rise to PCR products that were cloned and sequenced, thereby providing specific sequence information that was used to perform 5′ and 3′ RACE experiments on human testis cDNA. This procedure led to the characterization of the 2,526-bp full-length human *DNAI1* coding sequence together with parts of the 5′ and 3′ untranslated regions (UTR). This coding sequence was further confirmed by the sequence determination of the RT-PCR products generated from trachea and testis RNAs, with the use of two primers (P4 and P5) that bracket the full-length coding region. The 5′-UTR sequence, which contains two in-frame termination codons, includes 171 bp upstream of the translation start. The isolated 3′-UTR sequence (258 bp) contains a single putative polyadenylation signal (AATAAA) at position 2307 (nucleotide +1 corresponds to A of the ATG translation start codon), located 207 bp downstream of the stop codon. The predicted protein consists of 699 amino acids (fig. 1*A*). To better characterize the *DNAI1* cDNA, we integrated its corresponding amino acid sequence into an evolutionary approach by constructing a phylogenetic tree with the sequences of all outer arm dynein intermediate chains identified to date and with several cytoplasmic intermediate chains (fig. 2). The results of this analysis revealed that DNAI1 exhibits less conservation with DNCI1, the human cytoplasmic dynein intermediate chain (15% identity), than with axonemal intermediate chains from distantly related species (fig. 2). DNAI1 had a greater similarity with sea urchin IC2 and *Chlamydomonas* IC78 sequences than with sea urchin IC3 and *Chlamydomonas* IC69, which are two other closely related sequences; the deduced amino acid sequence of human *DNAI1* indeed displayed a significant homology to IC2 and IC78 (probability values calculated by BLAST: $P = 1.2 \times 10^{-272}$ and $P = 3.1 \times 10^{-187}$, respectively), with an overall sequence identity of 59% and 39% with IC2 and IC78, respectively. Regions of high similarity are located in the carboxy-terminal halves of these chains and primarily correspond to sequence elements known as tryptophan-aspartate (WD) repeats (fig. 1*A*). Five of the six WD consensus sequences present in the *Chlamydomonas* IC78 sequence (Wilkerson et al. 1995) are conserved in sea urchin IC2 (Ogawa et al. 1995) and in human DNAI1 sequences. The five human WD repeats share 29%–63% identity with the *Chlamydomonas* sequence and 51%–87% identity with the sea urchin sequence.

Expression studies of *DNAI1* provided additional evidence that this cDNA indeed encodes an axonemal protein. Hybridization of the full-length *DNAI1* cDNA probe to commercially available multiple-tissue northern blots revealed the presence of a specific and abundant transcript of ~2.5 kb in adult trachea and testis but not in other adult tissues examined, which are devoid of cilia (fig. 3). In trachea, an additional weakly expressed transcript of ~4 kb was also detected. To determine whether the 2.5-kb and 4-kb transcripts result from common *DNAI1* primary transcripts, we used four different probes consisting of different parts of the cDNA in similar northern blot experiments. All four probes recognized the two transcripts (data not shown), in keeping with a common origin of these two molecular species.

Genomic structure of the *DNAI1* coding region was elucidated by long-range PCRs between exons. The human *DNAI1* gene (fig. 1*B*) is composed of at least 20 exons and 19 introns. All exons and introns were sized, with each intron-exon boundary containing the gt-ag consensus sequences for eukaryotic splice donor and acceptor sites.

Chromosomal Localization of the Human DNAI1 *Gene*

A primer set was used for PCR screening of a panel of 24 hybrid somatic cell lines (human/rodent), each of which retained 1 of the 24 human chromosomes. An amplification product of expected size and sequence was detected in only one hybrid cell line, demonstrating the location of the human *DNAI1* gene on chromosome 9 (data not shown). The gene was sublocalized by FISH. Spots of specific hybridization were observed on metaphase spreads at band p13-21 (fig. 4).

DNAI1 *Mutation Analysis*

Patient II-1 from family 1 displayed a PCD phenotype characterized by an absence of outer dynein arms (fig. 5). All the coding regions and intron-exon boundaries of the DNA sample from this patient were amplified with *DNAI1*-specific primers and were run on SSCP gels or were sequenced directly. Two SSCP variants were identified in his genomic DNA; these variants were located in two PCR fragments spanning exon 5 (fig. 6*A*) and exon 1 (fig. 7*A*), respectively. Direct sequencing of these amplified products yielded blurred sequencing patterns (data not shown), a result suggesting the presence of heterozygous insertions or deletions.

