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Identification and molecular evolution of new dynein-like protein sequences in rat brain

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SUMMARY

RT-PCR cloning was performed to find unknown members of the dynein superfamily expressed in rat brain. Six kinds of degenerate primers designed for the dynein catalytic domain consensuses were used for extensive PCR amplifications. We have sequenced 550 plasmid clones which turned out to include 13 kinds of new dynein-like sequences (DLP1-8, 9A/B, 10-12) and cytoplasmic dynein heavy chain. In these clones, alternative splicing was detected for a 105 nt-domain containing the CFDEFNRI consensus just downstream of the most N-terminal P-loop (DLP9A and 9B). By using these obtained sequences, initial hybridization studies were performed. Genomic Southern blotting showed each sequence corresponds to a single copy of the gene, while northern blotting of adult brain presented more than one band for some subtypes. We further accomplished molecular evolutionary analysis to recognize their phylogenetic origins for the axonemal and non-axonemal (cytoplasmic) functions. Different methods (UPGMA, NJ and MP) presented well coincident phylogenetic trees from 44 partial amino acid sequences of dynein heavy chain from various eukaryotes. The trunk for all the cytoplasmic dynein heavy chain homologues diverged directly from the root of the phylogenetic tree, suggesting that the first dynein gene duplication defined two distinct functions as respective subfamilies. Of particular interest, we found a duplication event of the cytoplasmic dynein heavy chain gene giving rise to another subtype, DLP4, located between the divergence of yeast and that of *Dictyostelium*. Such evolutionary topology builds up an inceptive hypothesis that there are at least two non-axonemal dynein heavy chains in mammals.

Key words: axonal transport, motor protein, RT-PCR, dynein, microtubule-ATPase, flagellum, cilium, microtubule, phylogeny, molecular evolution

INTRODUCTION

The dynein gene family encodes a class of microtubule(MT)-dependent motor ATPase catalytic heavy chain proteins, which contain a characteristic motor domain. Two modalities in cell motility has been found for the dynein motor. One is the translocation of membrane organelles or chromosomes (reviewed by Hirokawa, 1993a,b; Vallee, 1993) and the other is the movement of cilia or flagella (Gibbons, 1981; Witman, 1992). From biochemical data, each subtype seems to have its specific function in each of these modalities (reviewed by Asai and Brokaw, 1993). The cytoplasmic dynein heavy chain (DHC) is the most unique DHC subtype so far identified in nonciliary cells, and functions as a MT minus-end-directed cytoplasmic translocater (Paschal et al., 1987; Lye et al., 1987; Neely and Boekelheide, 1988; Schroer et al., 1989; Schnapp and Reese, 1989; Gilbert and Sloboda, 1989; Corthésy-Theulaz et al., 1992). In contrast, eleven DHC subtypes have been identified in the axoneme of *Chlamydomonas* flagella, and eight and three of them serve as inner and outer dynein arms, respectively, to slide adjacent MT outer-doublets of the 9 + 2 structure (Luck and Piperno, 1989; Kagami and Kamiya, 1992). Cytoplasmic dynein is abundant in neurons, being thought to be a motor protein for fast retrograde axonal transport of membrane organelles (Hirokawa et al., 1990). In addition, it also localizes in the mitotic apparatus (Pratt, 1984; Pfarr et al., 1990; Steuer et al., 1990) and possibly plays a role in chromosomal segregation (Li et al., 1993; Eshel et al., 1993).

In this half decade, methods to elucidate the primary structure of DHCs have been accessible. Our group previously determined the complete primary structure of cytoplasmic DHC from rat brain (Zhang et al., 1993) independent of another group (Mikami et al., 1993). Cloning studies on *Dictyostelium* (Koonce et al., 1992); yeast (Eshel et al., 1993; Li et al., 1993); *Emericella nidulans* (Xiang et al., 1994); *Neurospora crassa* (Plamann et al., 1994); and *Caenorhabditis elegans* (R. J. Lye et al., unpublished) have confirmed that its N-terminal domain, about one-third of the molecule, has low homology with axonemal outer-arm DHC subtypes (Gibbons et al., 1991; Ogawa, 1991; Wilkerson et al., 1994; Mitchell and Brown, 1994), while the remaining two-thirds seems to be conserved. In the molecule's highly conserved central domain,

four well-conserved putative ATP-binding motif (P-loops: GXXXXGK(T,S,Q)) exist in all of the sequences ever cloned. The region around the most N-terminal P-loop of the four sequences is most conserved and contains some perfectly conserved motifs in addition to the P-loop, suggesting it has functional significance.

It is reasonable to suppose that these highly conserved motifs are characteristic of the dynein motor domain, so these sequences can be a key for further identification of the dynein gene family at molecular level. Recently, several genes (7 from *Drosophila* and 15 from sea urchin embryo) have been identified (Rasmusson et al., 1994; Gibbons et al., 1994), but their counterparts in mammals and their functional or evolutionary relationship with all other cloned DHCs are not known. That is, there still remains the question whether there are other DHC subtypes that serve as non-axonemal cytoplasmic translocation devices. Thus we have intended to elucidate the molecular nature of the mammalian dynein superfamily from this strategy in the present study. It is expected that unknown cytoplasmic translocater DHCs other than the cytoplasmic DHC could be resolved using a molecular approach, since there is good biochemical and morphological evidence to indicate that multiple kinds of motor molecules exist which provide cytoplasmic transports in the plus-end and minus-end direction of MTs (Hirokawa, 1982, 1993a,b). Actually, in the same way, our group has found multiple kinds of kinesin superfamily proteins (KIFs), which contain a kinesin-like motor domain as a cytoplasmic translocater acting in the brain (Aizawa et al., 1992; Kondo et al., 1994; Sekine et al., 1994; Nangaku et al., 1994; Noda et al., 1995). In this paper we identify mammalian dynein-like sequences from rat brain and characterize them with initial hybridization studies and extensive molecular phylogeny. The hybridization data suggest a complicated nature for their transcripts, which were first discovered in mammals, and their phylogeny gives more candidates for the cytoplasmic translocater DHC. These primary molecular descriptions of the mammalian dynein gene family will stimulate the molecular cell biology of DHCs in mammals, which will also contribute to clinical studies such as the genetic analysis of the WIC-Hyd rat, a model of immotile cilia syndrome.

MATERIALS AND METHODS

RT-PCR cloning of mammalian dynein-like proteins

Total RNA was prepared from adult rat brain by guanidium-CsTFA isopycnic purification (Okayama et al., 1987). Poly(A)$^+$ RNA was isolated with oligo(dT)-cellulose column chromatography (Pharmacia) and reverse-transcribed using the First-Strand cDNA Synthesis Kit (Pharmacia) with pd(N)$_6$ primer. This single strand cDNA mixture was used as the PCR templates.

