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Identification of Seven Rat Axonemal Dynein Heavy Chain Genes: Expression during Ciliated Cell Differentiation

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Axonemal dyneins are molecular motors that drive the beating of cilia and flagella. We report here the identification and partial cloning of seven unique axonemal dynein heavy chains from rat tracheal epithelial (RTE) cells. Combinations of axonemal-specific and degenerate primers to conserved regions around the catalytic site of dynein heavy chains were used to obtain cDNA fragments of rat dynein heavy chains. Southern analysis indicates that these are single copy genes, with one possible exception, and Northern analysis of RNA from RTE cells shows a transcript of ~15 kb for each gene. Expression of these genes was restricted to tissues containing axonemes (trachea, testis, and brain). A time course analysis during ciliated cell differentiation of RTE cells in culture demonstrated that the expression of axonemal dynein heavy chains correlated with the development of ciliated cells, while cytoplasmic dynein heavy chain expression remained constant. In addition, factors that regulate the development of ciliated cells in culture regulated the expression of axonemal dynein heavy chains in a parallel fashion. These are the first mammalian dynein heavy chain genes shown to be expressed specifically in axonemal tissues. Identification of the mechanisms that regulate the cell-specific expression of these axonemal dynein heavy chains will further our understanding of the process of ciliated cell differentiation.

INTRODUCTION

Dyneins represent a unique class of very large, microtubule-dependent, molecular motors. They exist as multi-subunit enzyme complexes consisting of a collection of heavy, intermediate, and light chains. The catalytic ATPase site is located on the dynein heavy chain (Johnson, 1985). Dyneins are divided into two functional classes: axonemal dyneins, which provide the force necessary for the beating of cilia and flagella (Witman, 1992), and cytoplasmic dyneins, which may contribute to the movement of chromosomes during cell division and the trafficking of intracellular organelles along microtubules (Schroer, 1994).

Axonemal dyneins are best characterized in organisms such as *Chlamydomonas* and *Paramecium*. Inner and outer dynein arms line the length of microtubules in the axoneme (Porter and Johnson, 1989; Mitchell, 1994). The number and complexity of the subunit chains comprising each type of dynein arm is dependent upon the species analyzed. *Chlamydomonas* outer arm dyneins contain three types of heavy chains, α, β, and γ, two intermediate chains, and at least 10 light chains (King and Witman, 1994), while *Paramecium* outer arms consist of three heavy chains, three intermediate chains, and eight light chains (Walczak and Nelson, 1993). The inner arm structures of *Chlamydomonas* are very complex. They are classified into three

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subtypes, which vary in heavy chain composition at different locations along the length of the axoneme (Piperno *et al.*, 1990; Witman, 1992). Little is known about the corresponding inner arm structures of *Paramecium*. The total number of inner and outer arm heavy chains remains undetermined in most species. At least 11 axonemal dynein heavy chains have been identified in sea urchin embryo (Gibbons *et al.*, 1994), while only six have been identified in *Drosophila* (Rasmusson *et al.*, 1994). Axonemal dyneins from mammalian species have yet to be characterized in any detail.

In contrast to axonemal dyneins, only two identical heavy chains are present in cytoplasmic dynein enzyme complexes (Schroer, 1994), and these heavy chains appear to be encoded by a single gene (Mikami *et al.*, 1993). Unique sequences surrounding the region encoding the catalytic site of dynein heavy chains have been reported to distinguish cytoplasmic and axonemal heavy chain genes (Asai and Brokaw, 1993; Asai *et al.*, 1994).

Deficiencies in axonemal dyneins are the most prevalent cause of primary ciliary dyskinesia (Sturgess, 1989), a genetic defect that results in abnormal axonemal structure and ciliary dysfunction. Individuals with primary ciliary dyskinesia suffer from repeated pulmonary infections, demonstrating the importance of ciliated cells and mucociliary clearance to the defense mechanisms of the airways. Ciliated cells are also damaged or lost following many types of environmental injury such as exposure to nitrogen dioxide (Carson *et al.*, 1993), formaldehyde (Hastie *et al.*, 1990), or smoke inhalation (Abdi *et al.*, 1990; Sisson *et al.*, 1994), and in disease states such as asthma (Laitinen *et al.*, 1985) and bronchitis (Lee and Forrest, 1991). Following injury to the upper airways, progenitor cells first proliferate and then differentiate to repair the damaged mucociliary epithelium. The regulatory mechanisms controlling differentiation into normal mucociliary epithelium or abnormal squamous or mucous cell hyperplastic epithelium are not well understood. In previous studies, we identified factors that regulate ciliated cell differentiation in a cell culture model of tracheal regeneration (Clark *et al.*, 1995; Ostrowski and Nettesheim, 1995; Davenport and Nettesheim, 1996). This model system provides a useful tool for studying ciliated cell differentiation, because the extent of differentiation can be influenced both positively and negatively. Although cilia contain over 200 proteins (Sturgess, 1989), molecular markers of the ciliated cell phenotype have yet to be identified. We were therefore interested in cloning axonemal dynein heavy chains from rat tracheal epithelial (RTE)[1] cells to use as molecular markers of ciliated cell differentiation.

