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Identification of Dynein Heavy Chain 7 as an Inner Arm Component of Human Cilia That Is Synthesized but Not Assembled in a Case of Primary Ciliary Dyskinesia*

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Although the basic structure of the axoneme has been highly conserved throughout evolution, the varied functions of specialized axonemes require differences in structure and regulation. Cilia lining the respiratory tract propel mucus along airway surfaces, providing a critical function to the defense mechanisms of the pulmonary system, yet little is known of their molecular structure. We have identified and cloned a dynein heavy chain that is a component of the inner dynein arm. Bronchial epithelial cells were obtained from normal donors and from a patient with primary ciliary dyskinesia (PCD) whose cilia demonstrated an absence of inner dynein arms by electron microscopy. Cilia from normal and PCD cells were compared by gel electrophoresis, and mass spectrometry was used to identify DNAH7 as a protein absent in PCD cilia. The full-length DNAH7 cDNA was cloned and shares 68% similarity with an inner arm dynein heavy chain from *Drosophila*. DNAH7 was induced during ciliated cell differentiation, and immunohistochemistry demonstrated the presence of DNAH7 in normal cilia. In cilia from PCD cells, DNAH7 was undetectable, whereas intracellular DNAH7 was clearly present. These studies identify DNAH7 as an inner arm component of human cilia that is synthesized but not assembled in a case of PCD.

The basic structure of the axoneme consisting of nine outer microtubule doublets surrounding a central pair (9 + 2 arrangement) is one of the most highly conserved structures in all of cell biology (1, 2). The 9 + 2 arrangement is found in many diverse organisms ranging from the flagella of *Chlamydomonas* and the gill cilia of freshwater mussels to the sperm flagella of sea urchins and respiratory tract cilia of humans. Despite the structural similarity, each of these axonemes is specialized for a particular function. Thus, the waveform of a sperm flagellum (3), which functions to propel the sperm forward, is very different from the coordinated beating of respiratory tract cilia, which function to transport mucus over airway surfaces (4). Moreover, the regulation of the activity of each specialized axoneme is also likely to be unique. For example, increases in Ca^{2+} have been shown to stimulate ciliary beating of tracheal epithelial cells (5), whereas *Paramecium* cilia respond to increased Ca^{2+} by reversing the direction of beating (6). These functional differences are a reflection of unique modifications of the structure and composition of each specialized axoneme. For example, *Chlamydomonas* flagella have three outer arm dynein heavy chains (DHCs)[1] (7), whereas outer arms from mammalian respiratory cilia have been reported to contain only two DHCs (8). Therefore, it is clear that although the basic structure and components of cilia and flagella are conserved, specialization of function has resulted in important variations of structure and regulation among the different motile axonemes.

The *Chlamydomonas* flagellum represents the most thoroughly characterized axonemal structure. Through the use of readily obtained mutants and computer-assisted image analysis of electron micrographs, the positions and functions of many of the proteins that make up the inner and outer dynein arms, which provide the force for axonemal motility, have been identified in this species. As noted above, the outer dynein arms of *Chlamydomonas* contain three DHCs known as α, β, and γ (7). The arrangement and composition of DHCs in the inner arms are more complex with at least eight different DHCs organized into seven distinct inner arm complexes (9). In contrast, our understanding of mammalian axonemes, including human respiratory tract cilia, is limited. This is partly because of the difficulties involved in obtaining sufficient material for biochemical characterization but also because, unlike *Chlamydomonas*, the development of mutants (*i.e.* murine knock-out models) is time- and labor-intensive. However, there is a naturally occurring disease in which individuals display an inherited defect in ciliary function known as primary ciliary dyskinesia (PCD) (10, 11). Cilia from PCD patients demonstrate an abnormal pattern of ciliary beating and in some cases are completely immotile. In most cases of PCD, the defects in ciliary function are correlated with abnormal ciliary structure with the absence of inner and/or outer dynein arms being the most frequently reported defect. Thus, cilia from these individuals are analogous to mutant flagella from *Chlamydomonas*, and studies of these defective cilia will probably yield new information about the structure of mammalian axonemes.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF327442.

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[1] The abbreviations used are: DHC, dynein heavy chain; HBE, human bronchial epithelial; PCD, primary ciliary dyskinesia; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PBS, phosphate-buffered saline; PBST, PBS plus Tween 20.

To further our understanding of the structure and regulation of human respiratory tract cilia, we have begun a detailed characterization of the components of this complex organelle. By culturing human bronchial epithelial (HBE) cells under conditions that support ciliated cell differentiation, we have obtained sufficient quantities of human ciliary axonemes for biochemical and molecular analysis. As an approach to identify specific components of the inner dynein arm, ciliary axonemes from normal cells were compared with ciliary axonemes from PCD cells lacking inner dynein arms. Proteins absent in the PCD cilia were identified by mass spectrometry of the corresponding protein from control cilia. This approach identified human DNAH7 as a likely component of the axonemal inner arm. To further characterize this DHC, the partial sequence data obtained by mass spectrometry were used to clone a full-length cDNA. The expression of DNAH7 during ciliogenesis was examined both by Northern blotting and by using specific antibodies developed against a recombinant fragment of the protein. Finally, the expression of DNAH7 was examined in HBE cells from the PCD patient.

EXPERIMENTAL PROCEDURES

Cell Culture—HBE cells were obtained from excess surgical tissue under protocols approved by the University of North Carolina Institutional Review Board. HBE cells were obtained from normal subjects (transplant donors), a patient with established PCD who required a lung transplant, and as disease controls, cystic fibrosis patients. No differences were observed between cilia from the normal and cystic fibrosis cells. Passage 1 or passage 2 HBE cells were grown at an air-liquid interface using previously described procedures (12, 13) with only minor modifications. 5–10 $\times$ 10^5 passage 1 or passage 2 HBE cells were plated on 30-mm-diameter Millicell-CM culture inserts (Millipore Corp., Bedford, MA) previously coated with rat tail collagen (BD PharMingen). After reaching confluence, medium was removed from the apical surface, and cultures were fed from only the basal surface for the remainder of the experiment. The medium was replaced every 2–3 days, and cilia were isolated from well differentiated cultures after 4–6 weeks.

Isolation of Cilia—Cilia were isolated essentially as described previously (14, 15). Heavily ciliated cultures were washed with phosphate-buffered saline (PBS) to remove mucus and cell debris. Deciliation buffer (14) was added to the surface, and the culture was rocked gently for 1 min. The supernatant was collected, and the procedure was repeated. The two washings were pooled and after pelleting debris at 1000 $\times$ *g*, the ciliary axonemes were collected by centrifugation at 16,000 $\times$ *g* and frozen at −80 °C. Protein concentrations were estimated using the BCA reagent (Pierce).

Gel Electrophoresis—For analysis of high molecular weight ciliary proteins, samples were electrophoresed on 4% Tris-glycine-SDS gels (Novex, San Diego, CA) according to the manufacturer's instructions. To facilitate analysis by mass spectrometry, proteins were visualized by staining with colloidal blue (Pro-Blue, Owl Separation Systems, Woburn, MA) according to the manufacturer with the omission of the first fixation step.

Mass Spectrometry Analysis—In-gel digestion with trypsin, liquid chromatography-tandem mass spectrometry (LC/MS/MS), and searching of data bases were all performed using standard techniques essentially as described previously (16).