As summarized in Fig. 1, we employed six degenerate synthetic oligonucleotides as primers as follows: d1, 5′-TAYGGNTTY-GARTAYYTNGG-3′ corresponding to the sense strand for YGFEYLG; d2, 5′-GTNCRRACNCCNYTNACNGA-3′ corresponding to the sense strand for V(Q/R)TPLTD; d3, 5′-CCNGCNG-GNACNGGNAARAC-3′ corresponding to the sense strand for PAGTGKT; d4, 5′-GGRTTCATNGTDATRAA-3′ corresponding to the antisense strand for FITMNP; d5, 5′-TTNARRTTRTCNG-GNARNTT-3′ corresponding to the antisense strand for NLPDNLK; d6, 5′-CKNARNCCRAARTCRTARTG-3′ corresponding to the antisense strand for HYDFGLR. These consensus sequences which lie around the most N-terminal putative ATPase catalytic site (P-loop) were resolved from a comparison of rat cytoplasmic DHC (Zhang et al., 1993; Mikami et al., 1993), *Dictyostelium* cytoplasmic DHC (Koonce et al., 1992), and sea urchin flagellar outer-arm β DHC (Gibbons et al., 1991; Ogawa, 1991).

PCR reactions were carried out on a 50 μl scale with a GeneAmp PCR System 9600 (Perkin-Elmer) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each of dNTP, 5 units of AmpliTaq polymerase (Perkin-Elmer), and 2 μM of each upstream and downstream primer. The following schedule was employed for 43 cycles: 94°C for 1 minute, 40°C for 2 minutes, and 72°C for 3 minutes with a 5 second extension per cycle. We performed nine series of PCR amplification in a round robin fashion using three upstream (d1-d3) and three downstream (d4-d6) primers so as not to miss subtypes which have only a few of the dynein motor consensus sequences. The annealing temperature used for each different set of primers was lower than the calculated t_m values.

An aliquot (20 μl) of each PCR product was analyzed by agarose gel electrophoresis. Proper length DNA fragments were eluted from the gel and treated with T4 polynucleotide kinase (Toyobo, Osaka) and Klenow fragment of DNA polymerase I (Toyobo), to be cloned into the *Eco*RV site of pBluescript II-SK(+) plasmid vector (Toyobo). *Escherichia coli* strain XL1-Blue (Stratagene) was transformed with the ligated samples and screened for inserts using Xgal and α-complementation of β-galactosidase (Sambrook et al., 1989). Plasmids from about 550 colorless colonies were purified to sequence with a Taq Dye Primer Cycle Sequencing Kit (Perkin-Elmer) and an autosequencer (model 373A, Perkin-Elmer). Resultant sequence data were assembled to several contigs using Macintosh AssemblyLign software (ver. 1.0.5, Eastman Kodak). The number of overlapping clones for each contig are summarized in Table 1. The homology between the contigs' deduced amino acid sequences and those of known DHCs was further searched with the accompanying software MacVector (ver. 4.1.4, Eastman Kodak).

Hybridization probes preparation and dot blot analysis

The probes for hybridization experiments were prepared from one of the obtained plasmid clones for each subtype (DLP1-12, CyDn). The portions employed for the probes are described in Fig. 4A. The DLP9 probe was selected from the region in common with DLP9A and DLP9B. The adequate restriction fragments were separated by agarose gel electrophoresis onto DEAE-cellulose membrane (DE81 paper, Whatman; Sambrook et al., 1989). They were subsequently oligolabelled with 3000 Ci/mmol [α^{32}P]dCTP (NEN) using a ^{T7}QuickPrime Kit (Pharmacia). Their crossreactivity was examined by a dot blot analysis. Heat-denatured DNA was spotted onto Hybond-N$^+$ membrane. A spot contained 10 ng of cloned plasmid DNA. The membrane was subsequently denatured and crosslinked by UV irradiation (Spectrolinker, Spectronics, Co.). Each strip spotted with all kinds of clones were prehybridized at 65°C and hybridized with each respective probe for 2 hours at 65°C in QuikHyb hybridization solution (Stratagene) supplemented with 100 μg/ml of salmon sperm single-stranded DNA (Sigma). They were subsequently washed twice for 30 minutes in 2× SSC (300 mM NaCl, 30 mM Na_3citrate) containing 0.1% (w/v) SDS at 65°C, then placed overnight on X-ray film (Fuji Photo film, Japan) at –80°C.

Genomic Southern blotting

Genomic DNA was prepared from the liver of an adult male Wistar rat according to standard methods (Enrietto et al., 1983). Briefly, excised liver was rapidly frozen in liquid nitrogen, crushed, treated overnight by proteinase K at 55°C, and extracted with phenol/chloroform/isoamyl alcohol. The product was digested completely with restriction enzyme *Bam*HI (New England Biolabs) or *Eco*RV (Boehringer Mannheim). 10 μg of aliquot per lane was separated by 0.8% agarose gel electrophoresis, denatured with alkaline solution,

and transferred by passive capillary diffusion to Duralon-UV membrane (Stratagene) according to the standard protocol described by Sambrook et al. (1989). The membrane was UV-crosslinked and hybridized with the adequate probes under the same conditions as that of dot blot analysis, then placed for 7 to 9 days on X-ray film at –80°C in the presence of enhancer screens (Kodak).

Northern blotting

Poly(A)$^+$ RNA was isolated from various tissues of adult rats as described above, and quantified by measuring the absorbance at 260 nm. The integrity of the RNA was checked by ethidium bromide staining after agarose gel electrophoresis. Northern blotting was basically performed as described by Sambrook et al. (1989). Briefly, the same amount of poly(A)$^+$ RNA was boiled and loaded on each lane of a 1% agarose gel containing 2.2 M formamide. After electrophoresis at 3 V/cm for 5 hours, the RNA was transferred by passive capillary diffusion to Hybond-N$^+$ membrane (Amersham) and immobilized by baking for 2 hours at 80°C. Hybridization and washing was performed under the same conditions as those of dot blot analysis.

Construction of phylogenetic trees

First, we aligned the amino acid sequences using the CLUSTAL W program (Thompson et al., 1994) compiled on a workstation (model 715/64, Hewlett Packard) with default parameters of the program. This program is the recent update version of the former CLUSTAL V program (Higgins et al., 1992). Together with deduced amino acid sequences of DLP1-12, we analyzed data released from the GenBank data base for cytoplasmic DHCs from rat (Zhang et al., 1993; Mikami et al., 1993), HeLa cells (Vaisberg et al., 1993), *Dictyostelium* (Koonce et al., 1992), yeast (Eshel et al., 1993; Li et al., 1993), *E. nidulans* (Xiang et al., 1994), *N. crassa* (Plamann et al., 1994); and *C. elegans* (R. J. Lye et al., unpublished); outer-arm DHCs and two unidentified PCR fragments from *Chlamydomonas* (Mitchell and Brown, 1994; Wilkerson et al., 1994); and PCR-detected DHC subtypes from *Drosophila* (Rasmusson et al., 1994) and sea urchin (Gibbons et al., 1994). PCR cloning studies from *Chlamydomonas*, *Drosophila*, and sea urchin were published during manuscript preparation. Table 2 summarizes applied sequence data.

Phylogenetic trees were constructed by the distance matrix method, neighbor-joining (NJ) method (Saitou and Nei, 1987) and unweighted pair group method using arithmetic average (UPGMA; Sokal and Michener, 1958), and maximum parsimony (MP) method (Eck and Dayhoff, 1966). We primarily utilized the NJ method due to its high efficiency (Saitou and Imanishi, 1989) and short run-time under CLUSTAL W. The distance matrix was calculated on a PAM250 matrix excluding positions with gaps. The confidence limits on trees were calculated by performing bootstrap resampling 1,000 times (Felsenstein, 1985; CLUSTAL W option).