To characterize these molecular variations, we cloned the corresponding PCR products. A 4-bp insertion was present in 10 of the 20 clones spanning exon 5 (fig. 6*B*). This short insertion, located at codon 95, resulted in the introduction of a *Vsp*I site, which was used to confirm the maternal origin of the mutated allele (fig. 6*C*). A similar experimental approach led to the identification of a 1-bp insertion in the exon 1–amplified product; this mutation, which involves the splice-donor site following exon 1, results in the introduction of a *Hpa*I site (fig. 7*B*). *Hpa*I digestion of the PCR-amplified genomic DNA spanning the mutation site showed that the 1-bp insertion was inher-

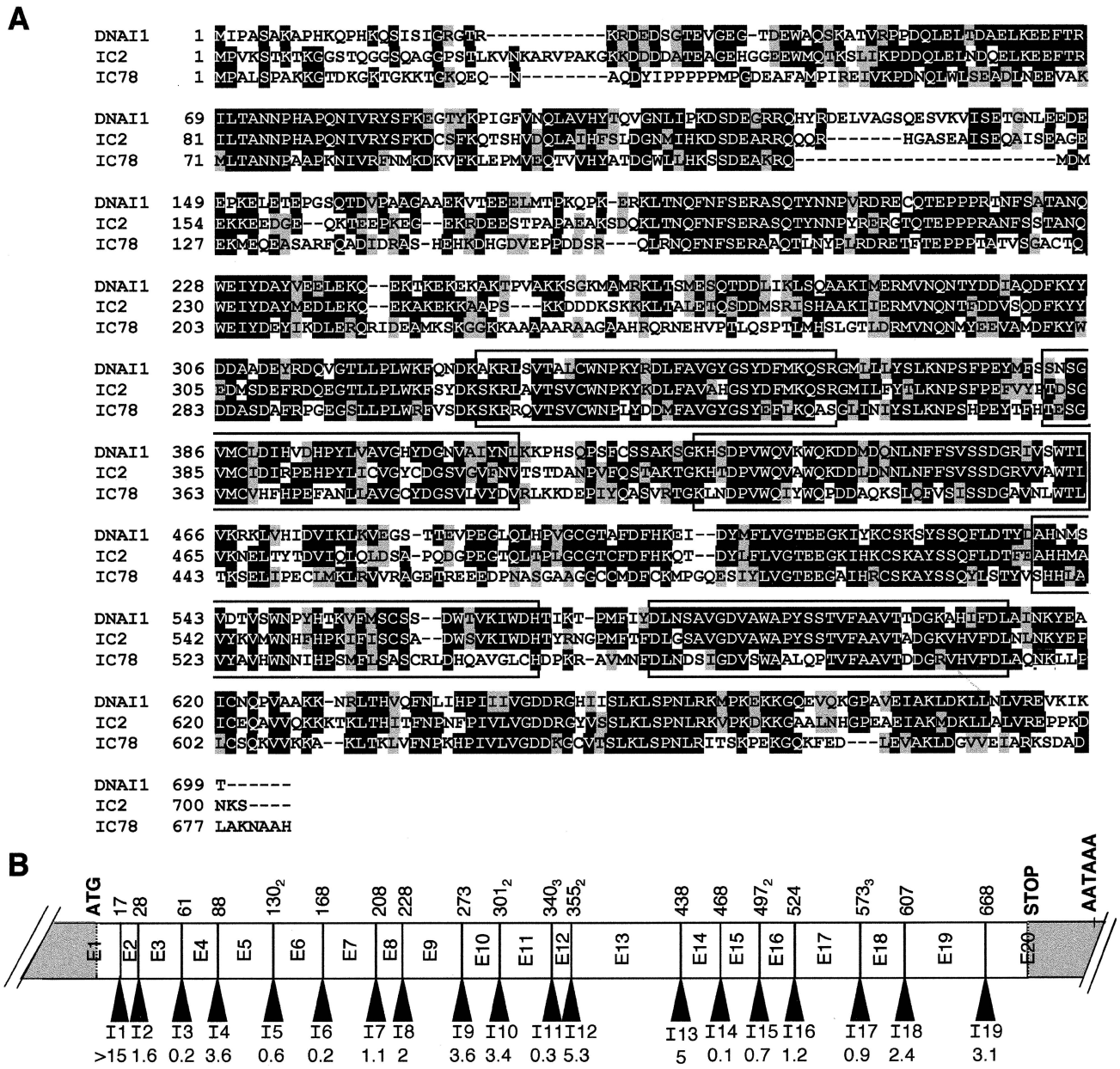

Figure 1 Predicted amino acid sequence and genomic organization of the human *DNAI1* gene. *A*, Comparison of deduced amino acid sequences of human *DNAI1*, sea urchin IC2, and *Chlamydomonas* IC78. Identical residues are denoted by a blackened background, whereas similar residues are shaded. The five WD repeats are within boxes. Gaps that were introduced to optimize the alignment are denoted by dashes. *B*, Intron-exon organization of the human *DNAI1* gene. The 20 exons (E1–E20) encoding the DNAI1 protein are indicated by unshaded or shaded boxes (translated and untranslated regions, respectively). The locations of the ATG initiating codon, the stop codon, and the polyadenylation signal are shown. The number of the first codon in each exon is indicated; exons beginning with the second or third base of a codon are indicated by subscript 2 or 3, respectively. The exons are drawn to scale. Intron-exon boundaries are denoted by blackened triangles; the size (kb) of each intron (I1–I19) is indicated at the bottom.