Cloning, Sequencing, and Analysis of DNAH7—Mass spectrometry of the high molecular weight protein identified a partial cDNA sequence (GenBank™ accession number Z83801) encompassing the P1-loop of a DHC. The sequence of DNAH7 that lies 5′ to the P1-loop was obtained by sequence walking employing successive steps of PCR with vector primers and DNAH7-specific primers obtained from verified sequences from the previous step. An oligo(dT) and random-primed cDNA library generated from heavily ciliated HBE cell RNA prepared by Stratagene in λZAP was used as template. The sequence of DNAH7 that lies 3′ to the P1-loop was obtained by amplification of the sequence between the P1-loop region and another partial cDNA sequence identified by mass spectrometry, KIAA0944 (GenBank™ accession number AB023161). In general, PCR was performed for 40 cycles (4 min at 94 °C initially followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 68 °C for 5 min) using a GeneAmp PCR System 9700 (PerkinElmer Life Sciences). Reaction conditions were as follows: 50 μl of total volume containing 2 μl of cDNA library supernatant, 25 pmol of each primer, 0.75 μl of enzyme mix (Expand Long Template PCR System, Roche Molecular Biochemicals) in the buffer system provided by the manufacturer. The resulting PCR products were cloned into pCR2.1TOPO (Invitrogen) and sequenced in the University of North Carolina at Chapel Hill Automated DNA Sequencing Facility.

Reverse transcription (RT)-PCR was used to verify the sequence obtained from the library and to search for mutations in the PCD patient. Primer pairs located throughout the sequence were used that yielded 24 overlapping sequences of 700–800 bp. Total RNA was isolated from well differentiated cultures of HBE cells using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, Ohio). First strand cDNA was prepared using 3 μg of total RNA and 250 ng of random primers with SuperScript II RNaseH− reverse transcriptase (Invitrogen) according to manufacturer's instructions. 1 μl of the first strand reaction was used in a 50-μl PCR mixture containing 10 pmol of each primer, 2.5 units of *AmpliTaq* DNA polymerase (PerkinElmer Life Sciences) in manufacturer's buffer with 2 mM $MgCl_2$. Reaction conditions were as follows: initial denaturation at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min with a final 10-min extension at 72 °C. RT-PCR products were gel-purified using QIAquick™ gel extraction kits (Qiagen, Valencia, CA). Products were directly sequenced in the University of North Carolina at Chapel Hill Sequencing Facility using DNAH7-specific sequencing primers.

To verify identified sequence variations on genomic DNA, genomic sequence from the data base (GenBank™ accession numbers AC068528, AC068919, and AC013274) was used to design primers on either side of the variation, usually one primer in the exon and one primer in the intronic region surrounding the exon. These primers were used to amplify the region of interest from genomic DNA (50 ng). PCR conditions and sequencing were as described above for RT-PCR.

Sequence assembly and analyses were performed using Vector NTI suite 6 (InforMax), GCG (Genetics Computer Group), and BLAST (NCBI) programs.

Antibody Production and Characterization—For antibody production, a 0.8-kb *Bgl*II/*Eco*RI cDNA fragment of DNAH7 consisting of amino acids 1531–1780 of our established sequence was cloned into the bacterial expression vector pET-28a (Novagen, Madison, WI) to generate plasmid pET-H7. This construct was used to produce a DNAH7 peptide that was designated H7. In addition, a peptide from a similar region of DNAH9 (GenBank™ accession number AF257737) consisting of amino acids 2057–2346 was obtained by PCR of the differentiated airway cell library using primers 5′-ATCAAGTCCGTGCTGGTGG-3′ and 5′-CAAGATCTGAACCATGCTCTGCTCTG-3′, which contains a *Bgl*II site. The product was cloned into the pET-28a vector using *Bgl*II and *Bam*HI (the *Bam*HI site was in the DNAH9 cDNA sequence) to produce plasmid pET-H9. This plasmid produced a peptide that was designated H9.

For antibody production, the H7 peptide was expressed and purified from *Escherichia coli* strain BL21(DE3) (Novagen) using a HisTrap purification kit (Amersham Biosciences) according to the manufacturer's instructions. The purified protein was further fractionated by polyacrylamide gel electrophoresis, and the band containing H7 peptide was excised and submitted to Aves Laboratories (Tigard, OR) for immunization to obtain chicken anti-DNAH7 antibody. The chicken IgY from the eggs of the immunized chickens was purified by the manufacturer.

For testing the specificity of the antibodies produced, [^{35}S]methionine-labeled H7 and H9 proteins were generated using TNT T7-coupled rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine (ICN Biomedicals, Costa Mesa, CA) according to the manufacturer's protocol using plasmids pET-H7 and pET-H9 as templates. Immunoprecipitation was conducted by mixing 1 μl of *in vitro* translated [^{35}S]methionine-labeled proteins with 1 μl of anti-DNAH7 chicken IgY. The mixture was then incubated with 25 μl of immobilized anti-chicken IgY (Promega) in 500 μl of PBS containing 1% Empigen BB detergent, 1 mM EDTA, and 0.1 mM dithiothreitol at 4 °C for 1 h. Unbound proteins were removed by several washes of the beads with the binding buffer. Bound proteins were eluted with SDS-PAGE loading buffer (50 mM Tris, pH,6.8, 2% SDS, 0.1 M dithiothreitol, 10% glycerol, 0.1% bromphenol blue) separated by gel electrophoresis and visualized by fluorography.

Western Blotting—Cultured cells were washed with cold PBS and scraped into SDS lysis buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol). Samples were then boiled for 5 min and passed through a 25-G needle 10 times to shear the genomic DNA. The lysate was clarified by centrifugation at 13,000 rpm for 5 min at 4 °C, and protein concentrations were determined using the BCA kit. One-tenth volume

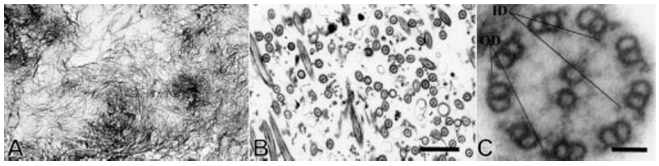


FIG. 1. **Isolated cilia from cultures of HBE cells.** Cilia were isolated from well differentiated cultures of HBE cells and examined by light (*A*) and electron (*B* and *C*) microscopy. The preparation consists of mostly intact ciliary axonemes with inner and outer dynein arms present. *A*, Richardson's stain, original magnification at ×40. *B* and *C*, fixed in 2% paraformaldehyde/2% glutaraldehyde with 1% tannic acid. *Scale bars* = 1.1 μm (*B*); 50 nm (*C*).

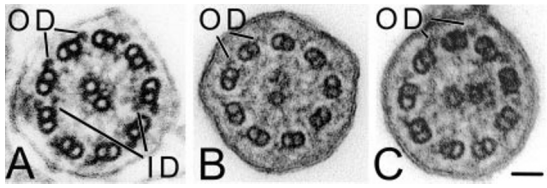


FIG. 2. **PCD cilia are deficient in inner dynein arms.** Cilia obtained from a nasal scrape of a control (*A*) and the PCD patient (*B* and *C*) were examined by electron microscopy. Although both inner and outer dynein arms are clearly visible in the control, cilia from the PCD patient demonstrated an almost complete absence of inner arm structures. *Scale bar* = 50 nm.

of 1 M dithiothreitol and 1% bromphenol blue were added before gel electrophoresis. The total cell lysates (50 μg) or aliquots of purified cilia were separated on precast gels (Novex, San Diego, CA) according to the manufacturer. Gels were transferred to polyvinylidene difluoride membranes (Amersham Biosciences) in NuPAGE transfer buffer at 30 volts for 2 h using the Novex Xcell blotting apparatus. Polyvinylidene difluoride membranes were blocked with BlockHen (Aves Laboratories) diluted 1:5 in PBST (0.58 M Na_2HPO_4, 0.017 M NaH_2PO_4, 0.68 M NaCl, 0.05% Tween 20) or with 5% milk in PBST followed by the incubation with the chicken anti-DNAH7 IgY (1:10,000 dilution) or anti-β-tubulin IV (1:4000 dilution, monoclonal, Biogenetex, Seabrook, TX) for 1 h. β-Tubulin IV is known to be localized in cilia (17) and was used as a control for axonemal protein loading. Membranes were then washed with PBST plus 1% milk and incubated with a 1:20,000 dilution of horseradish peroxidase-conjugated secondary antibodies (goat anti-chicken IgY from Aves Laboratories or rabbit anti-mouse IgG from Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactivity was detected using ECL-plus (Amersham Biosciences) according to the manufacturer's instructions.