We constructed NJ trees from six different truncated alignments (Fig. 2): (1) residues 47-88 for 47 sequences; (2) residues 47-146 for 44 sequences; (3) residues 21-146 for 33 sequences; (4) residues 47-220 for 28 sequences; (5) residues 21-220 for 24 sequences; and (6) residues 8-220 for 20 sequences. The number of applied sequences was limited by the varible length of the sequenced regions in each partial cloning study. For alignment (1), we also considered sea urchin DHC3C (Gibbons et al., 1994; GenBank accession number U03972) and *Paramecium tetraurelia* PCR-detected sequences (Asai et al., 1994; GenBank accession numbers L16962, L17050, L17132, L18801, L18802, L18803, and L18804). However, their precise relationships remain obscure solely based on these short data because of poor bootstrap values obtained for their branches. It is expected that a molecular cloning study to determine a longer region will give sufficient confidence limits which will lead to the correct assignment of their evolutionary relationships. After comparing all these trees, the one based on alignment (2) is considered most informative as judged by the number of sequences and confidence of the analysis.

The UPGMA tree was constructed from alignment (2) using the PROTDIST and NEIGHBOR programs contained in the Phylogeny Inference Package (PHYLIP, ver. 3.5p for Macintosh; Felsenstein, 1993).

MP was also performed on alignment (2) using the PROTPARS program in PHYLIP for 100 times in which the input order was randomized. Only rat and yeast sequences were analyzed and resulted in the two 'best' trees. Their branching patterns were different for DLP1, 3, 7, and 12, i.e. (DLP1, (DLP7, (DLP3, DLP12))), and (DLP3, (DLP1, (DLP7, DLP12))). We further analyzed these four sequences with MP, and subsequently found the former to be presented.

Associated figures were made with the RETREE and TREEDRAW programs (PHYLIP) in conjunction with their Macintosh HyperCard implementation TreeDrawDeck by D. G. Gilbert (1990). Since the NJ and MP methods generate unrooted trees, they were drawn with a root deduced by UPGMA analysis.

RESULTS

Molecular identification of new DLP sequences

To identify proteins containing a dynein-like motor domain, we initially performed extensive RT-PCR analysis using several degenerate primers according to the dynein motor consensus sequences (Fig. 1). As a result, we identified 13 new subtypes of dynein-like motor domain sequences (DLP1-8, 9A/B, 10-12) from adult rat brain poly(A)$^+$ RNA, along with the cytoplasmic DHC previously cloned (Zhang et al., 1993; Mikami et al., 1993). Their deduced amino acid sequences (Fig. 2) were confirmed by overlapping double clones (Table 1). Since several previously identified amino acid consensus motifs were also conserved in these sequences (Fig. 2), this region must be functionally essential.

In order to exclude the possibility that these obtained sequences are from contamination of genomic DNA, we performed mock PCR control amplifications. The same amount of starting RNA solution was amplified in a PCR reaction with just the same reaction conditions as the proper ones except the

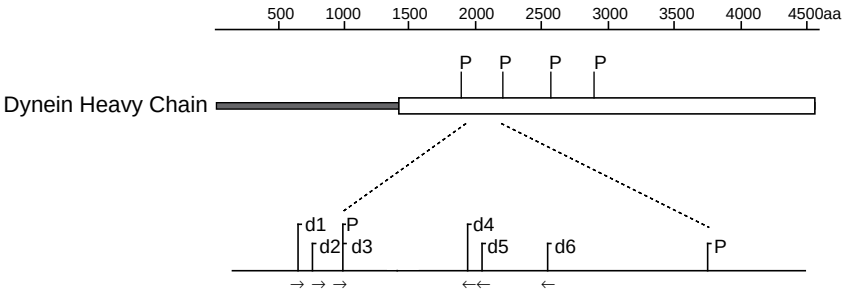

Fig. 1. Schematic presentation of the six primers synthesized for PCR amplification. Hatched and opened bars respectively indicate slightly- and highly-conserved domains among reported DHC sequences. 'P' indicates the position of a P-loop, putative ATP-binding consensus. Arrows indicate the locations and directions corresponding to the degenerate primers (d1-d6), whose sequences are described in Materials and Methods. The scale for rat cytoplasmic DHC was used (Zhang et al., 1993).

reverse-transcriptase treatment. The product was analyzed by agarose gel electrophoresis and proved that no visible amplified products were detected in the proper length (data not shown).

Of particular interest, we detected a portion of splice variation resulting from primary PCR cloning. The minor variant, DLP9B, had a 105 nucleotide deletion (corresponding to 35 amino acids just in frame) in comparison with DLP9A (Fig. 3). A cloning artifact is considered unlikely, because further PCR with primers specific for DLP9A/B, 5′-GCACTGGGCATCATGGTCTATGTG-3′ and 5′-AGCAATCGGGCTTCAATGAATCC-3′ (underlined in Fig. 3), also amplified these two subtypes (data not shown). In fact, their sequences in the overlapping regions were identical, and 'AG' motifs were present in both junctional regions (Fig. 3). Taken together, it appears that an exon was alternatively spliced out in accordance with the GT-AG rule (Padgett et al., 1986). The spliced domain is adjacent to the putative ATP-binding motif GPAGTGKT (P-loop) and contains the GXWX-CFDEFNR consensus. Further RT-PCR and RNase protection assays demonstrated that a tissue-specific splicing variation occurs in this region (data not shown). As shown later, DLP9A is the rat counterpart of sea urchin outer-arm β DHC so that such splicing may modulate the mechanochemical property of the outer dynein arm. This is the first report of the alternative splicing of the dynein motor domain ever known.

Copy number of DLP genes and their transcript length in the brain

Next we examined the copy number of each rat DLP sequences and their transcript length in the brain by blot hybridization experiments. Specific radioprobes were selected for each DLP subtype from the obtained sequences. To examine their cross-reactivity between each other, we performed a dot blot analysis to hybridize all sorts of obtained plasmids (Fig. 4A) as a control study. Major crosshybridization was not observed under these conditions while some probes weakly hybridized with the DLP1 plasmid.

Using these probes, we performed genomic Southern blotting to examine the copy number of rat DLP genes. Genomic DNA obtained from a male rat was digested separately with *Eco*RV and *Bam*HI. The probes do not contain any restriction sites for these two enzymes. Fig. 4B shows the results with *Eco*RV, which gives only one predominant band at a different height for each probe. Coincident results were obtained with *Bam*HI (data not shown). It suggests that only one rat gene strictly corresponds to each of the obtained sequences, similar to the case in *Drosophila* (Rasmusson, 1994). Thus there does not seem to have been any very recent gene duplication event in rat, which would have generated an unidentified gene very close to DLP1-12 and cytoplasmic dynein.