ited from the father (fig. 7*C*). The patient therefore carries two different *DNAI1* mutations, thereby demonstrating compound heterozygosity, a finding that is in keeping with the absence of consanguinity documented in this family.

The maternal 4-bp insertion results in a frameshift leading to a premature stop codon 24 amino acids downstream. To determine the consequences of the paternally-inherited splice-donor-site mutation on the processing of *DNAI1* transcripts, total RNA obtained from the nasal

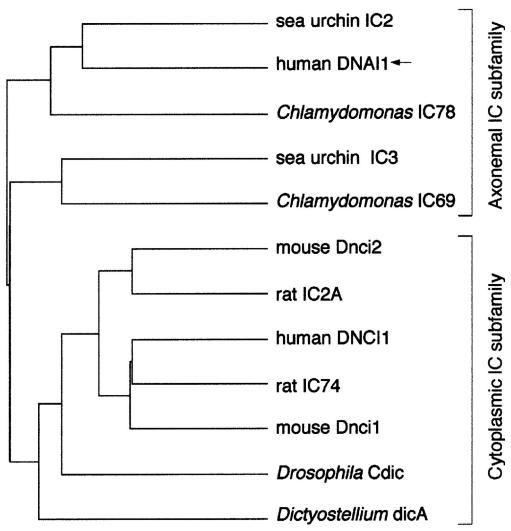

Figure 2 The deduced amino acid sequence of human *DNAI1* (arrow) integrated into an evolutionary tree of representative classes of dynein intermediate chains (IC): axonemal outer dynein IC and cytoplasmic dynein IC subfamilies. A parsimony tree for *Chlamydomonas* IC78 and IC69, sea urchin IC2 and IC3, rat IC74 (GenBank accession number X66845), rat dynein cytoplasmic intermediate chain–2A (IC2A [GenBank accession number U39044]), mouse Dnci1 (GenBank accession number AF063229) and Dnci2 (GenBank accession number AF063231), *Drosophila* Cdic (GenBank accession number AF070687), *Dictyostelium* cytoplasmic dynein intermediate chain (dicA [GenBank accession number U25116]), human DNCI1 (GenBank accession number AF063228), and the newly identified human DNAI1 was created by the PILEUP program.

epithelial cells of patient II-1 was reverse-transcribed. The resulting products were used as templates in a PCR assay performed with *DNAI1*-specific primers bracketing the splice junction (primers P6 and P7, located in exon 1 and 4, respectively) (fig. 7*D*). Two molecular species were generated: one of expected size (290 bp) and a 422-bp fragment, which was observed only in the patient's sample and not that of the control. Sequencing of these 290-bp and 422-bp PCR products revealed that the larger fragment contained a 132-bp insertion corresponding to the 5′ intronic sequence following exon 1, thereby indicating a cryptic splice-donor site in this intron at position 133 (fig. 7*E*). In addition, the same RNA sample from patient II-1 was subjected to RT-PCR with primers (P4 and P5) that bracket the full-length *DNAI1* coding sequence. The resulting PCR products were cloned and sequenced. Among 36 clones analyzed, 22 were found to carry the paternally inherited 132-bp insertion, whereas the 14 remaining clones contained the maternal mutation.

To further test whether these two mutations are responsible for the PCD phenotype, 50 unrelated control subjects were screened for these *DNAI1* variations. None of their 100 chromosomes contained such mutations. However, in the course of this study, we identified two intragenic nucleotide polymorphisms. One is located at nucleotide 42 (G→C) of intron 11, and the other one is a G→A transition at nucleotide 1003 that results in the V335I substitution.

Locus Heterogeneity in PCD

The two intragenic polymorphisms were used to test the involvement of *DNAI1* in the PCD phenotype identified in five additional unrelated families (families 2–6), in which the patients were born to consanguineous unions. The genotype at these two loci was characterized by genomic DNA sequencing. In family 2, the two affected children, who displayed an absence of outer dynein arms in all cilia, and the healthy older sister share the same *DNAI1* genotype, thereby demonstrating an exclusion of linkage between the *DNAI1* gene and the PCD phenotype. In family 5, the two affected children, who displayed a PCD phenotype characterized by an absence of both outer and inner dynein arms, carried different *DNAI1* genotypes at nucleotide 42 of intron 11. In the three remaining consanguineous families (families 3, 4, and 6), the patients were found to be heterozygous at one or both intragenic loci (data not shown).