Immunohistochemistry—For immunostaining, ciliated cultures of HBE cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min, and embedded in paraffin. The sections were deparaffinized twice in xylene and rehydrated in an ethanol series. Slides were then immersed in methanol containing 0.6% hydrogen peroxide for 15 min to inactivate endogenous peroxidase activities. Slides were incubated for 1 h in blocking buffer (5% goat or rabbit serum, 1% gelatin, 1% bovine serum albumin in PBS plus 0.05% Tween 20) and then for 1 h with the anti-DNAH7 (1:250 dilution in blocking buffer) or anti-β-tubulin IV (diluted 1:500) antibodies. Preimmune IgY from the same chicken that generated the anti-DNAH7 antibody was used as a control for anti-DNAH7 antibody at a 4-fold higher concentration. The sections were then washed with PBST followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:500) for 1 h. The signal was detected with SIGMA*FAST* DAB (Sigma) for 15 min. The sections were lightly counterstained with methyl green, dehydrated in ethanol, and coverslipped with permount.

Northern Analysis—15 μg of total RNA isolated as described above was separated in a 1% agarose-formaldehyde gel according to NorthernMax™ protocols (Ambion, Austin, TX). RNA was transferred to Hybond-XL membrane (Amersham Biosciences) by capillary blotting according to the manufacturer's instructions and cross-linked using a Stratalinker (Stratagene). A gel-purified PCR fragment containing nucleotides 51–812 of DNAH7 cDNA was used as a probe for detecting DNAH7 expression. A DNA fragment containing nucleotides 1–718 of the human intermediate chain dynein cDNA (IC78, GenBank™ accession number NM_012144), another protein known to be associated with cilia (18), was obtained by PCR from the differentiated airway cell library. This fragment was used as a probe to detect IC78 message. Probes were labeled with ^{32}P using Megaprime DNA-labeling system (Amersham Biosciences) and purified on MicroSpin™ G-50 columns (Amersham Biosciences). Hybridizations were performed in Rapid-hyb buffer (Amersham Biosciences) at 65 °C, and blots were washed according to manufacturer's instructions before being exposed to film at −70 °C with intensifying screens.

Image Analysis—Light microscope images were captured with a Hamamatsu C5810 color 3CCD camera (Hamamatsu Photonic Systems, Bridgewater, NJ) and processed in Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA). Electron micrographs and blots were scanned with a Umax powerlook III flatbed scanner (Umax Technologies, Inc., Fremont, CA) and also processed in Adobe Photoshop.

RESULTS

Identification of Inner Arm Axonemal Dynein Heavy Chains—Normal HBE cells were cultured at an air-liquid interface under conditions that favor mucociliary differentiation. Under these conditions, HBE cells differentiate to produce a heavily ciliated epithelium that resembles native tracheobronchial epithelium (12, 13). Cilia in these cultures become coordinated and are capable of actively transporting mucus (19). Cilia were isolated from these well differentiated cultures by using a non-ionic detergent in the presence of calcium (14, 15). These preparations are highly enriched for cilia (Fig. 1, *A* and *B*) and consist primarily of intact ciliary axonemes with the 9 + 2 microtubules, inner and outer dynein arms, and radial spokes clearly visible (Fig. 1*C*).

HBE cells were obtained from a PCD patient who underwent a lung transplant operation for severe bronchiectasis. Light microscopic examination of cilia obtained from a nasal scrape demonstrated abnormal ciliary motion. Electron microscopy revealed a number of ciliary abnormalities with the most consistent defect being a deficiency of inner dynein arms. Thus, although structures resembling outer dynein arms were observed on a majority of axonemal doublets, the inner arm structures were only rarely observed (Fig. 2, *A–C*). These PCD cells when cultured at an air-liquid interface differentiated normally to produce a mucociliary epithelium. These cultures also reproduced the phenotypic defect present in PCD patients. Whereas cilia of normal or non-PCD disease control (*i.e.* cystic fibrosis) cells exhibited abundant ciliary activity, which frequently was coordinated, the cilia in the PCD cultures appeared straight and rigid and beat only infrequently. Electron micrographs of cilia from cultured PCD cells also reproduced the deficiency of inner dynein arms observed in the freshly excised tissue.

To identify components of the inner dynein arm, cilia were isolated from differentiated cultures of normal and disease control (cystic fibrosis) HBE cells and compared with cilia isolated from differentiated cultures of PCD cells. Proteins that are components of the inner dynein arm, including the inner arm DHCs, would be expected to be absent or greatly reduced in the axonemes from the PCD cells. Because DHCs are too large to resolve by two-dimensional gel electrophoresis (molecular mass of ~500 kDa), we examined the DHC composition of these samples by SDS-PAGE on low percentage gels. Under these conditions, four bands were clearly resolved from the normal axonemes in the high molecular weight region of the gel where DHCs would be expected to migrate (Fig. 3*A*). In most samples, the two upper bands appeared more intense than the lower two bands and are likely to contain outer arm DHCs (14, 15). In contrast, axonemes isolated from the PCD cells showed a distinct absence of the lowest of the four high molecular weight protein bands. To verify that this was a specific reduction in the protein(s) present and not a sample preparation or loading artifact, different quantities of axonemal proteins isolated from normal and cystic fibrosis cultures were compared

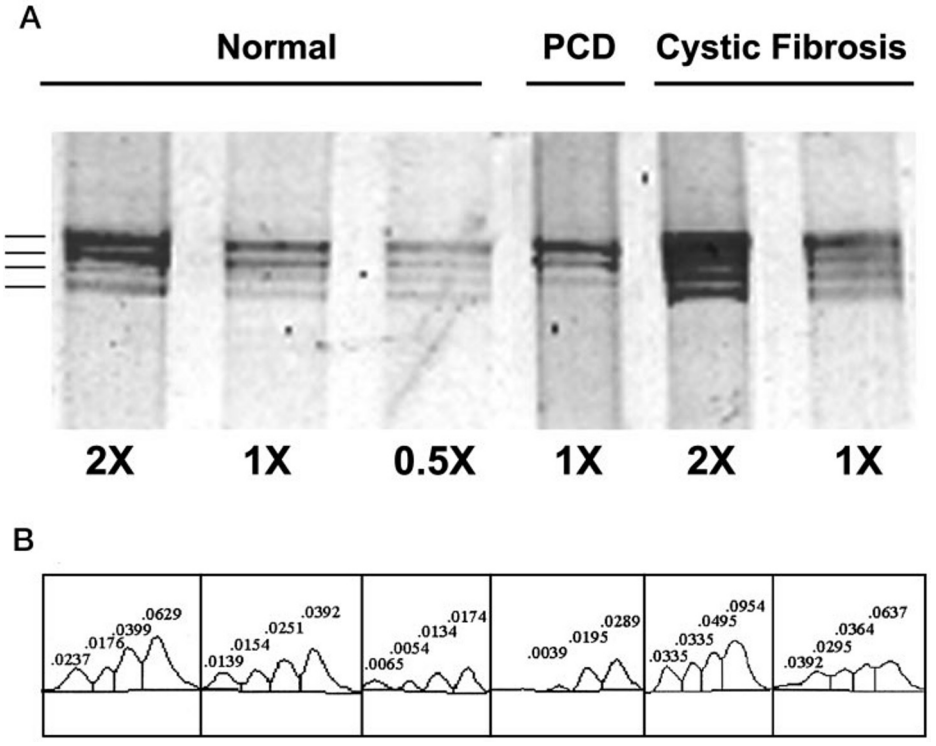


FIG. 3. **Analysis of axonemal proteins by gel electrophoresis.** *A*, different amounts of isolated axonemes from normal, cystic fibrosis, or PCD cells were electrophoresed on an acrylamide gel and stained with colloidal Coomassie Blue. *B*, densitometric tracings of the gel shown in *A*. The results demonstrate that the lowest of the four high molecular weight protein bands is undetectable in axonemes from the PCD patient. In contrast, four bands are clearly present in the control samples, even when less total protein is loaded.