We further performed northern blotting analysis using poly(A)$^+$ RNA obtained from adult rat brain to examine their transcript length. The results, shown in Fig. 4C, show that almost all of the DLPs have a very long transcript of about 15 kb, similar to that of cytoplasmic dynein. No apparent band was obtained for DLP8 from brain preparation even after a long exposure, but the DLP8 probe strongly hybridized with a single band of around 15 kb when mRNA from testis was used (data not shown). Since a control mock PCR experiment without RT gave no bands of the adequate length as described above, DLP8 is unlikely to be amplified from a contaminating pseudogene, but from a very small amount of mRNA which is under the detection level of northern blotting, by a detailed RT-PCR cloning.

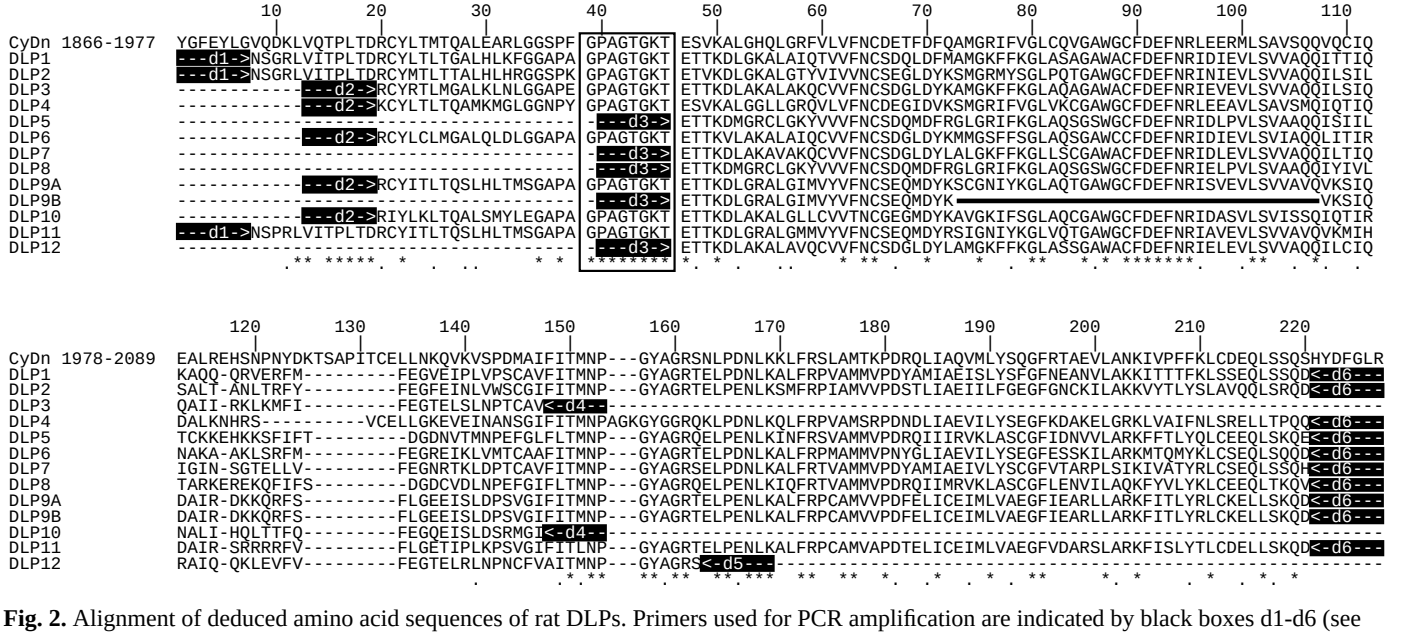

Fig. 2. Alignment of deduced amino acid sequences of rat DLPs. Primers used for PCR amplification are indicated by black boxes d1-d6 (see Fig. 1). For convenience, the numbers above the alignment begin with the first aligned position. Asterisks and periods (bottom) show that the residue's degree of conservation among the sequences is 100% and more than 50%, respectively. Note the perfectly conserved residues containing the most N-terminal P-loop motif (boxed). In DLP9B, the conserved region neighboring this P-loop was deleted compared to DLP9A (thick line, see Fig. 3 also). The sequence data of DLPs will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence data bases under accession number D26492-26504.

Table 1. Assembled DLP clones for each contig

Contig	Number of assembled clones
DLP1	37
DLP2	39
DLP3	6
DLP4	38
DLP5	11
DLP6	6
DLP7	6
DLP8	6
DLP9A	25
DLP9B	3
DLP10	7
DLP11	11
DLP12	2

Interestingly, some probes hybridized with more than one band in this analysis. DLP5 and DLP9 hybridized with apparently two separate populations of bands, whose difference was up to 5 kb. DLP4 also showed two minor bands of around 10 kb, in addition to the main band of about 15 kb. Since crosshybridization either between these subtypes or with other unknown gene transcripts is quite unlikely from the two hybridization experiments above, at least some must be the alternative spliced transcripts. Such a diversity of transcript length has never been observed for their counterpart molecules in *Drosophila* (Rasmusson, 1994) or sea urchin (Gibbons, 1994). Mammals seem to have developed a special splicing

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                E  T  T  K  D  L  G  R  A  L  G  I  M  V  Y  V  F
DLP9A   GAGACCACCAAGGACCTGGGCCGAGCACTGGGCATCATGGTCTATGTGTT
DLP9B   GAGACCACCAAGGACCTGGGCCGAGCACTGGGCATCATGGTCTATGTGTT

                  N  C  S  E  Q  M  D  Y  K  S  C  G  N  I  Y  K
DLP9A   TAACTGTTCCGAGCAGATGGACTACAAGTCCTGTGGCAACATCTACAAAG
DLP9B   TAACTGTTCCGAGCAGATGGACTACAAG----------------------

                G  L  A  Q  T  G  A  W  G  C  F  D  E  F  N  R  I
DLP9A   GCCTGGCTCAGACTGGTGCCTGGGGCTGTTTTGATGAATTTAACCGAATC
DLP9B   ---------------------------------------------------

                S  V  E  V  L  S  V  V  A  V  Q  V  K  S  I  Q  D
DLP9A   TCTGTGGAGGTCTTGTCAGTGGTGGCTGTGCAGGTGAAAAGCATCCAGGA
DLP9B   ----------------------------------GTGAAAAGCATCCAGGA

                  A  I  R  D  K  K  Q  R  F  S  F  L  G  E  E  I
DLP9A   CGCAATCAGAGACAAGAAGCAGAGGTTCAGCTTCCTTGGGGAGGAGATTA
DLP9B   CGCAATCAGAGACAAGAAGCAGAGGTTCAGCTTCCTTGGGGAGGAGATTA

                S  L  D  P  S  V  G  I  F  I  T  M  N  P  G  Y  A
DLP9A   GCCTTGACCCTTCTGTGGGCATCTTCATCACTATGAACCCGGGCTATGCT
DLP9B   GCCTTGACCCTTCTGTGGGCATCTTCATCACTATGAACCCGGGCTATGCT

                  G  R  T  E  L  P  E  N  L  K  A  L  F  R  P  C  A
DLP9A   GGCCGCACAGAACTGCCAGAAAACCTCAAGGCCCTCTTCAGGCCCTGTGC
DLP9B   GGCCGCACAGAACTGCCAGAAAACCTCAAGGCCCTCTTCAGGCCCTGTGC