Discussion

We have isolated a novel mammalian gene, named *DNAI1,* which is predicted to encode a protein related

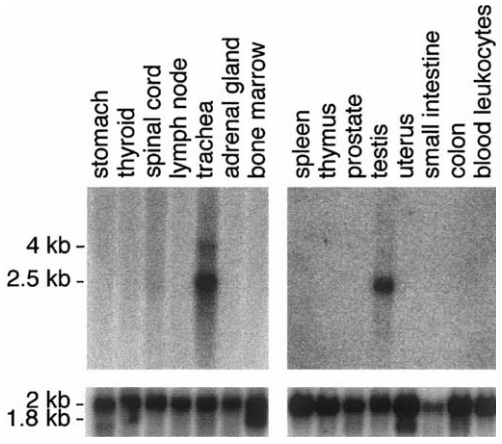

Figure 3 Expression analysis of the human *DNAI1* gene. Northern blot analysis was performed with the use of human multiple-tissue northern blots (Clontech) hybridized with the full-length coding sequence of *DNAI1* (*top*). A 2.5-kb transcript was detected in trachea and testis tissues. An additional transcript of ~4 kb was detected in trachea tissue only. Probing with β-actin used as a control (resulting in bands at 2.0 kb and 1.8 kb) is shown (*bottom*).

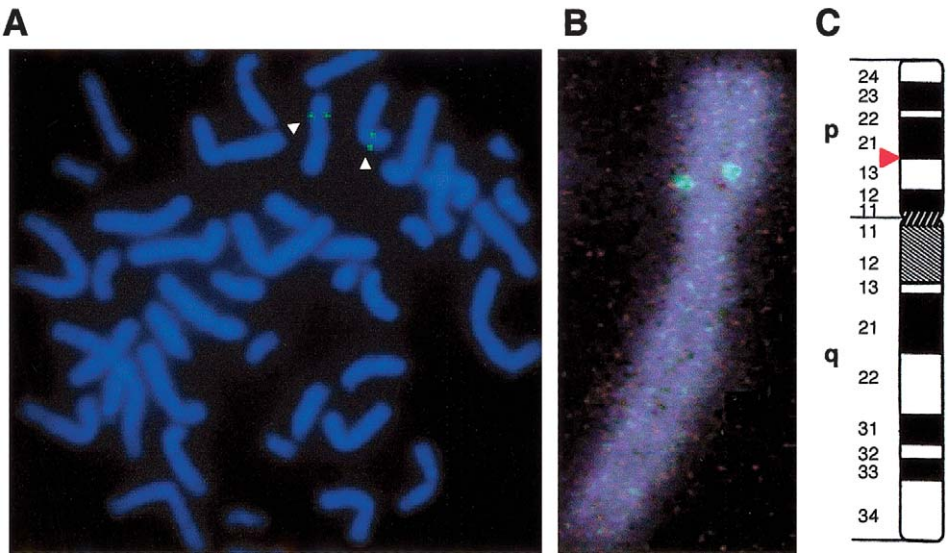

Figure 4 Chromosomal mapping of the human *DNAI1* gene. *A,* FISH showing localization of hybridization signals to both chromosomes 9 on metaphase spread (*arrowheads*). *B,* Magnification of chromosome 9 that allows the precise mapping of the *DNAI1* gene to the p13-p21 region on both chromatids. *C,* Ideogram of human chromosome 9 that shows the precise localization of the *DNAI1* gene (*red arrowhead*).

to a *C. reinhardtii* intermediate chain of dynein. We have demonstrated its involvement in human PCD, an as-yet-unexplained syndrome characterized by a severe respiratory disease associated with male infertility. This gene, which is highly expressed in trachea and testis, is composed of 20 exons located in the p13-p21 region of chromosome 9.