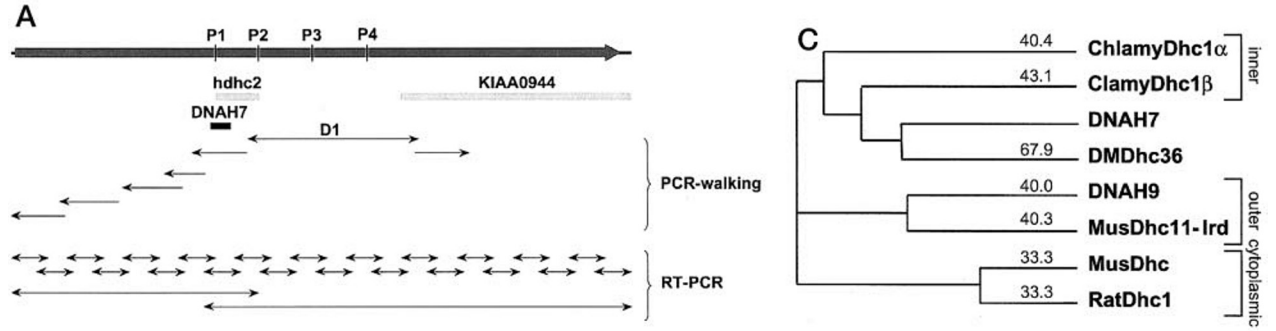
TABLE I
Peptide matches obtained from LC-MS/MS of the isolated high molecular weight protein

	Sequence	*m/z*	GenBank™ accession number	Description
1	GINAGADILMFEGTELK	889.8	Z83801	Human dynein heavy chain
2	YAYEYLGNSPR	666.8	U53532	Human dynein heavy chain
3	IGDST(L/I)EYAPDFR	742.3	P23098	Sea urchin dynein
4	MVVFVDDVNMPAR	754.7	U03970	Sea urchin dynein
5	ELQNQLNEIVELVR	848.9	U53532	Human dynein heavy chain
6	...NDGFVEADDLR	870.7	U03978	Sea urchin dynein
7	...SDDSDFEWKSQR	913.3	U53532	Human dynein heavy chain

with axonemes isolated from PCD cultures as noted above and analyzed by densitometry (Fig. 3*B*). This experiment shows that even when less protein was loaded from the control samples, all four protein bands were clearly detected. This result demonstrates that in the PCD cilia, a protein or proteins present in this lowest band are specifically reduced or absent.

Because this protein band is likely to represent inner arm components, we sought to identify the protein(s) present in this band. Isolated axonemes from control cultures were separated by SDS-PAGE as described above, and the lowest of the four high molecular weight protein bands was excised. To obtain sufficient sample for analysis by mass spectrometry, ~40 gel slices from several different cultures were pooled. The protein was digested with trypsin, and the purified peptides were analyzed by capillary LC/MS/MS. High quality amino acid sequence was obtained on >30 peptides. An initial search of the data bases using Mascot identified seven peptides that matched DHC sequences, including several with an identity to known human DHCs (Table I). These peptides matched sequences located near the conserved catalytic region of DHCs including two human dyneins identified as HDHC2 (GenBank™ accession number Z83801 (20)) and DHC3 (GenBank™ accession number U53532 (21)). These results were not unexpected, because the catalytic region around the first nucleotide-binding site (P-loop region) of the DHCs is highly conserved and is the region most represented in the data base. Both of these sequences are probably derived from inner arm axonemal DHCs. Because there are no other human full-length inner arm sequences available to date, the other peptides are most likely derived from sequences outside the conserved P-loop region and may represent additional inner arm DHCs. One sequence (Table I, *row number 1*) was uniquely specific for a human DHC previously identified as HDHC2 and is the subject of this report.

Cloning and Characterization of a Full-length cDNA for Axonemal Dynein DNAH7—To further characterize this human DHC and investigate its possible role in PCD, the full-length sequence was obtained by a combination of techniques. Anchored PCR was used to walk in the 5′ direction from the P-loop region of HDHC2 using a library constructed from heavily ciliated cultures of human airway cells (see "Experimental Procedures" for details). To obtain the 3′ end of the cDNA, we again took advantage of the mass spectrometry data. During the course of this work, the peptide sequences obtained by LC/MS/MS were used to search the data bases for newly deposited sequences. One of these searches resulted in the identification of a partial cDNA isolated from human brain designated KIAA0944 (GenBank™ accession AB023161 (22)), which contained sequences coding for several peptides from the isolated protein band. This partial cDNA is homologous to an inner arm DHC from sea urchin and is likely to represent one of the inner arm DHCs absent from the cilia of the PCD patient. To determine whether this cDNA was related to either of the dyneins previously identified by mass spectrometry, specific primers were designed to the 3′ ends of the HDHC2 and DHC3 sequences and to the 5′ end of the KIAA0944 sequence. PCR


A
P1
P2
P3
P4
hdhc2
KIAA0944
DNAH7
D1
PCR-walking
RT-PCR
C
40.4
ChlamyDhc1α
43.1
ClamyDhc1β
inner
DNAH7
67.9
DMDhc36
40.0
DNAH9
40.3
MusDhc11-Ird
outer cytoplasmic
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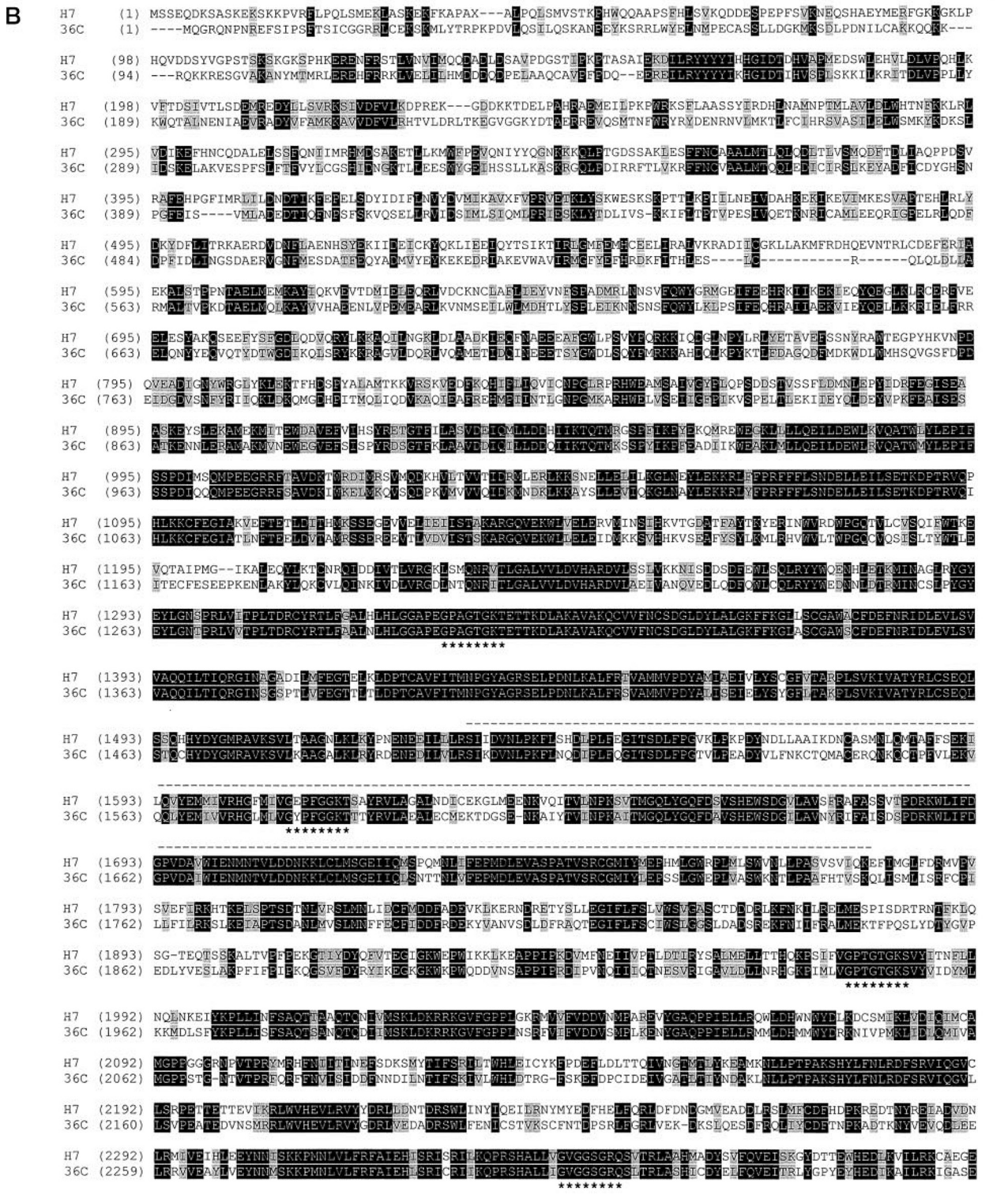
MusDhc
33.3
RatDhc1

