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A Family of Dynein Genes in *Drosophila melanogaster*

Korise Rasmusson,* Madeline Serr,* Janice Gepner,* Ian Gibbons,† and Thomas S. Hays*§

*Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108; and †Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822

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We report the identification and initial characterization of seven *Drosophila* dynein heavy chain genes. Each gene is single copy and maps to a unique genomic location. Sequence analysis of partial clones reveals that each encodes a highly conserved portion of the putative dynein hydrolytic ATP-binding site in dyneins that includes a consensus phosphate-binding (P-loop) motif. One of the clones is derived from a *Drosophila* cytoplasmic dynein heavy chain gene, *Dhc64C*, that shows extensive amino acid identity to cytoplasmic dynein isoforms from other organisms. Two other *Drosophila* dynein clones are 85 and 90% identical at the amino acid level to the corresponding region of the β heavy chain of sea urchin axonemal dynein. Probes for all seven of the dynein-related sequences hybridize to transcripts that are of the appropriate size, ~14 kilobases, to encode the characteristic high molecular weight dynein heavy chain polypeptides. The *Dhc64C* transcript is readily detected in RNA from ovaries, embryos, and testes. Transcripts from five of the six remaining genes are also detected in much lesser amounts in tissues other than testes. All but one of the dynein transcripts are expressed at comparable levels in testes suggesting their participation in flagellar axoneme assembly and motility.

INTRODUCTION

Microtubules assemble into a variety of structures that participate in many of the cell's most fundamental activities, including the transport and positioning of cytoplasmic components, the control of cell shape and migration, ciliary and flagellar motility, and cell division. At a basic level, the assembly, function, and regulation of microtubule-based structures depend on the motors that power movement. Microtubule-associated motors carry out their functions by converting the energy derived from nucleotide binding and hydrolysis into the sliding of microtubules and/or the movement of vesicles, organelles, and molecules relative to a microtubule lattice. The first microtubule motors to be identified were the dynein ATPases that provide the forces required for the bending of ciliary and flagellar axonemes (Gibbons, 1981; Porter and Johnson, 1989; Witman, 1992). The multitude of microtubule-based movements that occur within cells have suggested that, in addition to axonemal dyneins, other microtubule motors might exist. This hypothesis was confirmed after the development of in vitro microtubule motility assays (Vale *et al.*, 1985; Paschal *et al.*, 1987). These assays have permitted the identification of both kinesin and dynein motors in the soluble extracts of cells that contain no ciliary or flagellar axonemes (reviewed in Vallee and Shpetner, 1990; Bloom, 1992; Skoufias and Scholey, 1993).

In most cases, dyneins and kinesins can be distinguished on the basis of the size of their native enzyme complexes, as well as the opposing directionality of their movements along the microtubule lattice. Both axonemal and cytoplasmic dyneins contain multiple subunits, including unusually large heavy chain polypeptides of >500 kDa, and show directed movement toward the minus end of a microtubule. In contrast, the kinesin motors contain lower molecular weight heavy chain polypeptides and usually move toward the plus end of a microtubule. The sequence analysis of the *Drosophila* kinesin heavy chain polypeptide provided the first complete primary sequence of a microtubule motor (Yang *et al.*, 1989). Subsequent analysis of the motor domain of the kinesin heavy chain (Scholey *et al.*, 1989; Yang *et al.*, 1989, 1990) and sequence comparisons be-

§ Corresponding author.

tween kinesins from a variety of organisms have revealed that regions within the motor domain are highly conserved (Goldstein, 1991). This conservation has been exploited in polymerase chain reaction (PCR) and immunological strategies to identify genes that encode a superfamily of related kinesin isoforms (McDonald and Goldstein, 1990; Endow and Hatsumi, 1991; Goldstein, 1991; Stewart *et al.*, 1991; Cole *et al.*, 1992; Endow and Titus, 1992; Roof *et al.*, 1992; Sawin *et al.*, 1992; Skoufias and Scholey, 1993).