                  M  V  V  P  D  F  E  L  I  C  E  I  M  L  V  A
DLP9A   AATGGTAGTTCCAGACTTTGAGCTGATCTGTGAGATTATGCTGGTAGCAG
DLP9B   AATGGTAGTTCCAGACTTTGAGCTGATCTGTGAGATTATGCTGGTAGCAG

                E  G  F  I  E  A  R  L  L  A  R  K  F  I  T  L  Y
DLP9A   AAGGATTCATTGAAGCCCGATTGCTGGCCAGGAAGTTCATTACCCTTTAC
DLP9B   AAGGATTCATTGAAGCCCGATTGCTGGCCAGGAAGTTCATTACCCTTTAC

                  R  L  C  K  E  L  L  S  K  Q
DLP9A   CGGCTGTGTAAAGAACTTCTCTCCAAACAG
DLP9B   CGGCTGTGTAAAGAACTTCTCTCCAAACAG
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Fig. 3. Alternative splicing of DLP9. cDNA sequences of DLP9A and DLP9B are confirmed by secondary PCR cloning using specific primers designed for the underlined regions. Note the exon-intron consensus motif, AG, present at the two junctional regions.

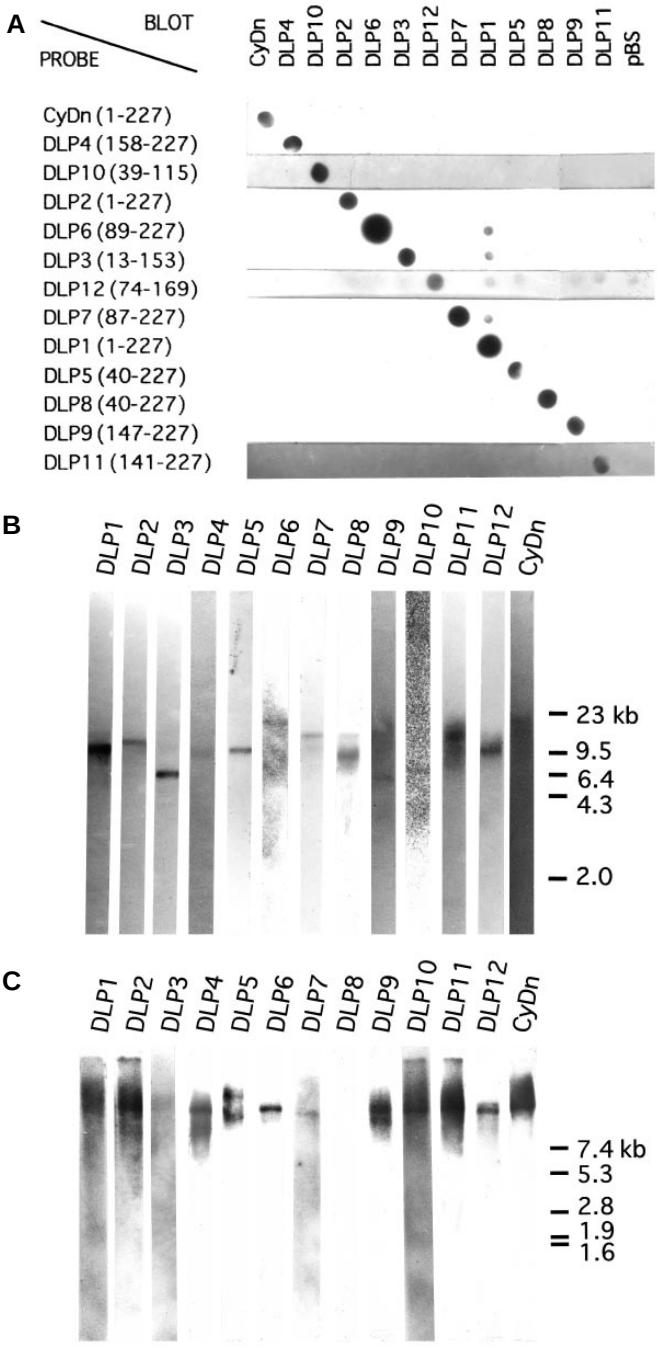

Fig. 4. Blot hybridization analyses of DLPs. (A) Dot blot analysis. The portions of the probes used are indicated by the residue number defined in Fig. 2. On each strip (horizontal lanes), 14 spots were blotted and immobilized. Each spot contains 10 ng of pBluescript clone dsDNA corresponding to each DLP subtype (indicated in the upper legend) or with no insert for a negative control (pBS). The strips were hybridized separately with each corresponding probe. Note that no major crosshybridization is observed. (B) Genomic Southern blotting. 10 μg of male rat genomic DNA per lane was hybridized with corresponding DLP probes. All of them present one predominant band. (C) Northern blotting. 10 μg of adult rat brain poly(A)$^+$ RNA per lane was hybridized. Almost all of them show a transcript of around 15 kb, and DLP4, 5 and 9 apparently hybridized with more than one band. The information presented here is not quantitative but qualitative, since the exposure time varies from lane to lane.

mechanism for DLP mRNAs which must provide some unknown regulation for dynein function in the brain.

Molecular evolution of the dynein ATPase superfamily

In order to reveal the evolutionary identity and genetic origin of DLPs, we carried out a phylogenetic analysis of the dynein ATPase superfamily, similar to that for the myosin ATPase superfamily (Padgett et al., 1986; Espreafico et al., 1992; Goodson and Spudich, 1993; Cheney et al., 1993) and kinesin ATPase superfamily (Goldstein, 1993; Goodson et al., 1994; Sekine et al., 1994). Our deduced amino acid sequences were analyzed together with various available DHC sequences (Table 2; see Materials and Methods).

We confirmed the accuracy of the resultant phylogenetic tree by comparing the trees deduced via the NJ (Fig. 5) and MP (Fig. 6) methods, which showed nearly identical branching patterns. The rooted UPGMA tree also had a similar pattern (data not shown). In addition, we proved that several NJ trees constructed from differently truncated regions gave almost identical patterns (see Materials and Methods). Bootstrap resampling (Felsenstein, 1985) was performed 1,000 times to test the confidence limits of each branch, and bootstrap probabilities over 90% were obtained for some important branches (Fig. 5). We found that a longer consensus length gave higher bootstrap values.