B



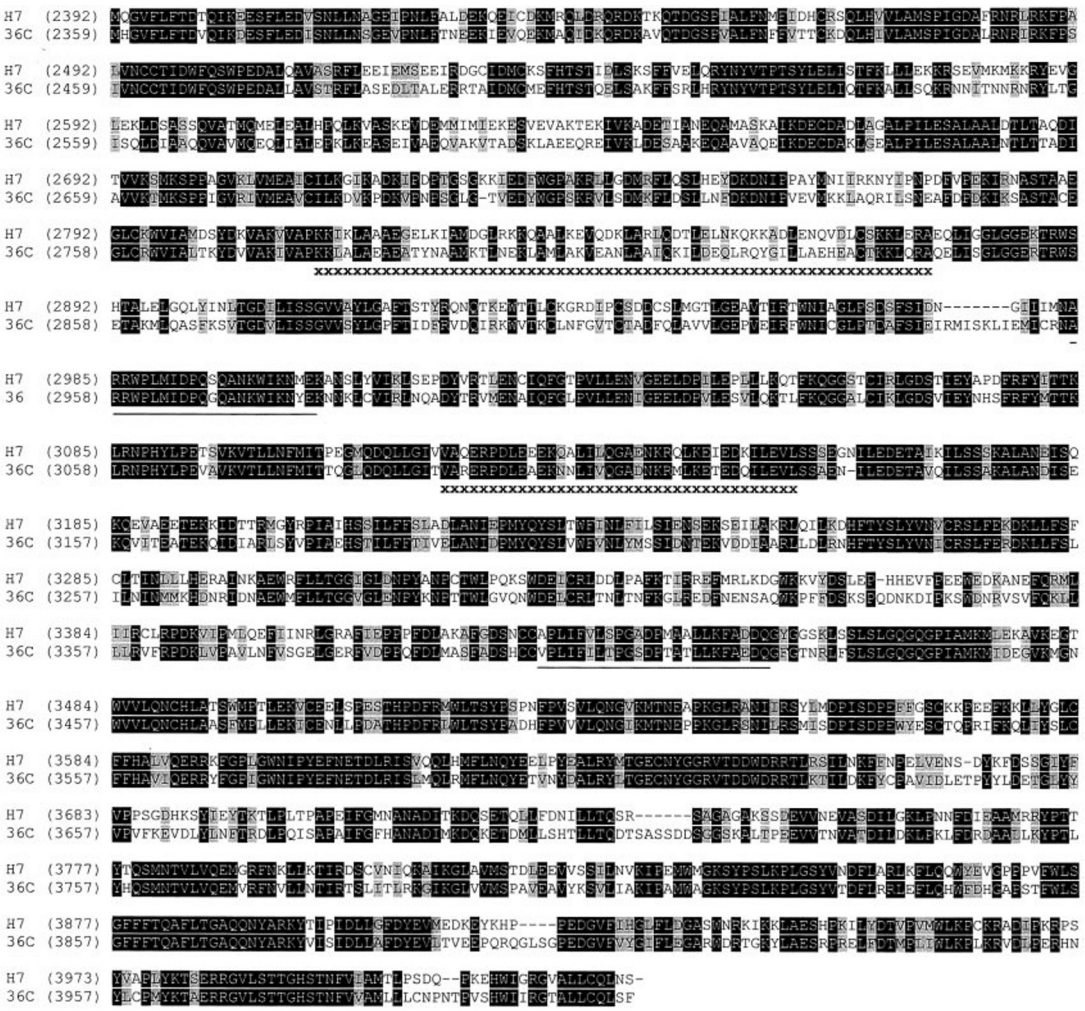


FIG. 4. *A*, schematic of the PCR-based cloning strategy. The assembled cDNA of DNAH7 is shown at the top with the location of the P-loops (ATP-binding sites) indicated. The fragment labeled D1 was derived by PCR from two sequences present in GenBank™ containing peptides that were detected by MS analysis, hdhc2 (accession number Z83801) and KIAA0944 (accession number AB023161) (*gray boxes*). This fragment completed the 3′ end of the sequence. The 5′ end of the gene was obtained by PCR walking from the hdhc2 sequence (walks indicated by *arrows*). The first PCR walk in the 5′ direction gave a sequence identical to another data base entry, DNAH7 (accession number HSA132084), indicated as the *black box*. This sequence homology solidified the designation of the sequence as DNAH7. *B*, alignment of deduced amino acid sequence of DNAH7 (*H7*) (GenBank™ accession number AF327442) with that of it closest known homologue, *Drosophila melanogaster* Dhc36C (*36C*) (GenBank™ accession number AAF53626). Amino acids identical in both species are shown with a *black background*, whereas similar residues are *shaded*. Gaps that were introduced to optimize the alignment are denoted by *dashes*. The *asterisks* indicate the four conserved P-loop regions. Additionally, two putative ATP-binding sites are *underlined*. The regions showing the highest probability of forming helical-coiled coils are indicated with an "*X*" underneath. The fragment of the protein expressed in bacteria and used for antibody generation is indicated by the *dashed overline*. *C*, phylogenetic tree showing the relationship of DNAH7 with other full-length DHCs. A representative set of full-length, cytoplasmic, inner, and outer arm DHCs were aligned to DNAH7 (Hu-DNAH7) with Align-X of Vector NTI suite using default parameters. DNAH7 maps to the axonemal inner arm family. The percentage of similarity of DNAH7 to each DHC in a pairwise comparison is indicated. The GenBank™ accession numbers of the dyneins are as follows: *Chlamydomonas reinhardtii* inner dynein arm 1-α dynein heavy chain-IA (*ChlamyDhc1*α), accession number CAB56598; *C. reinhardtii* inner dynein arm 1-β dynein heavy chain-IA (*ChlamyDhc1*β), accession number CAB99316; *D. melanogaster* dynein heavy chain dhc36c (*DMDhc36c*), accession number AAF53626; human outer dynein arm heavy chain DNAH9 (*DNAH9*), accession number CAB94756; mouse outer dynein arm heavy chain DhC11 (left-right dynein) (*MusDhc11-lrd*), accession number NP_034190; mouse cytoplasmic dynein heavy chain (*MusDhc*), accession number AAF91078; Rat cytoplasmic dynein heavy chain 1 (*RatDhc1*), accession number NP_062099.

amplification of aliquots of the cDNA library was performed with different combinations of these primers. Only the HDHC2 primer combined with the KIAA0944 primer yielded a product (Fig. 4*A*, *fragment D1*). The cloning and sequencing of this 3.8-kbp fragment demonstrated the expected identity to HDHC2 and KIAA0944 at both ends. Additional primers were designed to the newly obtained internal sequence of this fragment and used to amplify additional fragments from the cDNA library in both the 3′ and 5′ directions. The 3′ products obtained were identical to KIAA0944, confirming the sequence of this region. However, clones obtained in the 5′ direction showed identity to another partial dynein sequence, DNAH7 (GenBank™ accession AJ132084 (23)), while showing four nucleotide differences with HDHC2. These differences resulted in two amino acid changes between DNAH7 and HDHC2 at positions 1353 (Asn > Asp) and 1355 (Ser > Pro) of the completed sequence (see below). No other DHC sequence containing the Asp and Pro residues predicted by the DHC2 sequence was found in a search of the data bases. Because the original sequence of HDHC2 was obtained by PCR amplification using degenerate P-loop primers, it is not clear whether the sequence of HDHC2 represents another gene with nearly identical sequence to DNAH7 or whether the original sequence of HDHC2 was the result of a polymorphism. However, because the sequence we obtained was identical to the sequence of DNAH7, we will refer to the sequence obtained here as DNAH7. The entire 5′ sequence of DNAH7 was obtained from the cDNA library by several rounds of PCR walking.