There are considerable biochemical data that demonstrate the existence of multiple dynein heavy chain polypeptides in flagellar and ciliary axonemes (Gibbons and Gibbons, 1987; Goodenough *et al.*, 1987; Kamiya *et al.*, 1989; Piperno *et al.*, 1990; Witman, 1992). For example, high resolution sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis has shown that there are at least nine distinct dynein heavy chain polypeptides in *Chlamydomonas reinhardtii* axonemes, including three outer arm dyneins and six inner arm dyneins (Piperno *et al.*, 1990). The low level of immunologic cross-reactivity between these heavy chain polypeptides has suggested that they are encoded by separate genes, rather than isoforms that result from alternative splicing or posttranslational modifications (Gibbons, 1988). Moreover, several unlinked mutations have been isolated in *Chlamydomonas* that result in defective flagellar motility and the loss of different dynein heavy chain polypeptides from the axoneme (Huang *et al.*, 1979; Brokaw and Kamiya, 1987; Kamiya, 1988; Kamiya *et al.*, 1989; Sakakibara *et al.*, 1991; Porter *et al.*, 1992). The recent sequence analyses of the β heavy chain from sea urchin axonemal dynein (Gibbons *et al.*, 1991; Ogawa, 1991) and cytoplasmic dynein heavy chains from *Dictyostelium discoideum* (Koonce *et al.*, 1992), rat brain (Mikami *et al.*, 1993), and yeast (Eshel *et al.*, 1993; Li *et al.*, 1993) indicate the existence of at least two distinct classes of dynein heavy chain genes, but the extent of the dynein gene family within a single organism has not been directly examined.

In this paper, we provide evidence for a family of seven dynein heavy chain genes in *Drosophila melanogaster*. Using PCR, we have amplified dynein-related sequences in *Drosophila* genomic DNA using degenerate oligonucleotide primers. The primers used correspond to the predicted amino acid sequences near the putative hydrolytic ATP-binding site of the β heavy chain polypeptide of sea urchin axonemal dynein (Gibbons *et al.*, 1991). In addition to a dynein sequence related to cytoplasmic dyneins from other organisms, we identify six additional *Drosophila* sequences that encode putative dynein motor proteins. A companion paper (Gibbons *et al.*, 1994) describes the analysis of an analogous dynein gene family in sea urchin and considers the sequence relationships between the *Drosophila* and sea urchin dynein genes. These studies represent an important step toward an analysis of dynein motor diversity and function in the development of multicellular organisms.

MATERIALS AND METHODS

PCR Amplification and Sequence Analysis of Drosophila Dynein Heavy Chain Sequences

The PCR was used to identify members of a dynein heavy chain gene family. Four degenerate oligonucleotide primers corresponding to amino acid sequences near the putative hydrolytic ATP-binding site of the β heavy chain polypeptide of axonemal dynein from sea urchin embryo (Gibbons *et al.*, 1991; Ogawa, 1991) were synthesized. The sequences of each primer are listed below and are written 5′ to 3′. The positions of the four primers relative to the published sea urchin amino acid sequence are given in parentheses and are shown in Figure 1. Primer 1, a sense primer: (1827)-AT(A/C/T)-AC(A/C/G/T)-CC(A/C/G/T)-CT(C/G/T)-AC(A/C/G/T)-GA(C/T)-(A/C)G-(1833). Primer 2, a sense primer: (1854)-GC(A/C/G/T)-GG(A/C/T)-AC(A/C/G/T)-GG(A/C/T)-AA(A/G)-AC(A/C/G/T)-GA-(1860). Primer 3, an antisense primer: (1908)-C(G/T)-(A/G)TT-(A/G)AA-(C/T)TC-(A/G)TC-(A/G)AA-(A/G)CA-(1902). Primer 4, an antisense primer: (1957)-CC(A/C/G/T)-GG(A/G)-TTC-AT(A/C/G/T)-GT(A/G/T)-AT(A/G)-AA-(1951). Oligonucleotide primers were synthesized on an Applied Biosystems 392 DNA synthesizer (Foster City, CA) or purchased from Oligos, Etc. (Wilsonville, OR).