[1] Abbreviations used: CT, cholera toxin; EGF, epidermal growth factor; RTE, rat tracheal epithelial.

Using degenerate primers to conserved regions of known axonemal dynein heavy chains, we have identified seven mammalian axonemal dynein heavy chains in RTE cells. Primers were designed to specifically amplify axonemal dynein heavy chains, and this was confirmed by their tissue-specific amplification of PCR products only in tissues containing cells with axonemal structures. In RTE cell cultures, the expression pattern of the individual axonemal dynein cDNA clones correlated with the presence of the ciliated cell phenotype. In addition, factors that increase ciliated cell differentiation also increased expression of these dynein heavy chain genes, suggesting that they will serve as excellent molecular markers for ciliogenesis. This report provides the first analysis of axonemal dynein heavy chain gene expression during ciliogenesis in mammalian cells.

MATERIALS AND METHODS

Materials

Oligonucleotides were ordered from Genosys (The Woodlands, TX) or synthesized on an ABI 392 DNA/RNA Synthesizer (Advanced Biotechnologies, Columbia, MD). All amplifications were performed in a DNA Thermal Cycler 480 or GeneAmp PCR System 9600 (Perkin-Elmer, Branchburg, NJ). [α^{32}P]dCTP and [^{35}S]dATPαS were obtained from Amersham (Arlington Heights, IL) and ^{35}Sequetide was obtained from DuPont NEN Research Products (Boston, MA). Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture media and reagents were obtained from Sigma Chemicals (St. Louis, MO) with the exception of epidermal growth factor (EGF), transferrin, and rat tail collagen, which were purchased from Collaborative Research (Bedford, MA). Bovine pituitaries were purchased from Pel Freeze (Rogers, AR).

Cell Culture

Our procedure for culturing primary RTE cells has been described in detail elsewhere (Kaartinen *et al.*, 1993; Clark *et al.*, 1995; Ostrowski *et al.*, 1995). Briefly, tracheas were isolated from 10- to 14-wk old Fisher 344 rats, filled with a solution of 1% pronase, and incubated overnight at 4°C. Dissociated cells were washed from the trachea and plated on type I collagen coated Transwell-col inserts in 6-well tissue culture plates (Costar, Cambridge, MA). The cells were submerged in growth factor–supplemented DMEM/F12 medium until they reached confluence on day 7–8, at which time an air/liquid interface was formed by feeding the cells from the basal compartment only. Under these conditions, cells grew as a poorly differentiated monolayer until confluent, at which time differentiation into a pseudostratified mucociliary epithelium occurred. Ciliated cells were first observed around day 12 and increased in number over the next several days. Cultures were also grown submerged in media for the entire culture period, or in media without EGF and cholera toxin (CT) for the last 7 days of culture. Additional cultures were grown in the presence or absence of retinoic acid for 14 days followed by culture for 3 days in medium with or without EGF and bovine pituitary extract. These cultures were harvested on day 17 for preparation of RNA. The various culture conditions decrease or increase ciliogenesis in primary RTE cell cultures (Kaartinen *et al.*, 1993; Clark *et al.*, 1995; Ostrowski and Nettesheim, 1995).

Isolation of RNA and DNA

Cultured cells were removed from the collagen membranes by incubation in a solution containing 1.5 g/l trypsin, 0.6 g/l EDTA, and 0.1% (w/v) pronase at 37°C. Pelleted cells were resuspended in guanidinium thiocyanate for RNA isolation as described by Chomczynski and Sacchi (1986). The same procedure was used to isolate RNA from rat heart and tracheal epithelium with the following exceptions. The heart tissue was frozen, and then minced and homogenized in guanidinium thiocyanate, while tracheas were simply flushed with the guanidinium thiocyanate solution as described elsewhere (Ostrowski *et al.*, 1993). Liver, testis, and brain RNA were gifts from Dr. K. Guzman and Dr. M. Eddy.

To isolate genomic DNA for Southern analysis, freshly isolated rat lung tissue was treated with proteinase K and SDS according to established procedures (Ausubel *et al.*, 1987). Briefly, frozen lung tissue was powdered under liquid nitrogen and incubated overnight at 50°C in a buffer containing 0.1 mg/ml proteinase K and 0.5% SDS. The sample was RNase A treated, nucleic acids were extracted with phenol/chloroform, and DNA was precipitated with sodium acetate and ethanol.

Partial Cloning and Sequencing of Rat Dynein Heavy Chain Genes

Two different day 17 cell cultures were utilized for partial cDNA cloning of dynein heavy chain genes. One set of cells was cultured under standard conditions, while the other set was cultured under standard conditions until day 14, at which time EGF and bovine pituitary extract were removed from the medium. Removal of EGF and bovine pituitary extract has been shown to enhance the development of ciliated cells (Clark *et al.*, 1995).