At least 13 DHC subtype clusters were identified, and in almost all of these clusters, rat, sea urchin, and *Drosophila* orthologues diverged in a manner representing the phylogenetic order (shaded regions in Fig. 5). Orthologues from other eukaryotes can also be identified for some subtypes. In most cases, the branch between a cluster and the node differentiating another subtype was long and showed relatively high bootstrap values, which is important, since this indicates that most of the detected 13 DHC subtypes are fairly well differentiated and that multiple gene duplications of DHC may have occurred earlier in the phylogeny of higher eukaryotes. Since some subtypes were cloned in order to experimentally

Table 2. Dynein-like protein sequences applied to molecular evolutionary analysis

Species	Accession number	Nomenclature	Cloning	Reference
Rattus norvegicus	D13896/L08505	Rat CyDn	Complete	Zhang et al., 1993; Mikami et al., 1993
	D26492	Rat DLP1	Partial	Present paper
	D26493	Rat DLP2	Partial	Present paper
	D26494	Rat DLP3	Partial	Present paper
	D26495	Rat DLP4	Partial	Present paper
	D26496	Rat DLP5	Partial	Present paper
	D26497	Rat DLP6	Partial	Present paper
	D26498	Rat DLP7	Partial	Present paper
	D26499	Rat DLP8	Partial	Present paper
	D26500	Rat DLP9A	Partial	Present paper
	D26502	Rat DLP10	Partial	Present paper
	D26503	Rat DLP11	Partial	Present paper
	D26504	Rat DLP12	Partial	Present paper
Tripneustes gratilla	Z21941	Sea urchin DHC1A	Partial	Gibbons et al., 1994
	U03969	Sea urchin DHC1B	Partial	Gibbons et al., 1994
	U03970	Sea urchin DHC3A	Partial	Gibbons et al., 1994
	U03971	Sea urchin DHC3B	Partial	Gibbons et al., 1994
	U03973	Sea urchin DHC4	Partial	Gibbons et al., 1994
	U03977	Sea urchin DHC5A	Partial	Gibbons et al., 1994
	U03974	Sea urchin DHC5B	Partial	Gibbons et al., 1994
	U03976	Sea urchin DHC5C	Partial	Gibbons et al., 1994
	U03975	Sea urchin DHC6	Partial	Gibbons et al., 1994
	U03978	Sea urchin DHC7A	Partial	Gibbons et al., 1994
	U03979	Sea urchin DHC7B	Partial	Gibbons et al., 1994
	U03980	Sea urchin DHC7C	Partial	Gibbons et al., 1994
	X59603	Sea urchin β DHC	Complete	Gibbons et al., 1991
Anthocidaris crassispina	D01021	Sea urchin β DHC	Complete	Ogawa, 1991
Drosophila melanogaster	L23195	*D. melanogaster* CyDn	Complete	Li et al., 1993
	L23196	*D. melanogaster* DHC62B	Partial	Rasmusson et al., 1994
	L23197	*D. melanogaster* DHC16F	Partial	Rasmusson et al., 1994
	L23198	*D. melanogaster* DHC93AB	Partial	Rasmusson et al., 1994
	L23199	*D. melanogaster* DHCYh3	Partial	Rasmusson et al., 1994
	L23200	*D. melanogaster* DHC98D	Partial	Rasmusson et al., 1994
	L23201	*D. melanogaster* DHC36C	Partial	Rasmusson et al., 1994
Homo sapiens	L23958	HeLa cell HDHC1	Partial	Vaisberg et al., 1993
Dictyostelium discoideum	Z15124	*D. discoideum* CyDn	Complete	Koonce et al., 1992
Saccharomyces cerevisiae	Z21877/L15626	Yeast CyDn	Complete	Eshel et al., 1993; Li et al., 1993
Caenorhabditis elegans	L33260	*C. elegans* CyDn	Complete	R. J. Lye et al., unpublished
Emericella nidulans	U03904	*E. nidulans* CyDn	Complete	Xiang et al., 1994
Neurospora crassa	L31504	*N. crassa* CyDn	Complete	Plamann et al., 1994
Chlamydomonas reinhardtii	L26049	*C. reinhardtii* α DHC	Partial	Mitchell and Brown, 1994
	U02963	*C. reinhardtii* β DHC	Complete	Mitchell and Brown, 1994
	–	*C. reinhardtii* γ DHC	Complete	Wilkerson et al., 1994
	–	*C. reinhardtii* PCR1	Partial	Wilkerson et al., 1994
	–	*C. reinhardtii* PCR2	Partial	Wilkerson et al., 1994

determine their encoding proteins, this enables us to predict the identity of their counterpart molecules recognized by RT-PCR analyses.

As shown in Fig. 5, the axonemal outer-arm DHCs from sea urchin and *Chlamydomonas* are clustered. The number of DHCs comprising the outer arm varies per species, e.g. two DHCs (α, β) were detected in sea urchin sperm and three (α, β, γ) in *Chlamydomonas* flagellum (reviewed by Asai and Brokaw, 1993). DLP5, 8, 9, and 11 are thought to be their rat counterpart molecules. Since ependymal cells and choroid plexus have many cilia (Agduhr, 1932), the detection of axonemal DHCs from rat brain preparation should be expected. Our preliminary RNase protection assay revealed a differential expression of DLP5 and 8 in rat tissue; thus, these similar DHCs may serve in different types of axonemes. On the other hand, eight inner-arm DHCs were biochemically identified in *Chlamydomonas* flagella (Kagami and Kamiya, 1992). However, their counterparts from the unidentified cluster DLP 1, 2, 3, 6, 7, 10, and 12 cannot be identified since no inner-arm DHC has been cloned yet. Studies on sequence-mutant rela-