Genomic DNA templates for PCR amplification were prepared from single male flies of a previously isogenized *y; cn bw sp* stock, called *iso-1* (Tamkun *et al.*, 1991), according to the method of Engels *et al.* (1990), except that the resulting homogenate was incubated at 37°C for 20 min. PCR amplification reactions (100 μl) contained 2 μl genomic DNA homogenate, 0.2 mM each dNTP, 0.6–1.0 μM of each of two degenerate oligonucleotide primers, 1× reaction buffer (10 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), and 0.025 U/μl Taq DNA polymerase (Promega, Madison, WI). These reactions underwent 30–40 cycles at 50°C for 2 min, 72°C for 3 min, and 95°C for 1 min.

Initially, two sets of primers were used for amplification of *Drosophila* genomic DNA. One set (primers 1 and 3) was expected to amplify fragments of ~250 base pairs (bp) and the other set (primers 1 and 4), fragments of ~400 bp. Two microliters of the resulting PCR products were visualized in ethidium bromide-stained, 1% agarose/2% NuSieve (FMC, Rockland, ME) gels. In each case, amplification reactions resulted in a predominant product of the expected size. PCR reaction products were ethanol precipitated, treated with T4 polynucleotide kinase and Klenow, and then purified in low melt agarose gels. PCR-amplified fragments were ligated into the *Eco*RV site of pBluescript SK- (Stratagene, La Jolla, CA) and transformed into the host *Escherichia coli*, XL-1 Blue (Stratagene) by standard procedures. Twelve transformants containing 250-bp inserts and twelve containing 400-bp inserts were picked at random and screened by PCR for the presence of a consensus phosphate-binding (P-loop) motif using the nested primers 2 and 3 (see Figure 1). Seven of the 400-bp inserts and all of the 250-bp inserts produced the expected 160-bp fragment, further indicating that these inserts contained dynein-related sequences.

All 19 of the PCR-derived clones were then sequenced and seven different dynein-related sequences were distinguished (Figure 2). Six different dynein-related sequences were present among the cloned 250-bp inserts. Among the cloned 400-bp inserts, three dynein-related sequences were present, one of which was not found among the 250-bp inserts. To recover longer 400-bp versions of all seven sequences, a second PCR screen was carried out. PCR amplification was repeated with primers 1 and 4 using a lower annealing temperature (45°C versus 50°C). Transformants were again picked at random, and additional representatives of each of the seven dynein-related sequences

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                    ********
ITPLTDRCYITLTQSLHLVMSGAPAGPAGTGKTETTKDLGRALGIMVYVFNCSEQMDYKSCGNIY
Primer1                      Primer2

KGLSQTGAWGCFDEFNRISVEVLSVVAVQVKCVQDAIRDKKERFNFMGEEISLIPSVGIFITMNPG
          Primer3                                                Primer4
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Figure 1. Locations of PCR primers relative to the ATP-binding site of sea urchin axonemal dynein. The deduced amino acid sequence of a region of the β heavy chain of axonemal dynein from sea urchin embryos is shown (residues 1827–1957 from Gibbons *et al.*, 1991). Degenerate oligonucleotide primers were derived from the underlined amino acid residues. *, amino acid residues in the P-loop consensus motif (GXXXXGKT) (Walker *et al.*, 1982; Saraste *et al.*, 1990). Primers 1 and 2 are sense, and primers 3 and 4 are antisense.

were selected on the basis of their restriction map or on their ability to be amplified using sequence-specific primers. For each of the seven dynein-related sequences, at least two independent 400-bp inserts were sequenced. Minipreps of plasmid DNAs were prepared using the method of Medberry, Lockhart, and Olszewski (1990). DNA sequencing was performed using Sequenase, version 2.0 (United States Biochemical, Cleveland, OH) according to the supplier's instructions. For convenience in later analyses, primers 1 and 4 with appended *Eco*RI sites were synthesized and used to amplify each of the dynein sequences from the above plasmid DNA templates. The resulting PCR products were gel purified and ligated into the *Eco*RI site of the plasmid, pGEX-1 (AMRAD, Victoria, Australia).