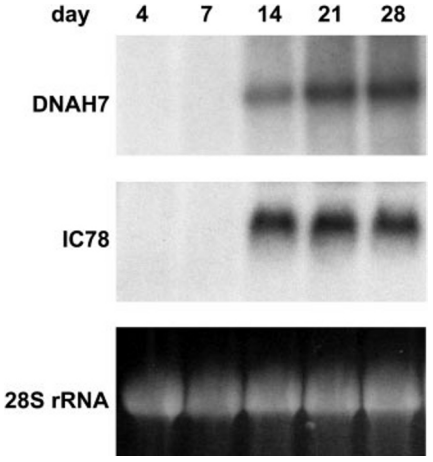


FIG. 5. **Northern analysis of DNAH7 expression during differentiation.** RNA was isolated from normal HBE cultures at the times indicated and analyzed for DNAH7 expression. The filter was reprobed with IC78, an intermediate dynein chain, as a control for axonemal gene expression. The ethidium bromide staining of the 28 S rRNA (*bottom panel*) demonstrates equivalent loading. DNAH7 and IC78 are not expressed in the undifferentiated cultures (days 4 and 7), whereas both genes are expressed in differentiated ciliated cultures (days 14, 21, and 28).

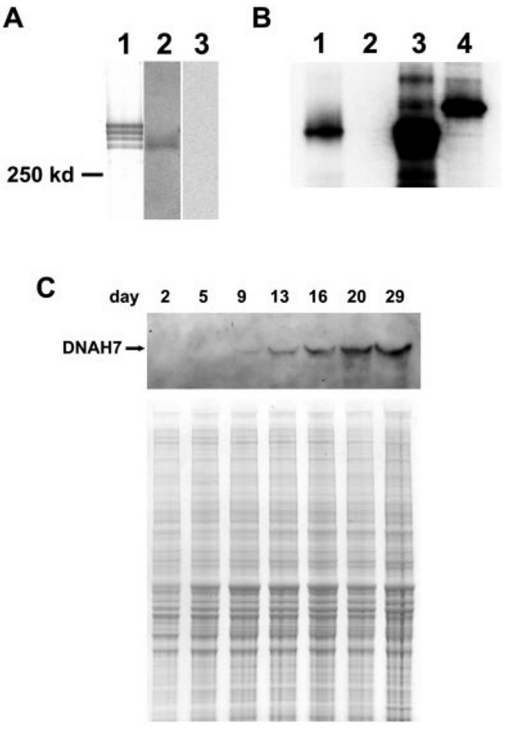


FIG. 6. **Analyses of DNAH7 protein expression.** *A*, Western blotting of axonemal proteins with an anti-DNAH7 antibody. The antibody reacts specifically with the lowest of the four proteins bands (*lane 2*), whereas preimmune antibody shows no reactivity (*lane 3*). *Lane 1* was silver-stained to visualize the protein pattern. *B*, the specificity of anti-DNAH7 antibody was demonstrated by immunoprecipitation of the H7 (*lane 1*) but not the H9 (*lane 2*) peptide produced by *in vitro* translation. The labeled, translated H7 and H9 peptides before immunoprecipitation are shown in *lanes 3* and *4*, respectively. *C*, expression of DNAH7 protein during differentiation. Total cell lysates were prepared from cultures of normal HBE cells at the indicated time points. DNAH7 was not detected early in culture but was induced during ciliated cell differentiation. The l*ower panel* shows a replicate gel stained with Coomassie Blue to demonstrate equal loading.

Because of the highly conserved nature of DHCs, additional experiments were performed to verify that the completed sequence was derived from a single DHC. First, RT-PCR was carried out directly on RNA isolated from well differentiated cultures of human airway cells. Over 20 individual overlapping products were generated and sequenced (see Fig. 4*A* and "Experimental Procedures" for details). These results verified that the clones obtained from the cDNA library were part of a single message. Second, the entire coding region of DNAH7 was amplified in two large overlapping fragments. Finally, nucleotides 3707–11530 of DNAH7 were found to align to a completely assembled genomic clone (GenBank™ accession number AC068919), further supporting the integrity of the sequence. To further confirm the presence of DNAH7 in the isolated protein band, the full-length sequence was searched with the original peptide sequences obtained by mass spectrometry. This resulted in 38 peptides being matched to the predicted coding sequence providing conclusive evidence that DNAH7 was present in the sample. These data also provide additional evidence for the correctness of the deduced coding sequence.

The assembled DNAH7 sequence (GenBank™ accession number AF327442) contains a single long open reading frame encoding a protein of 4025 amino acids with a predicted molecular mass of 461,190 Daltons (Fig. 4*B*). An in-frame stop codon is present upstream of the putative open reading frame, suggesting that the first AUG is the start codon. Of the full-length dynein sequences available, DNAH7 was most similar to the *Drosophila* gene product Dhc36C, sharing 54.6% identity and 67.9% similarity overall (Fig. 4*B*). An analysis of the predicted amino acid sequence demonstrated that DNAH7 shares many of the conserved features of dynein heavy chains (24). Four well conserved P-loops are located in the central domain of the molecule (Fig. 4*B*), and two additional degenerate ATP-binding sites are found in the C-terminal region. Two coiled coil domains, which may form the "stalk" of the dynein molecule, are predicted downstream from the fourth P-loop.

A phylogenetic analysis of DNAH7 compared with representative examples of available full-length inner, outer, and cytoplasmic DHC sequences (Fig. 4*C*) revealed that DNAH7 is most probably an inner arm DHC, because it is more closely related to inner arm DHCs of *Chlamydomonas* than to an outer arm DHC of human or cytoplasmic DHCs from rat and mouse.

DNAH7 Is Induced during Ciliogenesis of HBE Cells and Is Incorporated into the Ciliary Axoneme—The expression of DNAH7 mRNA was examined during ciliogenesis of normal HBE cells in culture. Initially, these cultures consist of a monolayer of undifferentiated basal-like cells. With time, the cultures become pseudo-stratified and differentiate into a mucociliary epithelium very similar to an *in vivo* airway. DNAH7 expression was not detected at early time points (Fig. 5, *days 4* and *7*) but was clearly induced during the period when differentiation occurs and ciliated cells develop (*days 14–28*). This pattern of expression has been observed for other axonemal proteins (15, 25) and supports the conclusion that DNAH7 is an axonemal DHC.

Specific antibodies were generated to allow characterization of the DNAH7 protein. A fragment of DNAH7 containing amino acids 1531–1780 was expressed in bacteria. To reduce the possibility of cross-reactivity with other DHCs, the portion of DNAH7 expressed was chosen not to contain the highly conserved first P-loop region (see Fig. 4*B*). The recombinant fragment was purified, injected into chickens, and IgY antibodies were obtained. When used to probe Western blots of isolated cilia, the antibody reacted with a single band of the expected size (Fig. 6*A*), demonstrating that under these conditions the antibody did not cross-react strongly with other DHCs. The specificity of the purified antibodies was further examined by expressing a similar portion of another human axonemal DHC, DNAH9 (26), and testing the antibody against both peptide fragments in an immunoprecipitation experiment. As shown in

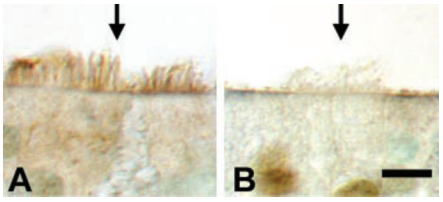


FIG. 7. **Immunohistochemistry of DNAH7 in HBE cells.** Paraffin sections of well differentiated HBE cells were stained with either the anti-DNAH7 antibody (*A*) or a 4-fold excess of preimmune IgY (*B*). The anti-DNAH7 antibody reacted strongly with the cilia, whereas the preimmune antibody showed no appreciable staining. *Scale bar* = 10 μm.