Primers were designed to specifically amplify either cytoplasmic or axonemal rat dynein heavy chains based upon unique sequences surrounding the catalytic site (Asai and Brokaw, 1993). The "axonemal-specific" upstream primer was 5'-ACIGGIAARACIGARACIAC-3' (primer 1), while the "cytoplasmic-specific" upstream primer was 5'-ACAGGAAAGACGGAGTCTGT-3' (primer 2) (Mikami *et al.*, 1993; Zhang *et al.*, 1993). A nonspecific upstream primer used to generate slightly larger products was 5'-ATCACICCIYTIACIGAYMG-3' (primer 4). The downstream primer for these amplifications was 5'-CCIGGRTTCATIGTDATRAA-3' (primer 5). Alternate downstream primers used in some amplifications were 5'-RTTRAAYTCRTCRAARCA-3' (primer 3) and 5'-AAGCCCAGGCGCCTGAAGAAG-3' (primer 6).

One microgram of RNA from the cultured rat epithelial cells was subjected to a polymerase chain reaction (PCR) with these primer sets using the reagents and protocol of an RNA PCR kit (Perkin-Elmer). The PCR cycling conditions were 2–5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C, with a final additional extension time of 7 min at 72°C. After amplification the samples were diluted 1:3, 1:10, and 1:30 for ligation into the vector pCRII according to the procedures of the TA Cloning Kit (Invitrogen, San Diego, CA). Plasmid DNA was isolated from colonies containing inserts and sequenced using a Sequenase sequencing kit (Amersham) and Sequagel-6 (National Diagnostics, Atlanta, GA). Sequences were aligned and an evolutionary analysis was performed with the Genetics Computer Group sequence analysis package (GCG, Version 8, September 1994, Madison, WI). ClustalV was also used in alignments (Higgins and Sharp, 1988, 1989).

RNA PCR Analysis of Primer Specificity

One microgram of RNA from rat heart, liver, testis, and trachea, and 0.3 μg of RNA from rat brain were reverse transcribed and PCR amplified with the RNA PCR kit (Perkin-Elmer). Following reverse transcription, the cDNA samples were divided in half for the addition of either the axonemal-specific primer (primer 1) or the cytoplasmic-specific primer (primer 2) during the PCR amplification. The downstream primer used in these amplifications was primer 3. The PCR cycling conditions were 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension time of 7 min at 72°C. An aliquot of each reaction was electrophoresed through a 2% NuSieve/SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME) and analyzed by ethidium bromide staining.

Southern Analysis

Rat lung genomic DNA was digested separately with *Bam*HI, *Eco*RI, and *Hin*dIII restriction enzymes. Twenty micrograms of digested DNA per lane was electrophoresed through a 0.8% agarose gel and visualized by ethidium bromide staining. The gel was then soaked for 15 min in 0.25 N HCl followed by two washes for 15 min each in a solution of 0.6 M NaCl/0.4 M NaOH. The DNA was transferred from the gel to a Nytran Plus membrane (Schleicher and Schuell, Keene, NH) by capillary blotting overnight with 0.6 M NaCl/0.4 M NaOH. The DNA was UV cross-linked to the membrane with a Stratalinker (Stratagene, La Jolla, CA), then prehybridized and hybridized in QuikHyb hybridization solution (Stratagene) according to the manufacturer's instructions. Nonspecifically bound radioactivity was removed by twice washing the membrane in a 2× SSC (300 mM NaCl, 30 mM sodium citrate) solution containing 0.1% SDS for 15 min at room temperature, followed by a 30-min wash in 0.1× SSC containing 0.1% SDS at 50°C. The blots were exposed to Hyperfilm-MP (Amersham,) at −80°C with intensifying screens for less than 2 days.

Northern Analysis

Northern analysis was performed using standard procedures, essentially as described (Ausubel *et al.*, 1987). Fifteen to twenty micrograms of RNA isolated from various tissue culture samples was electrophoresed through a 1% agarose-2.2 M formaldehyde gel. Ethidium bromide staining demonstrated approximately equal sample loading. The gel was immersed in 50 mM NaOH in 1× SSC for 15 min, then washed twice in 10× SSC for 15 min. RNA was transferred from the gel to a Nytran membrane (Schleicher and Schuell) by capillary blotting overnight with 10× SSC. RNA was UV cross-linked to the membrane as described for Southern analysis. Prehybridization, hybridization, washes, and autoradiography were also performed as described for Southern analysis. Membranes were probed twice. The removal of the original probe by a 30-min incubation at 65°C in 6× SSPE (1.1 M NaCl, 60 mM $NaPO_4$, 6 mM EDTA)/50% formamide was confirmed by autoradiography.

Generation of Probes for Southern and Northern Analyses

Radioactively labeled probes were generated by PCR amplification of cDNA inserts. [α^{32}P]dCTP was incorporated into the DNA during amplification of cDNA inserts from 100 pg plasmid DNA with dynein heavy chain primers. The amplification was carried out according to the protocol included in the PCR Radioactive Labeling Kit (Life Technologies, Gaithersburg, MD). The cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles as follows: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min; a 7-min extension time at 72°C completed the reaction. The probes were purified on NucTrap columns (Stratagene) before addition to hybridization solutions.