Several lines of evidence support the hypothesis that *DNAI1* encodes an axonemal intermediate chain of dynein. First, BLAST analysis of the *DNAI1* cDNA sequence revealed that its product was highly homologous to sea urchin IC2 and *Chlamydomonas* IC78 sequences. At least two subclasses of axonemal intermediate chains of dynein have been identified in lower eukaryotic organisms: *Chlamydomonas* IC78 and sea urchin IC2 proteins define one subclass, whereas *Chlamydomonas* IC69 and sea urchin IC3 proteins belong to the second subclass of intermediate chains (Ogawa et al. 1995). On the basis of the results of sequence analysis, DNAI1 is likely to belong to the IC78/IC2 subclass of axonemal intermediate chains; such a conclusion is further supported by the following phylogenetic data: when compared with axonemal intermediate chains and cytoplasmic intermediate chains isolated in different species (Paschal et al. 1992; Nurminsky et al. 1998; Crackower et al. 1999), including the human DNCI1 chain (Crackower et al. 1999), DNAI1 is still closer to both *Chlamydomonas* IC78 and sea urchin IC2. Second, the predicted protein sequence (699 residues) contains several short motifs known as "WD repeats," which are highly conserved through evolution among all axonemal intermediate chains identified to date (Ogawa et al. 1995; Wilkerson et al. 1995; Yang and Sale 1998). These motifs, which are located in the carboxy-terminal halves of the intermediate chains (Ogawa et al. 1995; Wilkerson et al. 1995), are thought to be involved in protein-protein interactions within multicomponent complexes (Neer et al. 1994; Smith et al. 1999). Third, the full-length *DNAI1* cDNA sequence, which was used as a probe on northern blots with various human adult tissues, revealed a signal only in testis and trachea; since these two tissues contain numerous axonemal structures, such hybridization data further support the hypothesis that the identified sequence indeed encodes an axonemal protein. A major transcript (~2.5 kb) was found in both tissues, whereas an additional weakly expressed transcript (~4

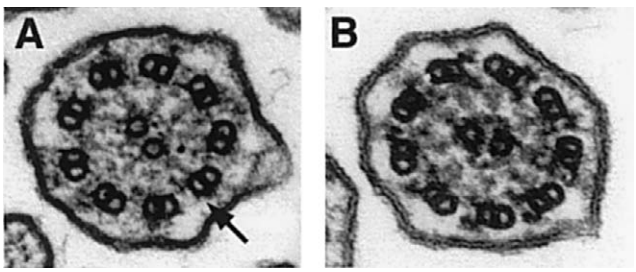

Figure 5 Electron micrograph of cross-sections of respiratory cilia (original magnification ×90,000). *A,* Absence of outer dynein arms (*arrow*) is observed on all the peripheral doublets of the ciliary sections obtained from PCD patient II-1. *B,* Normal ciliary ultrastructure from a control subject.