Fig. 6*B*, the anti-DNAH7 antibodies clearly immunoprecipitated the labeled DNAH7 peptide while showing no cross-reactivity with the DNAH9 peptide.

To examine the expression of DNAH7 during differentiation, total cell lysates were prepared from normal HBE cell cultures at different times and analyzed by Western blotting. As expected for an axonemal protein and in agreement with our Northern analysis, no signal was detected at early time points when the HBE cells were mostly undifferentiated (Fig. 6*C*, *days 2* and *5*). DNAH7 protein was first detectable at day 9, and the level of DNAH7 increased over the next 2–3 weeks during the time when ciliated cell differentiation occurs.

To directly test for the presence of DNAH7 in intact cilia, cultures of well differentiated HBE cells were examined by immunohistochemistry with the anti-DNAH7 antibodies. The cilia of these cultures stained intensely with the anti-DNAH7 IgY (Fig. 7) while the cultures stained with the control IgY at a 4-fold higher concentration showed only faint nonspecific staining. These results directly demonstrate that DNAH7 is present in the cilia of HBE cells, again supporting the classification of DNAH7 as an axonemal DHC.

DNAH7 Is Synthesized in PCD Cells but Is Not Assembled into the Ciliary Axoneme—Because cilia from the PCD cells showed a reduced level of the high molecular weight protein band containing DNAH7 (Fig. 3), we also examined the expression of DNAH7 in cultures of PCD cells. Northern blot analysis of well differentiated cultures of PCD demonstrated expression of DNAH7 RNA (Fig. 8*A*), and Western blot analysis of total cell lysates demonstrated that immunoreactive DNAH7 was present in PCD cells (Fig. 8*B*). Although the level of DNAH7 RNA and protein appears reduced in the PCD cells, this most probably reflects a difference in the extent of ciliated cell differentiation (note that the level of IC78 also appears reduced in the PCD cells). However, these results suggest that full-length DNAH7 RNA was produced and the DNAH7 protein was being translated correctly in the PCD cells. In contrast, Western blotting of ciliary axonemes isolated from the cultured PCD cells produced no signal, even though probing the sample with an anti-β-tubulin IV antibody, a marker for ciliary axonemes, revealed that ample material had been loaded on the gel (Fig. 8*C*). These results suggest that DNAH7 was being synthesized by the PCD cells but either was not incorporated or was incorporated at low levels into the cilia.

To test this hypothesis further, well differentiated cultures of PCD cells were examined by immunohistochemistry. As shown in Fig. 9*A*, the anti-DNAH7 antibody produced no appreciable staining of the PCD cilia, whereas the control antibody (anti-β-tubulin IV) stained the cilia strongly (Fig. 9*B*). Interestingly, the anti-DNAH7 antibody stained the cytoplasmic region of the PCD cells with equal or slightly greater intensity than the control cells.

The data above would be consistent with a mutation in the DNAH7 protein that prevented proper assembly of the cilia in

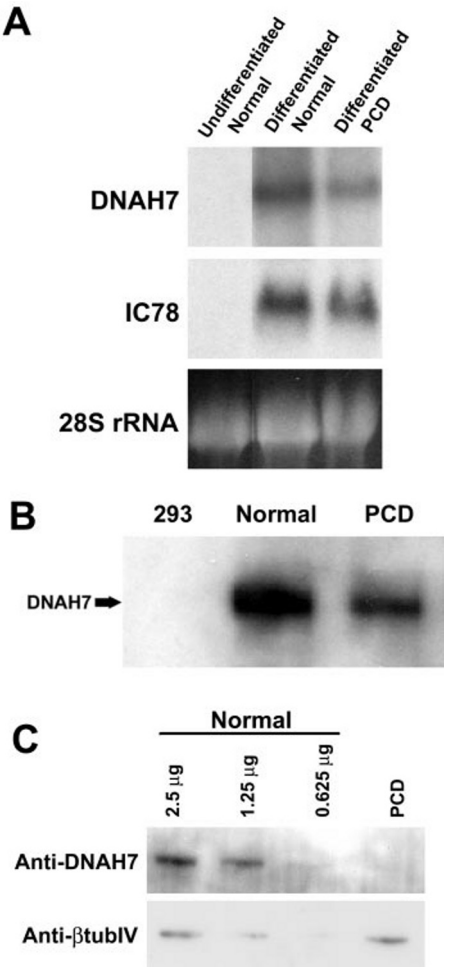


FIG. 8. **DNAH7 expression in PCD cells.** *A*, Northern blot analysis of differentiated PCD cells demonstrates clear expression of DNAH7 RNA. The PCD sample was included on the gel shown in Fig. 5. *B*, Western blot analysis of total cell lysates from well differentiated cultures of control and PCD cells. The 293 cell lysate was included as a negative non-ciliated control. DNAH7 protein is readily detected in PCD cells. *C*, Western blot analysis of axonemal proteins isolated from normal and PCD cells. Differing amounts of protein from the normal axonemes were loaded as indicated, and Western blots were performed with the chicken anti-DNAH7 or the mouse anti-β-tubulin IV (*Anti-βtubIV*). The amount of PCD cilia loaded was determined from a previous experiment to match the signal intensity obtained for β-tubulin IV at 2.5 μg of normal cilia. These results indicate at least a 4-fold decrease in DNAH7 in the cilia from the PCD patient.

the PCD patient. To determine whether mutations in DNAH7 were responsible for the PCD phenotype in this patient, RNA was isolated from well differentiated cultures of the PCD cells. RT-PCR was then used to amplify overlapping fragments of the entire DNAH7-coding region. These products were sequenced directly and compared with the previously obtained sequence from control cells to detect possible mutations and allelic variants. Any sequence variants were confirmed by sequencing fragments amplified from genomic DNA and, if necessary, by sequencing individual clones of PCR products. No deletions, insertions, or premature stop codons were identified in the sequence obtained from the PCD patient. Single nucleotide variants were identified at seven locations in the coding region of DNAH7 (Table II); however, only the C–T at position 1729 results in an amino acid substitution (Arg-Cys). To determine whether any of these variants is associated with the occurrence of PCD, additional unrelated control subjects were examined. For each of the variants listed, non-PCD controls were identified that were homozygous for the variant allele, indicating

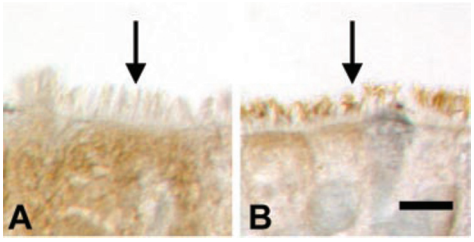


FIG. 9. **Immunohistochemistry of DNAH7 in PCD cells.** Paraffin sections of well differentiated PCD cells were stained with either the anti-DNAH7 antibody (*A*) or the mouse anti-β-tubulin IV (*B*). The anti-DNAH7 antibody stained the apical cytoplasm of the ciliated cells but did not stain the cilia, whereas the control anti-β-tubulin IV antibody reacted strongly with the cilia. *Scale bar* = 10 μm.

that these variants are most likely polymorphisms with no functional consequence.