RESULTS

To identify and clone rat axonemal dynein heavy chain cDNAs, degenerate primers were designed based upon conserved sequence motifs surrounding the catalytic site (P-loop) of dynein heavy chains (Asai and Brokaw, 1993). Sequence differences in these mo-

tifs have been observed to distinguish axonemal and cytoplasmic dynein heavy chains in *Paramecium* (Asai *et al.*, 1994). We designed a degenerate upstream primer (primer 1, based upon the amino acid sequence TGKTETT) to specifically amplify axonemal dynein heavy chains from RTE cells; a second upstream primer (primer 2, based upon the amino acid sequence TGKTESV) was designed to amplify cytoplasmic dynein heavy chains.

The specificity of these primers for amplification of rat axonemal dynein heavy chains over their cytoplasmic counterparts was analyzed by RNA PCR (Figure 1). The amounts of dynein heavy chain transcripts amplified after 35 cycles with each primer set were compared in tissue samples that did or did not contain axonemes. With the cytoplasmic-specific primer, transcripts were detectable in each tissue analyzed. However, with the axonemal-specific primer, transcripts were only detected in tissues known to contain axonemes: the trachea, testis, and brain. No product was observed in the heart or liver samples with the axonemal-specific primer, demonstrating its specificity for dynein heavy chains expressed by cells containing axonemes.

Having established the selectivity of the axonemal-specific primer for axonemal dynein heavy chains, RNA was isolated for cDNA cloning from well-differentiated RTE cell cultures containing mature ciliated cells. A series of different primer combinations (Figure 2A) was used to amplify either cytoplasmic or axonemal dynein heavy chain cDNAs from these samples by RNA PCR. The PCR products were cloned and sequenced; the results of the sequence analysis are presented in Figure 2B as putative amino acid translations.

Seven unique, putative axonemal dynein heavy chain sequences were identified in RTE cells. These unique sequences have been designated here as axo a through axo g. No cytoplasmic dynein heavy chain clones were detected among the 55 cDNA clones sequenced from the axonemal-specific primer (primer 1) amplifications. A single cDNA clone of axo e was isolated using a degenerate P-loop primer (based upon the amino acid sequence PAGTGKTE) and primer 3. Additional clones of axo e were obtained with an axo e–specific primer (primer 6) used in conjunction with primer 4 to generate the sequence shown. Axo f cDNAs were only obtained with the primer 4/primer 5 combination, most likely because axonemal-specific primer 1 was found to contain a 2-bp mismatch at the 3′ end with the axo f sequence (putatively translated to the amino acid sequence TGKTETV instead of the primer-based sequence TGKTETT). Additional residues outside the catalytic P-loop that were conserved between all the axonemal sequences but were distinct from the cytoplasmic sequences were also identified (Figure 2B, boxed amino acids). An isolated cytoplasmic dynein heavy chain cDNA clone obtained with the cytoplasmic-specific primer 2 contained a 30-bp (10 amino acid) insert, in agreement with previously described sequences for rat brain cytoplasmic dynein heavy chain (Mikami *et al.*, 1993; Zhang *et al.*, 1993).

Southern blots of rat lung genomic DNA digested independently with *Bam*HI, *Eco*RI, and *Hin*dIII restriction endonucleases were probed with individual cDNA clones or a probe representing a "pool" of axonemal dynein heavy chain cDNAs (Figure 3). The "pooled" probe was generated by labeling the product obtained by amplification of RTE cell RNA with the axonemal-specific primer. At least eight bands were detected with the "pooled" probe in the *Hin*dIII digest. Some of these bands were not detected with any of the individual axonemal or cytoplasmic dynein heavy chain probes, suggesting additional axonemal dynein heavy chains were expressed in the RTE cells.

A single band was observed in each digest with the axo b through axo f cDNA clones, indicating that these axonemal dynein heavy chains are encoded by single copy genes. With the axo a probe a single band was observed in the *Hin*dIII digest, while three bands of equal intensity were detected in the *Bam*HI and *Eco*RI digests. The ~300-bp cDNA probe for axo a did contain an *Eco*RI restriction site, but not a *Bam*HI restriction site. Therefore, these additional bands may be due to the presence of introns within this region of the axo a gene, cross hybridization with other as yet undescribed heavy chain genes, or multiple copies of the

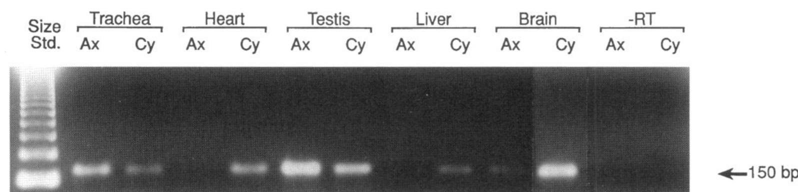

Figure 1. Axonemal-specific primers amplified dynein heavy chain transcripts only in tissues containing axonemal structures. One microgram of RNA from rat trachea, heart, testis, and liver and 0.3 μg of RNA from rat brain were subjected to 35 cycles of PCR amplification with either axonemal- or cytoplasmic-specific dynein heavy chain primers as described in MATERIALS AND METHODS. PCR products were visualized with ethidium bromide staining following electrophoresis through a 2% agarose gel. The amplified products were of the predicted size for these primer combinations (150 bp). Control amplifications with no reverse transcriptase (−RT) did not yield any products. PCR products amplified with the cytoplasmic-specific primer (Cy) were detectable in each tissue analyzed, while the axonemal-specific primer (Ax) only amplified transcripts from tissues containing axonemal structures: rat trachea, testis, and brain.