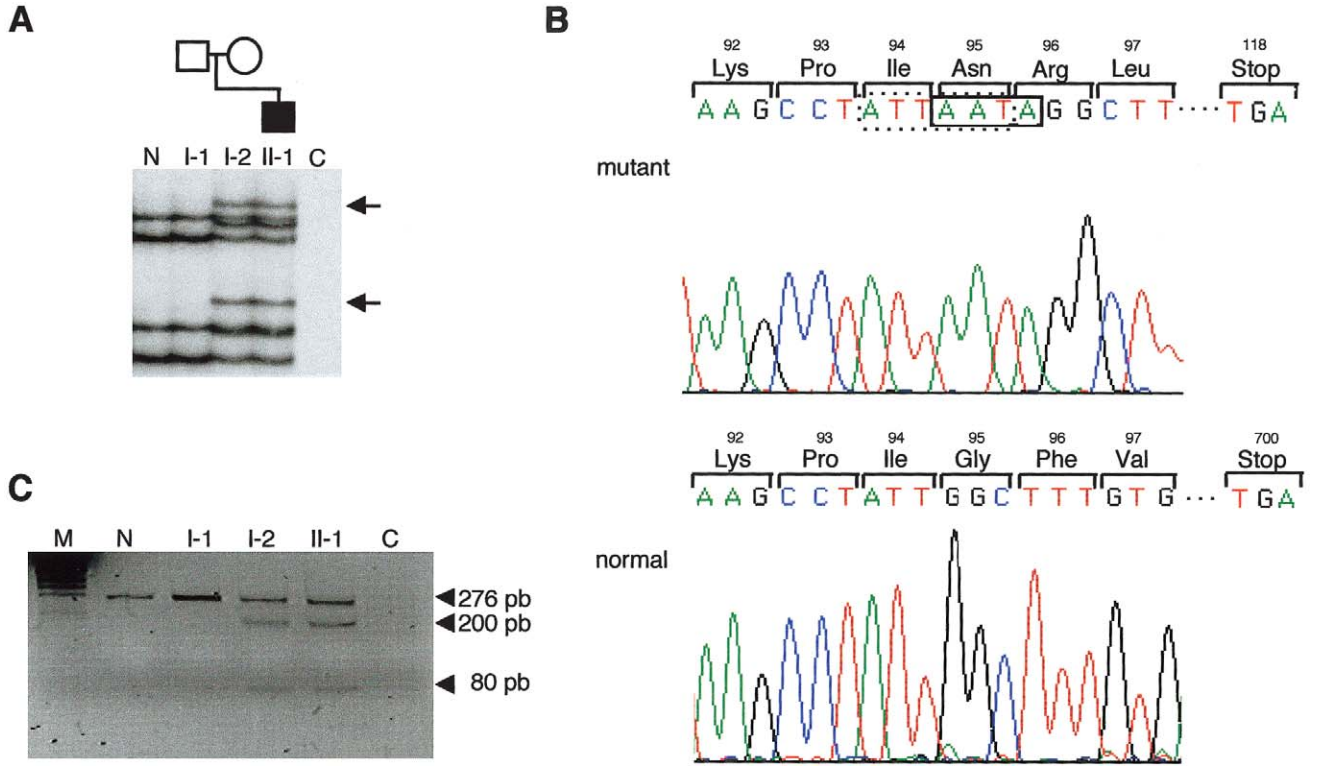

Figure 6 The exonic insertion identified on the maternal *DNAI1* allele of patient II-1 from family 1. *A,* SSCP analysis of the PCR products of exon 5 from three members (I-1, I-2, and II-1) of this family and from a control subject (N), showing bandshifts (*arrows*) both in patient II-1, who is denoted by a blackened square, and in his mother (I-2). Lane C corresponds to a negative control (PCR without genomic DNA). *B,* Nucleotide sequence of the mutant (*top*) and normal (*bottom*) *DNAI1* alleles. The insertion of four nucleotides in exon 5 is denoted by the area outlined by a solid line. The recognition sequence for the *Vsp*I restriction endonuclease is denoted by the area outlined by a dashed line. *C,* Segregation analysis of the 4-bp insertion within the family. The presence of the mutation in the genomic DNA from all three family members was assessed by *Vsp*I digestion of PCR products generated with primers P8 and P9. The affected child (II-1) and his mother (I-2) yield a banding pattern consistent with the presence of both the normal (276-bp) and the mutant (80-bp and 200-bp) alleles, whereas both his father (I-1) and a control subject (N) display only normal alleles. Lane C corresponds to a negative control (PCR without genomic DNA). The size marker (M) is a 1-kb ladder from Gibco BRL.

kb) was visualized only in trachea tissue. This latter molecular species, which was detected with different probes covering either the 5′ part or the 3′ part of the cloned cDNA, is highly likely to correspond to a *DNAI1* transcript with a larger 3′ UTR resulting from the use of a second polyadenylation site.

We postulated that the human *DNAI1* gene identified in this study was an excellent candidate sequence for investigation in patients with a PCD phenotype characterized by an absence of outer dynein arms. *Chlamydomonas* IC78 indeed belongs to outer dynein arms (King et al. 1991; Wilkerson et al. 1995); its sequence similarity with DNAI1 therefore suggests that, in humans, the DNAI1 protein may also participate in the composition of outer dynein arms. In addition, in *Chlamydomonas,* protein cross-linking studies indicated that IC78 is in direct contact with α-tubulin in the axoneme (King et al. 1991, 1995), thereby suggesting that IC78 may be involved in anchoring the outer arm to the peripheral microtubules. Furthermore, a lack of outer dynein arms has been clearly documented in the axonemes of *Chlamydomonas* strains, in which the IC78 gene is either deleted or disrupted by a large insertion (Wilkerson et al. 1995). Two different transallelic germline *DNAI1* mutations leading to frameshifts were indeed identified in one patient presenting with a PCD phenotype associated with an absence of outer dynein arms. The 4-bp insertion identified on the maternal *DNAI1* allele, which probably arose as a result of slippage replication, is predicted to produce a frameshift introducing a premature stop codon located 24 amino acids downstream. The paternal mutation is a splice defect; if translated, the abnormal *DNAI1* transcript, which retains the first 132 nucleotides of intron 1, would result in a premature TGA stop codon at position 73. Both the maternal mutation and the paternal mutation should, therefore, generate severely truncated polypeptides lacking 85% and 95%, respectively, of the DNAI1 protein, with