DISCUSSION

Human respiratory tract cilia are complex axonemal structures consisting of hundreds of different proteins, precisely assembled and regulated to efficiently provide the force for mucus clearance. Although the basic structure of nine outer doublets and two central microtubules has been highly conserved in motile axonemes, a specialization of function requires that different axonemes have unique structures and be regulated by different mechanisms. Thus, although significant progress has been made in understanding the detailed molecular structure of *Chlamydomonas* flagella, it is obvious that to understand human cilia, a similar detailed analysis of human material will need to be performed.

Previous investigations have used RT-PCR with degenerate primers targeted to highly conserved regions of DHCs to amplify partial cDNAs of many human DHCs. For example, Chapelin *et al.* (27) identified five axonemal DHCs expressed in trachea, whereas Neesen *et al.* (20) identified seven axonemal DHCs from testis. Based on homology to dyneins from other species and phylogenetic analysis, these DHCs have been grouped as inner or outer arm DHCs with no supporting biochemical analysis. To date, a complete sequence has been assembled for only one human axonemal DHC, DNAH9 (26), and evidence provided by Reed *et al.* (15) support the classification of this DHC as an outer arm component. However, no similar studies have been reported for any human inner arm DHCs.

In this work, we have begun to characterize components of the inner dynein arm of human respiratory tract cilia. By comparing normal cilia with cilia from a PCD patient who was diagnosed by analysis of electron micrographs with an absence of inner dynein arms, we have identified and characterized the first human full-length inner arm DHC. This approach is similar to that used for studies of *Chlamydomonas* mutants that lack specific axonemal structures. As a first step in our analysis, the composition of DHCs in normal cilia was compared with that in the PCD cilia. Gel electrophoresis demonstrated that one of the four major DHC protein bands was clearly reduced in the PCD cilia, and mass spectrometry identified DNAH7 as one component of this band. Mass spectrometry also identified another DHC, DHC3, which is also likely to be an inner arm component. Several other peptides have not yet been identified, suggesting that additional inner arm DHCs may migrate in this region. The results suggest that by comparing cilia from control and PCD patients using one- and two-dimensional gel electrophoresis coupled with tandem mass spectrometry, additional protein components of human cilia can be identified and their position in the axoneme localized.

To further characterize DNAH7, the entire cDNA was cloned and sequenced from a HBE cell cDNA library. This finding represents the first full-length sequence of a human inner arm axonemal DHC and only the second full-length human axonemal DHC that has been assembled. DNAH7 shares all the previously described features of axonemal DHCs, including four conserved P-loop regions and two downstream coiled coil domains. A comparison to other available full-length DHC sequences shows that DNAH7 is most homologous to a *Drosophila* inner arm DHC, Dhc36C, and is more homologous to other inner arm DHCs from *Chlamydomonas* then to the outer arm DHC from human, DNAH9. As shown by Northern and Western analyses, DNAH7 was not expressed in undifferentiated HBE cells but was strongly induced in differentiated ciliated cells. Immunostaining of control HBE cells demonstrated that DNAH7 was localized to the cilia, whereas cilia from the PCD cells did not react with the anti-DNAH7 antibodies. Together with the electron micrographs of the PCD cilia demonstrating a lack of inner dynein arms, these data support the conclusion that DNAH7 is a member of the inner arm family of DHCs.

TABLE II
Sequence variations in DNAH7 identified in the PCD patient

Position	Normal		PCD	
1409	AGT	Ser	AGT	Ser
	AAT	Asn		
1729	CGT	Arg	CGT	Arg
			TGT	Cys
1991	TGC	Cys	TCC	Ser
	TCC	Ser		
3582	GCA	Ala	GCA	Ala
			GCC	Ala
5656	GTC	Val	ATC	Ile
	ATC	Ile		
8521	GTA	Val	ATA	Ile
	ATA	Ile		
12188	TTC		TCC	
	TCC			
12199–12200	T----T		T----T	
			TTTATT	
12230–12234	ATATT		ATATT	
	A----T		A----T	
12364–12369	GAAATA		GAAATA	
	G----A			

The inability to correctly assemble DNAH7 onto the ciliary axoneme is likely to be at least partially responsible for the defective ciliary function and resultant disease in this PCD patient. Therefore, we explored the possibility that mutations in the *DNAH7* gene were responsible for preventing the proper assembly of the inner dynein arm. Northern analysis demonstrated that DNAH7 mRNA transcripts were present at approximately normal levels, and RT-PCR confirmed that both alleles were expressed, indicating that there were no large deletions or truncations of the *DNAH7* gene. Western blotting and immunohistochemistry with a specific antiserum confirmed the lack of DNAH7 in mature PCD cilia, whereas the DNAH7 protein was present in total cell lysates of differentiated PCD cells. This observation suggested that a point mutation or small insertion/deletion in DNAH7 might be responsible for preventing its assembly into the axoneme. However, although the analysis of the entire coding sequence of DNAH7 in the PCD patient revealed several variations from the original sequence, further screening of control subjects identified non-PCD individuals who were homozygous for each of the variant sequences, indicating that these differences most probably represent polymorphisms.

Our finding that DNAH7 is synthesized but not incorporated into the cilia of the PCD cells indicates that the causative mutation must be located in another gene. Similarly, a putative outer arm DHC, DNAH9, was excluded as the gene responsible for PCD in a population of patients by Bartoloni *et al.* (26). In

a very recent report, mutations in DNAH5 have been identified in six PCD patients by sequencing the entire coding region from a large group of affected individuals (28). Therefore, it is possible that the sequencing of DNAH7 from a large number of PCD patients with inner arm defects will identify mutations in DNAH7 as well. Other inner arm DHC genes, including *DHC3* as well as genes coding for light and intermediate chain proteins, would be probable candidates in these patients. Mutations in an outer dynein arm intermediate chain, IC78, have recently been identified in a small number of PCD patients with outer arm defects (18, 29, 30). An analysis of the *IC78* gene in the patient studied in this report did not identify any mutations (30), supporting the conclusion that IC78 mutations result in a loss of the outer dynein arms. A comparison of isolated cilia from cultures of normal and PCD cells by two-dimensional gel electrophoresis may identify additional inner arm proteins that are absent in the PCD cilia. These proteins would also be likely candidates for the site of a causative mutation.

Alternatively, the genetic defect responsible for PCD in this patient may be in a protein involved in the transport or assembly of DNAH7 into the dynein arms. Mutations in proteins that play a role in the assembly of axonemes have been described in several animal models. For example, the targeted deletion of kinesin-II subunits in mouse models results in animals that lack nodal cilia (31, 32). The absence of functional nodal cilia in this and other models has been associated with a randomization of left-right asymmetry. A randomization of left-right asymmetry is also a common feature of PCD with ~50% of patients demonstrating complete *situs inversus* (11, 33). The recent results demonstrating the importance of nodal cilia in left-right determination provide a mechanism whereby a single mutation in an axonemal protein could cause *situs inversus*, infertility, and the chronic respiratory infections observed in PCD. In another animal model, mutations in the *Tg737* gene, which is the homologue of a *Chlamydomonas* gene involved in intraflagellar transport, results in situs abnormalities, shortened primary cilia, and polycystic kidney disease (34, 35). Additional studies will be required to identify the genetic defect responsible for the lack of DNAH7 assembly and the resultant disease in this PCD patient.

In summary, by comparing cilia from normal and PCD cells using gel electrophoresis and mass spectrometry, we have identified an axonemal inner arm DHC. The entire coding sequence of DNAH7 was obtained, and the expression of DNAH7 was examined during ciliogenesis. Specific antibodies were used to demonstrate that DNAH7 was synthesized and assembled into the cilia of normal airway epithelial cells. In contrast, DNAH7 was synthesized but was not assembled into the cilia of a PCD patient. No mutations were present in the coding region of DNAH7 from the patient, although seven sequence variants (polymorphisms) were identified. The identification of these polymorphisms will be useful for linkage analysis studies to investigate the role of DNAH7 in other PCD patients with a deficiency of inner dynein arms. These studies suggest that a mutation in another inner arm component or in a protein involved in the assembly or transport of DNAH7 may be responsible for a subset of PCD.

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