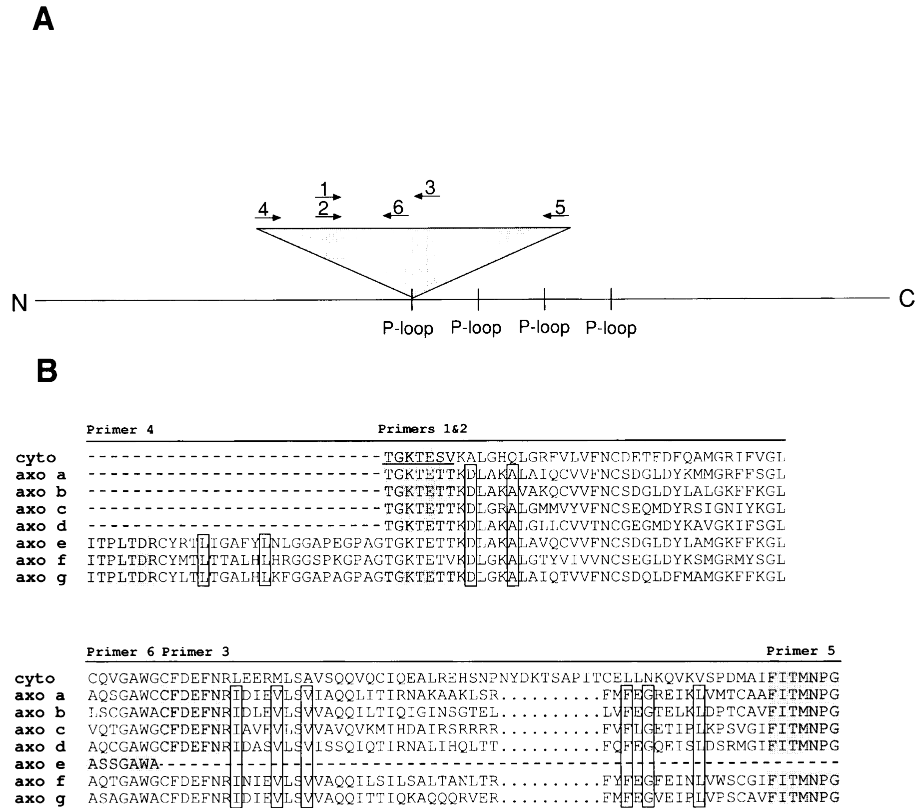

Figure 2. Seven axonemal dynein heavy chain cDNAs were identified in RTE cell cultures. Degenerate and specific primers were designed to conserved sequences adjacent to the catalytic site of dynein heavy chains (the first P-loop). (A) The location of the region amplified is indicated based upon predicted similarity with a completely sequenced sea urchin outer arm dynein heavy chain (Gibbons *et al.*, 1991; Ogawa, 1991). The orientations of six different primers used in various primer combinations to amplify dynein heavy chain sequences are also indicated. (B) RNA from well-differentiated RTE cell cultures were subjected to RNA PCR amplification of dynein heavy chain genes, and the cDNA products were cloned and sequenced. Compiled sequence data is presented here as the predicted amino acid translations of nucleotide sequences from a combined total of 64 cDNA clones. Primer combinations that amplified a particular cDNA sequence are represented as shaded amino acids. One cDNA sequence (cyto) was amplified with a cytoplasmic-specific upstream primer (primer 2, underlined), while seven unique cDNA sequences (axo a through axo g) were identified using axonemal-specific (primer 1) and degenerate upstream primers. Amino acids unique to the axonemal sequences are boxed.

axo a gene itself. The distinctive banding patterns observed between the various axonemal heavy chain cDNAs suggests cross hydridization was minimal. A Southern blot was also probed with cytoplasmic dynein heavy chain cDNA (Figure 3). The banding pattern observed with this cytoplasmic probe did not correspond with the pattern detectable with any of the putative axonemal cDNAs.

The size of transcripts detectable with individual axonemal cDNA clones was analyzed on Northern blots of RNA from RTE cell cultures grown in the presence or absence of retinoic acid (Figure 4). In the