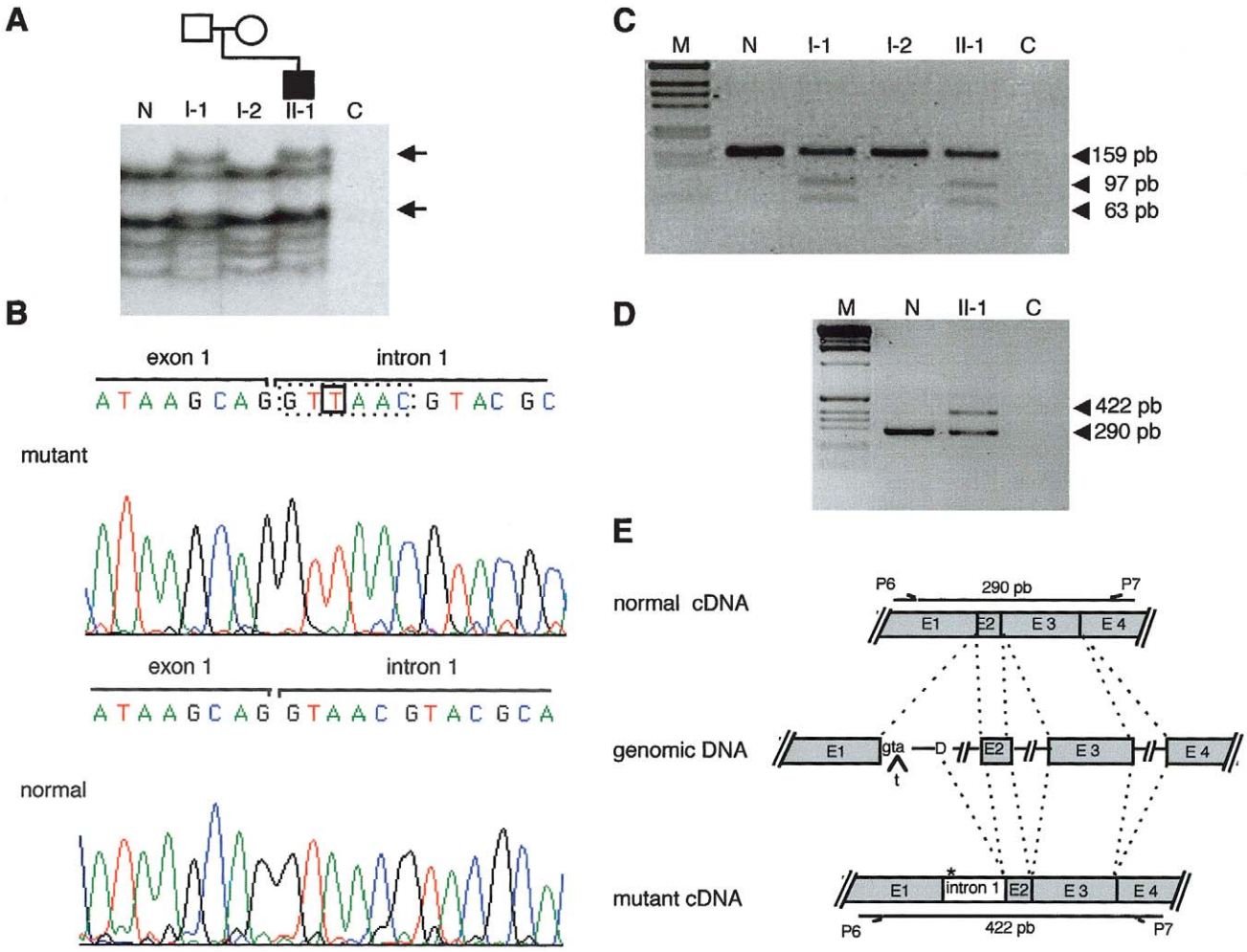

Figure 7 The splice mutation identified on the paternal *DNAI1* allele of patient II-1 from family 1. *A,* SSCP analysis of the PCR products of exon 1, which were obtained from three members of this family and from a control subject (N), show bandshifts (*arrows*) both in patient II-1 (denoted by the blackened square) and in his father (I-1). Lane C corresponds to a negative control (PCR without genomic DNA). *B,* Nucleotide sequence of the mutant (*top*) and normal (*bottom*) *DNAI1* alleles. The 1-bp insertion (T) at nucleotide +3 of the intronic sequence following exon 1 is outlined by a solid line. The recognition sequence for the *Hpa*I restriction endonuclease is denoted by the area outlined by a dashed line, and the position of the exon-intron boundary is indicated above the sequence. *C,* Segregation analysis of the 1-bp insertion within the family. The presence of the mutation in the genomic DNA from all three family members was assessed by *Hpa*I digestion of PCR products generated with primers P6 and P11. The affected child (II-1) and his father (I-1) yield a banding pattern consistent with the presence of both normal (159-bp) and mutant (97-bp and 63-bp) alleles, whereas both his mother (I-2) and a control subject (N) display only the normal alleles. Lane C corresponds to a negative control sample (PCR without genomic DNA). The size marker (M) is a 1-kb ladder from Gibco BRL. *D,* Functional consequences of this 1-bp insertion on *DNAI1* RNA splicing. RT-PCR amplifications of total RNA obtained from nasal cells from a control subject (lane N) and from the patient (lane II-1), with the use of primers P6 and P7. The RNA sample from the control subject generates a 290-bp product, whereas the RNA sample from patient II-1 generates two products, one of normal size (290 bp) and one arising from abnormal splicing (422 bp). Lane C contains a control without RNA sample. The size marker (lane M) is a 1-kb ladder from Gibco BRL. *E,* Schematic representations of the splicing mechanisms leading to normal (*top*) and abnormal (*bottom*) *DNAI1* transcripts, from the corresponding genomic DNA fragments (*middle*). The 1-bp insertion at the third nucleotide of intron 1 is indicated below the genomic sequence (*arrowhead*). Exonic sequences (E1–E4) and intronic sequences are denoted by gray boxes and thick solid lines, respectively. In the intronic sequence following exon 1, lowercase letters indicate the 5′ splice consensus site normally used in primary *DNAI1* transcripts; letter D denotes the cryptic splice-donor site used in the presence of the 1-bp insertion. The sizes of the normal (*top*) and mutant (*bottom*) RT-PCR products generated with primers P6 and P7 (*arrows*) are indicated. In the presence of the 1-bp insertion (*asterisk*), the 5′ part of the intron (*open box*) following exon 1 is retained in the mutant cDNA (*bottom*).

these large deleted regions including all five WD repeat motifs. Taken together, these data strongly suggest that such truncated proteins, even if synthesized, could not play their key role in the outer dynein arm assembly. We therefore conclude that these mutations, which were absent from 100 control chromosomes, underlie the ultrastructural phenotype observed in this patient with PCD. The documented immotile cilia are also consistent with the absence of outer dynein arms; this may be responsible for the mucociliary impairment that has led to the severe chronic respiratory symptoms observed in this patient.