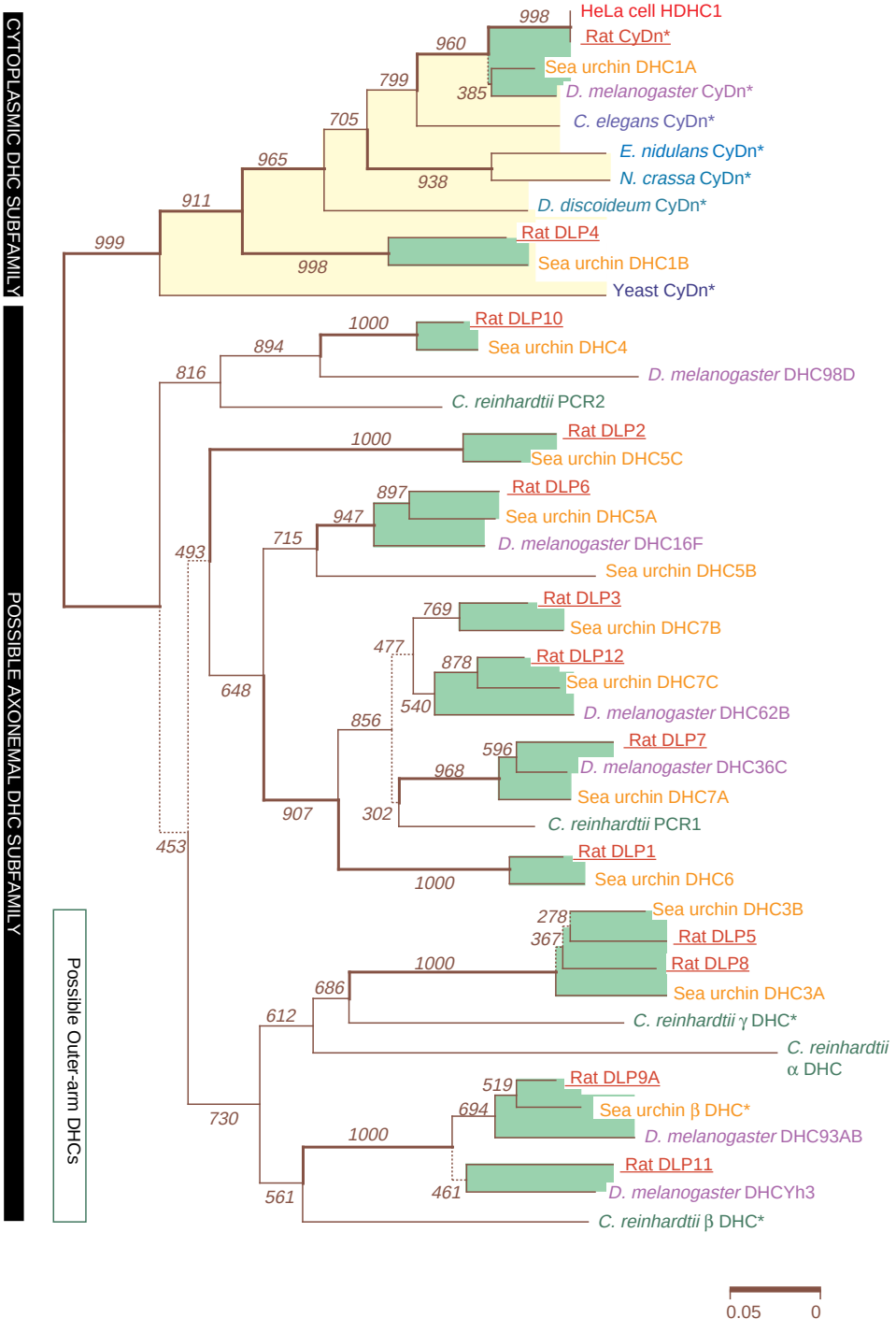

Fig. 5. Phylogenetic tree of the dynein ATPase superfamily as constructed by the neighbor-joining (NJ) method in which 44 DHC sequences are classified (see Materials and Methods). Cytoplasmic DHC (CyDn) and axonemal outer-arm DHC clusters were resolved using completely cloned sequences (asterisks). Abbreviations, references, and accession numbers are summarized in Table 2. The scale indicates the number of amino acid differences per residue. The thickness of a branch represents the bootstrap value following 1,000 trials, which is also shown in italics. Thick and dotted lines represent branches with >90% and <50% bootstrap probabilities, respectively. The tree was artificially rooted based on UPGMA analysis. Each subtype cluster from various species is indicated by shading.

tionship in *Chlamydomonas* flagellar mutants will give a good key to identify the functions of these sequences. Recently, human HDHC3 gene which seems to be the homologue of DLP12 is found to be expressed in non-ciliated human cell lines (Vaisberg, 1994). Some member of this unidentified cluster could also be served for non-axonemal function.

Of particular interest, the cluster of cytoplasmic DHCs showed a direct divergence from the root deduced by UPGMA, which subsequently suggests that the first gene duplication resulted in two distinct functions of dynein. From this trunk, cytoplasmic DHC from yeast *Saccharomyces cerevisiae* first diverged, being sequentially followed by those from the soil amoeba *Dictyostelium discoideum*, the ascomycetes *Emericella nidulans* and *Neurospora crassa*, the nematode *Caenorhabditis elegans*, the anthropod *Drosophila melanogaster*, the sea urchin *Tripneustes gratilla*, rat brain, and human HeLa cells. This order coincides well with those from past sequence comparisons of various proteins (Loomis and Smith, 1990; Hasegawa et al., 1993) and, therefore, the root defining the two subfamilies (translocater and axonemal DHC subfamily, respectively, in Fig. 5) should have occurred prior to the divergence between mycota and animalia. If so, cytoplasmic DHC could not have been 'borrowed' from the axonemal structure during evolution, but instead must have diverged before the wide expansion of axonemal DHCs. This would partially support the endosymbiont hypothesis of cilia (Margulis and Sagan, 1986).

Although the evolution of the cytoplasmic DHC subfamily looks monotonous in contrast to that of the axonemal DHC subfamily, one gene duplication event was found to have occurred during this evolution, and it gave rise to another subtype cluster containing rat DLP4 and sea urchin DHC1B. The detection of rat DLP4 made this subtype clustered, confirming its evolutionary identity. This duplication event seems to have occurred between the divergence of yeast and that of *Dictyostelium*. Yeast dynein is definitely cytoplasmic based on the following: (1) it does not have any cilia or flagella; (2) yeast DHC is not only highly homologous to rat cytoplasmic DHC in the dynein motor domain, but also in the subtype-specific N-terminal region comprising one-third of the protein (Li et al., 1993; Eshel et al., 1993); and (3) mutation of the dynein gene causes abnormal segregation of the mitotic spindle (Li et al., 1993; Eshel et al., 1993). According to the phylogenetic order, the ideal ancestory gene of both DLP4 and rat cytoplasmic DHC (on branch AB, Fig. 6) should have characteristics in common with rat and yeast cytoplasmic DHCs, i.e. the property to perform cytoplasmic translocation. Hence, its descendant DLP4 subtype is also likely to provide a MT-dependent translocation service for subcellular objects.

This evolutionary characterization was based on the phylogenetic order of nodes, where the divergence of yeast (node A, Fig. 6) occurs before the gene duplication for DLP4 (node B, Fig. 6). This order was reliable up to a 90% bootstrap probability in the NJ tree (Fig. 5), and was also proven by MP analysis (Fig. 6). If no yeast DHC existed in this tree, one could only have postulated that DLP4 is the 'closest' molecule to cytoplasmic DHC. Then it could have been said that the 'subcellular translocater' function might first have occurred after the divergence of DLP4, i.e. DLP4 might have served for axonemal movement. We can say that there is almost no doubt that DLP4 is another member of the cytoplasmic dynein subfamily from the present phylogenetic tree.

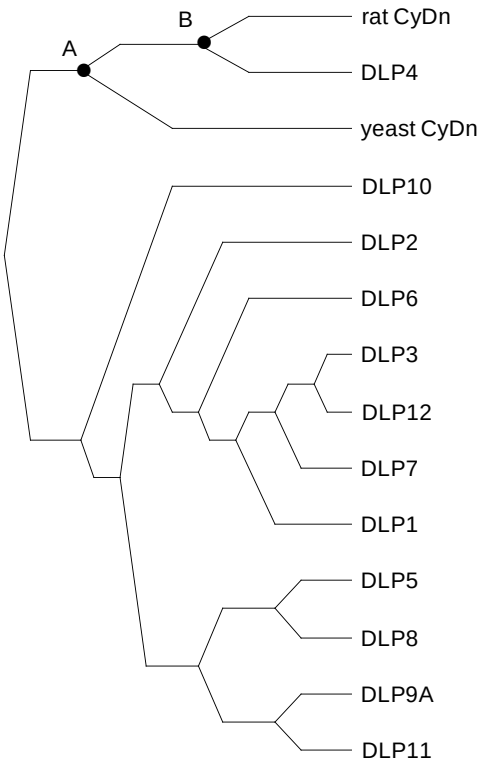

Fig. 6. Phylogenetic tree of rat and yeast DLPs constructed by the maximum parsimony (MP) method (see Materials and Methods). Note the branching pattern completely coincides with that of Fig. 5. The tree was artificially rooted based on UPGMA analysis. Branch lengths have no meaning. CyDn, cytoplasmic dynein heavy chain. The significance of nodes A and B is discussed in the text.