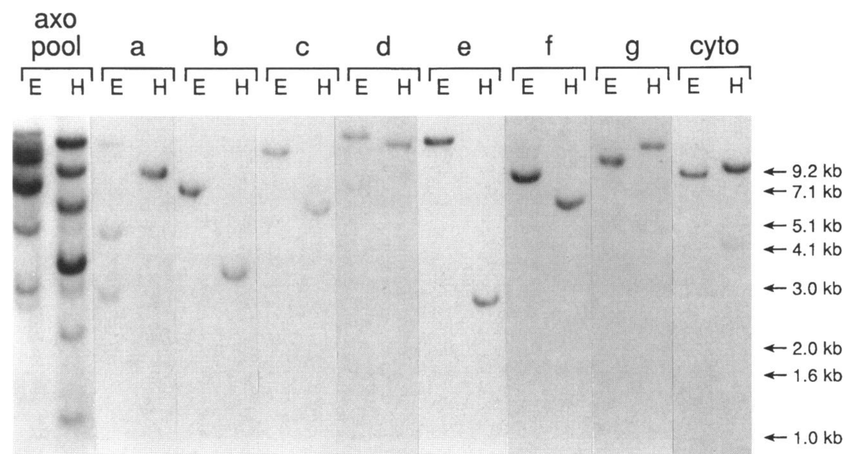

Figure 3. Southern analysis of individual axonemal cDNAs indicated that they are predominately single copy genes. Rat lung genomic DNA (20 μg) was digested with *Eco*RI (E) and *Hin*dIII (H) restriction endonucleases, separated by gel electrophoresis, and blotted onto a nytran membrane. Individual strips were probed with each of the cloned axonemal dynein heavy chain cDNAs, axo a through axo g (a–g), as well as with a cytoplasmic dynein heavy chain cDNA (cyto), and a population of dynein heavy chain cDNAs amplified with the axonemal-specific dynein heavy chain primer by RNA PCR (axo pool). Single bands were detected in each of the digests with the individual cDNAs, with the exception of three bands detected with the axo a probe in the *Eco*RI digest. The single banding pattern in these restriction digests suggests these dynein heavy chain cDNAs, with the possible exception of axo a, are encoded by single copy genes. Comparison of the banding pattern produced with the probe containing a "pool" of cDNAs to the individual bands observed with different axonemal cDNAs suggests other axonemal dynein heavy chain gene transcripts may also be expressed in cultured RTE cells.

presence of retinoic acid, RTE cells undergo mucociliary differentiation, while in its absence RTE cells undergo squamous differentiation with no evidence of ciliated cell formation (Kaartinen *et al.*, 1993). Transcripts of approximately 15 kb were detectable with each of the axonemal cDNAs (axo a through axo g) only in the cultures grown in the presence of retinoic acid. In contrast, cytoplasmic dynein heavy chain was expressed at similar levels in cultures grown in the presence or absence of retinoic acid (Figure 4). These results show that axo a through axo g are only expressed at detectable levels under conditions permissive for ciliogenesis.

To determine whether these genes would be good markers for ciliogenesis, we analyzed the expression of these genes under culture conditions that are known to inhibit or promote ciliogenesis. Submersion inhibits development of ciliated cells (Ostrowski and Nettesheim, 1995), while the removal of EGF and CT from the medium stimulates ciliogenesis four- to five-fold by day 12 of culture (Clark *et al.*, 1995). Cells were harvested at various time points from control cultures, which were grown in complete medium and switched to an air/liquid interface on day 8. Cells were also harvested at time points from cultures that had remained submerged in medium during the entire time course and from cultures that had both EGF and CT removed from the medium on day 8, concurrent with the formation of an air/liquid interface.

Northern blot analysis (Figure 5) of RNA isolated from these cells with the individual axonemal dynein heavy chain cDNAs (axo a through axo g) detected transcripts by day 13 to day 15 in cells grown under control conditions. Transcripts were generally not detectable with these probes in RNA from cultures that had remained submerged throughout the time course. The single exception was the presence of axo f transcripts at later time points under submerged conditions. This dynein heavy chain, whose expression clearly correlates with the development of the mucociliary phenotype (Figure 4; Figure 5, control), may have unique regulatory mechanisms that are not influenced by submersion. However, in cultures that had EGF and CT removed on day 8, a strong induction (2- to 16-fold) of axonemal dynein heavy chain transcripts was observed by day 13 when compared with control cultures for each of the seven axonemal dynein heavy chains. When probed with a cytoplasmic dynein heavy chain cDNA, approximately equivalent amounts of transcripts were detected in every sample (Figure 5). Axonemal dynein heavy chain gene expression is therefore regulated by conditions that regulate ciliated cell differentiation.

DISCUSSION

Axonemal dynein heavy chains have been well characterized in organisms such as *Chlamydomonas* and *Paramecium*, while no mammalian axonemal dynein heavy chain gene has been described in detail. We were interested in identifying rat axonemal dynein heavy chain genes as molecular markers of ciliogenesis. We designed primers to preferentially amplify axonemal as opposed to cytoplasmic dynein heavy chains from rat tracheal cells. The primers were based upon unique sequences surrounding the catalytic site (Asai and Brokaw, 1993), which distinguish axonemal from cytoplasmic dynein heavy chains in *Paramecium* (Asai *et al.*, 1994). The specificity of these primers was verified using RT-PCR to amplify dynein heavy chains from various rat tissues. Although the cytoplasmic-specific primer amplified a product in each tissue