In the course of this study, in addition to these two disease-causing mutations, we have detected two nucleotide polymorphisms in *DNAI1,* a finding that has allowed us to demonstrate an exclusion of linkage between the *DNAI1* gene and the PCD phenotype in five unrelated consanguineous families. These linkage data, which provide the first clear-cut demonstration of locus heterogeneity in this condition, also reveal a higher complexity level of heterogeneity than was first expected, since patients with identical ultrastructural defects may have mutations in other as-yet-unidentified genes; it is tempting to speculate that these latter genes may encode functional partners of DNAI1 involved in dynein arm assembly. Given the documented clinical, ultrastructural, and genetic heterogeneity in PCD, it is, therefore, not surprising that genetic analyses performed in different sets of families did not allow establishment of reproducible genetic linkage between the disease phenotype and a given chromosomal region (Chapelin et al. 1997*a*; Witt et al. 1999). However, in theory, this kind of approach could be fruitful, if applied to the study of separate families, with each being considered individually. Nevertheless, such genetic linkage studies have been hampered by the small size of affected families, in which male patients are usually infertile. As shown in the present study, this is the reason why approaches made on the basis of the investigation of candidate genes represent powerful alternatives. These candidate genes include the dynein gene family; however, the great number and the huge size of several of these genes make the ultrastructural phenotype the most pertinent feature for the identification of the best candidate(s). This study illustrates the use of one particular *Chlamydomonas* flagellar mutant as an excellent model for axonemal abnormalities observed in PCD; as several other light (LeDizet and Piperno 1995*a*), intermediate (Mitchell and Kang 1991; Perrone et al. 1998), and heavy (Sakakibara et al. 1991; Sakakibara et al. 1993; Porter et al. 1994; Rupp et al. 1996; Myster et al. 1997) chains of dynein have been implicated in other *Chlamydomonas* flagellar mutants, we consider the corresponding genes to be good candidates for other subsets of PCD and related developmental diseases.

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Electronic-Database Information

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

BLAST Sequence Similarity Search, http://www.ncbi.nlm.nih.gov/BLAST/

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for *DNAI1* cDNA, deduced protein sequence [accession number AF091619], intronic boundaries and sequences of primers [accession numbers AF190477–AF190496], *Chlamydomonas* IC78 [accession number U19120] and IC69 [accession number X55382], sea urchin IC2 [accession number D38538] and IC3 [accession number D28863], rat IC74 [accession number X66845], rat dynein cytoplasmic intermediate chain–2A [accession number U39044], mouse Dnci1 [accession number AF063229] and Dnci2 [accession numberAF063231], *Drosophila* Cdic [accession number AF070687], *Dictyostelium* dicA [accession number U25116], and human DNCI1 [accession number AF063228])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PCD [MIM 242650] and Kartagener syndrome [MIM244400])

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