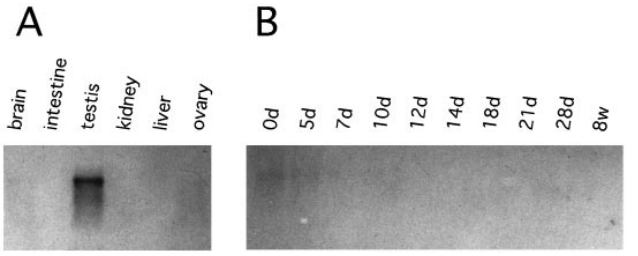

Fig. 7. Transcription level of DLP4 in rat. (A) Tissue distribution of DLP4 message in adult male rat. 20 μg of total RNA from each tissue was separated by 0.8% agarose gel electrophoresis. Transferred membrane was hybridized with the DLP4 probe characterized in Fig. 4A. A prominent signal is detected from testis, and weaker hybridization is observed from brain and ovary. (B) The developmental course of DLP4 message in male rat brain. 10 μg of total RNA per lane from each developmental stage was hybridized under the same conditions as (A). The postnatal age is described in the text. The signal was stronger in the juvenile stages.

Then we further examined the level of DLP4 messengers by quantitative northern blotting (Fig. 7). In adult rat, DLP4 message was most abundant in testis, but also detected in brain and ovary (Fig. 7A). We also examined the developmental course in rat brain (Fig. 7B), and subsequently found that the DLP4 message is relatively abundant in the juvenile brain.

DISCUSSION

In this study, an initial molecular approach to searching the mammalian dynein gene family was performed. We have found 13 dynein-like sequences from rat brain poly(A)$^+$ RNA preparation by a detailed RT-PCR cloning (Figs 1-3), and their molecular nature was examined by hybridization analyses and molecular evolutionary techniques (Figs 4-7). As a result, new information on the molecular diversity of outer-arm- and cytoplasmic DHCs was obtained.

The molecular nature of outer-arm DHCs has long been thought not to be conserved very well in evolution. That is, biochemical and morphological data suggest that the number of DHCs comprising the outer arm varies per species, e.g. two DHCs (α, β) were detected in sea urchin sperm and three (α, β, γ) in *Chlamydomonas* flagellum (reviewed by Asai and Brokaw, 1993). Our phylogenetic study can give a partial explanation of the origin of its diversity. In Fig. 5, DLP5 and DLP8 make a cluster together with sea urchin DHC3A and DHC3B. Their *Drosophila* counterpart has not been identified. There are at least two subtypes in both organisms so similar that may be duplicated in the later stage of higher eukaryote evolution, and correspond to *Chlamydomonas* γ DHC or α DHC. On the other side, the DLP9 subtype cluster containing sea urchin β DHC and *Drosophila* DHC93AB; together with the DLP11 subtype cluster containing *Drosophila* DHCYh3 encoded on the Y chromosome (Rasmusson, 1994) make a large significant cluster, which corresponds to *Chlamydomonas* β DHC. These two clusters may represent the biochemical classes of two main subtypes (α, β) in sea urchin, while each of them contains at least two gene products both in sea urchin and rat. Our observation presented a tissue type-specific expression pattern for DLP5 and DLP8 genes (data not shown), so that some functional differentiation can be made by recent gene duplication for brain cilia and sperm flagella.

In addition, we found diversity of the transcript lengths of DLP5 and DLP9 (Fig. 4C), both of which seemed to result from an alternative splicing of a single gene transcript. The specificity of the probes were proved by two control hybridization studies (Fig. 4A and B), which suggests that crosshybridization of other gene transcripts is unlikely. Actually, we detected alternative splicing of the DLP9 gene, DLP9A and DLP9B, at the sequence level (Fig. 3). These two are confirmed by a secondary RT-PCR cloning and RNase protection assay (data not shown). Such alternative splicing of DHC has never been reported for their homologues defined above in sea urchin and *Drosophila* (Gibbons et al., 1994; Rasmusson et al., 1994). Thus mammals may have developed a special splicing mechanism to add further diversity to their outer-arm DHC molecules, which should be considered when one interprets the information established from other organisms for mammalian cilia.

For the cytoplasmic DHC subtype, we have also expanded our knowledge on its molecular diversity. Our prediction that DLP4 must be another translocater DHC is quite stimulating because only one translocater DHC has so far been biochemically identified in mammals. DLP4 may have been missed in previous biochemical or immunological investigations due to its relatively low abundance in brain (Fig. 7) and its similar molecular mass to cytoplasmic DHC (Fig. 4C). Recently, their human homologue HDHC2, primarily detected from RT-PCR analysis of HeLa cell RNA, is expressed in non-ciliated cell lines (Vaisberg et al., 1994). These data strongly support our hypothesis established in our present study.

Since DLP4 is not a brain-dominant DHC like cytoplasmic DHC (Fig. 7; Zhang et al., 1993), it might play a less important role than cytoplasmic DHC in neuronal retrograde axonal transport. Or rather, it could take partial charge of a ubiquitously distributed cytoplasmic translocation system, e.g. motility occurring in the mitotic apparatus or subcellular trafficking of membrane organelles, mRNA, and/or structural proteins. In sea urchin eggs, the DHC1B transcript is reported to increase by decilliation experiment (Gibbons et al., 1994), suggesting its relevance to ciliary function. The high abundance of its transcript in testis (Fig. 7) supports this hypothesis. Thus, another possible function is that it serves some unknown mechanism for ciliary maintenance. Actually, the *Chlamydomonas* FLA10 gene product, KHC1, is found to be both responsible for cell division and flagella maintenance (Lux et al., 1991; Walther et al., 1994), while it is a homologue of mouse axonal transporter KIF3A/B (Kondo et al., 1994; H. Yamazaki et al., unpublished). A related mechanism can be naturally speculated for this dynein family protein since bidirectional intraflagellar transport is observed in *Chlamydomonas* (Kozminski et al., 1994). Complete molecular cloning and further cell biological investigations are expected to elucidate the function of this novel candidate as an intracellular translocater ATPase.

For DLP10, 2, 6, 3, 12, 7 and 1, there has been resolved only a limited information for now. At least some of them must be used as some of the eight kinds of inner-arm DHCs. A human counterpart of DLP12, HDHC3, is recently reported to be expressed in non-ciliated cells (Vaisberg et al., 1994). Thus some of them may also serve cytoplasmic functions.

In summary, we have reported many new aspects of the diversity of the mammalian dynein gene family initially presented at the molecular level. Particularly, our primary suggestions on the molecular diversity of the cytoplasmic dynein subfamily is quite stimulating for cell biologists who are working for cell motility and the microtubule system. In human, the dynein mutation is reported to be the cause of immotile cilia syndrome or Kartagener's syndrome, which causes situs inversus, respiratory complaints, and male infertility (Kartagener, 1933; Pedersen and Rebbe, 1975; Afzelius et al., 1975; Pedersen and Mygind, 1976). WIC-Hyd rat has been established as its animal model (Torikata et al., 1991). Our first report of the mammalian dynein family sequences gives a new direction in the clinical investigation of this well-known disease at the genetic level.

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