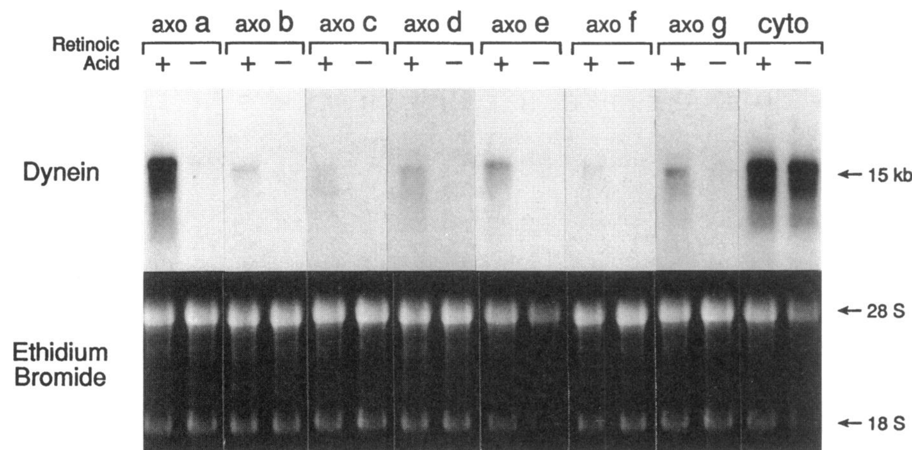

Figure 4. Northern analysis of individual axonemal cDNAs indicated their expression coincided with mucociliary differentiation. RNA was isolated from RTE cells grown in the presence (+) or absence (−) of retinoic acid. Fifteen micrograms of RNA was electrophoresed through an agarose-formaldehyde gel and blotted onto a nytran membrane. Membrane strips were probed with individual axonemal dynein heavy chain cDNAs (axo a through axo g) and a cytoplasmic dynein heavy chain cDNA (cyto). No messages were detected with any of the axonemal cDNAs in the absence of retinoic acid (squamous differentiation), while in the presence of retinoic acid (mucociliary differentiation) single transcripts of approximately 15 kb were detected with each axonemal cDNA. In contrast, cytoplasmic dynein heavy chain messages (~15 kb) were detected in both squamous and mucociliary RTE cell cultures. Ethidium bromide staining demonstrated equal loading of the gel. The selective expression of axo a through axo g messages only in cultures grown in the presence as opposed to the absence of retinoic acid supports their classification as axonemal dynein heavy chain genes.

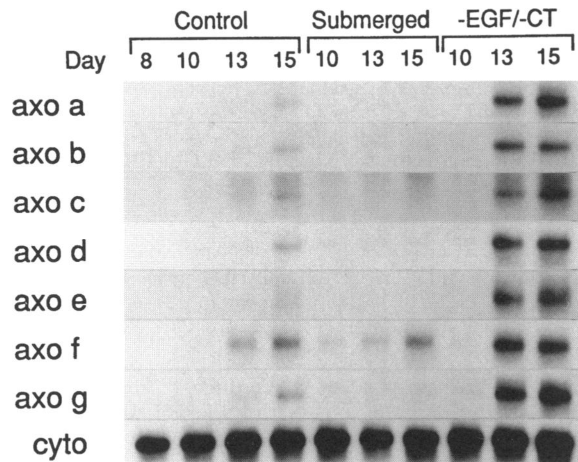

Figure 5. Culture conditions that inhibit or stimulate ciliogenesis similarly influence the expression of axonemal dynein heavy chain genes. RNA was harvested from RTE cells at different time points under control culture conditions and under conditions that inhibit (submerged) or stimulate (-EGF/-CT) ciliogenesis. Twenty micrograms of each sample was separated on an agarose-formaldehyde gel and transferred to a nytran membrane. Multiple Northern blots were generated in parallel; ethidium bromide staining demonstrated equal sample loading on all blots. The expression of axonemal dynein heavy chain transcripts (axo a through axo g) was strongly induced by day 13 in the cultures grown in the absence of EGF and CT when compared with control cultures. In contrast, expression of axonemal dynein heavy chain transcripts was inhibited in submerged cultures with the exception of axo f. A cytoplasmic dynein heavy chain cDNA probe (cyto) detected similar amounts of message at all time points. The size of each of these heavy chain transcripts was approximately 15 kb. In this model the expression of axonemal dynein heavy chains correlated with ciliogenesis.

tested, the axonemal-specific primer only amplified a product in tissues containing axonemal structures (Gabrion *et al.*, 1988; Yoshida *et al.*, 1992), namely trachea, testis, and brain. The small amount of product detected in the brain with the axonemal-specific primer may reflect the low percentage of ciliated ependymal cells in brain tissue. These results demonstrate that sequences that distinguish axonemal from cytoplasmic dyneins have been conserved throughout evolution, and may be important to the different functions of these two enzymes.

We amplified seven unique dynein heavy chain sequences from cultured RTE cells undergoing ciliated cell differentiation in vitro. These sequences were identified as axonemal dynein heavy chains by several criteria. First, as noted above, the tissue distribution of products detected with the axonemal specific primer was consistent with the expected distribution of axonemal dyneins. This demonstrates that this primer is specific for axonemal dynein heavy chains, and that products amplified from RTE cells with this primer are most likely axonemal. Second, the individual axonemal cDNA probes detected an approximately 15-kb transcript only at late time points on Northern blots from differentiating RTE cultures. Thus the expression of these genes during differentiation of RTE cells in culture occurs at the time ciliated cells are developing (Clark *et al.*, 1995), and the size of the transcripts detected is consistent with that of axonemal dynein heavy chains (Gibbons *et al.*, 1994; Rasmusson *et al.*, 1994). Third, conditions that inhibit (submersion and absence of retinoic acid) or promote (removal of EGF and CT from the media) ciliated cell differentiation (Kaartinen *et al.*, 1993; Clark *et al.*, 1995; Ostrowski and Nettesheim, 1995), resulted in parallel decreases or increases in the expression of these genes. These changes in axonemal dynein heavy chain expression were in sharp contrast to the relatively constant expression of cytoplasmic dynein heavy chain observed under all conditions. Finally, direct sequence comparisons show that the seven partial cDNAs reported here are most likely axonemal dynein heavy chains. Phylogenetic analysis (our unpublished results) indicated that all seven sequences reported here are more closely related to the axonemal dynein heavy chain sequences of other species than to the cytoplasmic dynein heavy chain gene of any species.

While this manuscript was in preparation, Tanaka *et al.* reported the partial cloning of 13 "dynein-like" sequences from rat brain using a similar procedure (Tanaka *et al.*, 1995). The seven axonemal dynein heavy chain sequences reported here, axo a through axo g, correspond to clones DLP6, DLP7, DLP11, DLP10, DLP12, DLP2, and DLP1, respectively, of Tanaka *et al.*[2] Their phylogenetic analysis also suggests these seven sequences represent axonemal as opposed to cytoplasmic dynein heavy chains. Our results, which show that the sequences are only expressed in tissues or cells containing axonemes, provide experimental data that these seven sequences represent axonemal dynein heavy chains. Although axo c shows sequence homology to known outer arm dynein heavy chains, conclusive assignment of the heavy chain isoforms to a particular inner or outer arm class of dyneins awaits further biochemical and microscopic characterization of axonemal dyneins in mammalian tissue.

Southern analysis of rat lung genomic DNA indicated that each of the cloned axonemal cDNAs appeared to be encoded by a single copy gene, with the possible exception of axo a. Southern analysis with a "pooled" probe in conjunction with probes for the

[2] Our data extends the sequence of axo e by 33 amino acids in the 5′ direction. Axo a, axo b, and axo f differed from the sequences reported by Tanaka *et al.* by two amino acids, three amino acids, and one amino acid, respectively. The new sequence data reported here for axo a, axo b, axo e, and axo f are available in the GenBank database under accession numbers U32181, U32180, U32182, and U32179, respectively.

individual cloned cDNAs indicated there may be more than seven axonemal dynein heavy chain sequences expressed in RTE cells. Tanaka *et al.* postulated that there may be as many as 11 axonemal dynein heavy chain genes expressed in rat brain (Tanaka *et al.*, 1995). A similar PCR cloning approach has identified at least 11 axonemal dynein heavy chain sequences in sea urchin (Gibbons *et al.*, 1994) and six in *Drosophila* (Rasmusson *et al.*, 1994). Additional studies are needed to determine the total number of rat axonemal dynein heavy chain genes and to determine if their expression is different between various tissues containing axonemes (i.e., brain versus trachea).

Our primary goal for cloning these axonemal dynein heavy chains was to define a molecular marker of ciliated cell differentiation. Ciliated cells of the upper airways are believed to develop through the terminal differentiation of a precursor cell (Randell, 1992). Although some studies suggest secretory cells are the major precursor for ciliated cells (Gordon and Lane, 1984; Breuer *et al.*, 1990), the pathways of airway cell differentiation and their regulation have not been completely defined. Markers of a differentiated cell phenotype are therefore important tools for studying the differentiation process. Although two antibodies have been described that bind to ciliated cells (Shimizu *et al.*, 1992; Aitken *et al.*, 1993), these antibodies primarily recognize a component of the membrane on the apical surface of these cells. We were interested in developing a molecular marker for ciliated cells, which does not rely on the binding of antibodies to uncharacterized epitopes. The results presented here demonstrate that axonemal dynein heavy chain gene expression correlated with ciliated cell differentiation. These genes will therefore be excellent molecular markers for studying the regulation of ciliated cell differentiation, and may be useful in identifying the precursor cell type(s) of ciliated cells in adult airways.

In summary, we have identified seven unique axonemal dynein heavy chain genes in cultured RTE cells. While we have identified these genes as putative axonemal dynein heavy chains on the basis of phylogenetic analysis, we have also provided strong experimental evidence supporting this hypothesis. The expression of these genes correlated with the appearance of the ciliated cell phenotype during differentiation of RTE cells in culture, and factors that influence ciliogenesis caused a corresponding change in axonemal dynein heavy chain gene expression. Molecular cloning of the promoters for these genes will allow characterization of transcription factors that regulate dynein heavy chain gene expression during the process of ciliogenesis. A better understanding of the regulation of ciliated cell differentiation may improve strategies for treatment of environmental and disease-related injury to the upper airways.

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