Genomic DNA Preparation and Southern Blot Analyses

Genomic DNA was prepared from *iso-1* adults. Anesthetized flies were ground in a buffer containing 100 mM Tris-HCl pH 9, 100 mM EDTA, 1% SDS. The homogenate was incubated at 65°C for 20 min. After addition of potassium acetate to a concentration of 1 M, the mixture was chilled on ice for 1 h, then centrifuged. Nucleic acids were precipitated from the resulting supernatant by the addition of 0.5 volumes isopropanol. The pellet was washed with 70% ethanol, dried, and resuspended in TE (1 mM EDTA, 10 mM Tris-HCl pH 8). RNAse A was added to a concentration of 25 μg/ml, and the mixture was incubated 30 min at 37°C. DNA was precipitated in the presence of 0.5 volumes 7.5 M ammonium acetate plus 2 volumes ethanol, dried, and resuspended in TE.

DNA samples (7 μg per lane) were digested with restriction enzymes, separated in 0.9% agarose gels, and transferred to nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Richmond, CA) according to standard methods (Sambrook *et al.*, 1989). Membranes were UV-cross-linked (Stratalinker, Stratagene) and dried in a vacuum oven for 1.5 h at 80°C. Seven replicate samples of the digested DNAs were fractionated on two gels, blotted to membrane, and cut into seven panels. Each panel was hybridized separately with a single dynein probe. Hybridizations with the appropriate ^{32}P-labeled probe (see below) were carried out overnight at 45°C in the presence of 50% formamide and 10% dextran sulfate as described by Wahl *et al.* (1979). After hybridization, membranes were washed at high stringency with 2× SSC (300 mM NaCl, 65 mM sodium citrate), 0.1% SDS at room temperature for 30 min and then three times with 0.2× SSC, 0.1% SDS at 65°C for 20 min. Autoradiograms were exposed in the presence of enhancer screens (Dupont, Wilmington, DE) at −80°C.

In Situ Hybridization

Salivary glands were dissected from third instar *Oregon-R* larvae raised at 22°C. Polytene chromosome squashes were prepared, and hybridizations were performed by the method of J. Lim, essentially as described by Engels and coworkers (1986), except that coverslips were removed as in Pardue (1986). The hybridization signal of digoxigenin-labeled DNA probes was detected using NBT and X-phosphate according to the directions of the manufacturer (Boehringer Mannheim, Indianapolis, IN). To verify correct cytological positioning, the polytene chromosomes from at least two flies were analyzed for each of the seven dynein probes.

RNA Preparation and Northern Blot Analyses

RNA for Northern analysis was isolated from *Oregon-R* flies. Ovaries and testes were dissected in *Drosophila* EBR saline solution (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid pH 6.9), flash frozen, and stored at −80°C. Embryos were flash frozen, stored at −80°C, and then ground with a mortar and pestle on dry ice immediately before RNA extraction. RNA was prepared by homogenizing anesthetized whole flies or frozen tissues in a 1:1 mixture of homogenizing buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 M NaCl, 1% SDS) and water-equilibrated phenol (pH 5.2) using a glass-Teflon homogenizer. The homogenate was mixed with 0.1 volumes chloroform and centrifuged at 4°C; the aqueous phase was recovered. RNA was extracted two more times with equal volumes of a 1:1 mixture of phenol:chloroform, ethanol precipitated twice, and stored at −80°C in diethyl pyrocarbonate-treated TE.

Total RNA (25 μg per lane) was size fractionated on 0.75% agarose-formaldehyde gels according to standard methods, essentially as described in Sambrook *et al.* (1989). Glycerol was omitted from the sample buffer, and 0.25 mg/ml ethidium bromide was included in the samples before heat denaturation to allow visual evaluation of the integrity of the ribosomal RNAs. Replicates of RNA samples were fractionated on the same gel, and the resulting blots cut into seven or more strips to obviate stripping and reprobing of blots. Transfer to nylon membranes, hybridization, and posthybridization washing procedures and conditions were exactly as for Southern blot analysis of genomic DNA.

Reverse Transcription (RT)-PCR Analysis of the Distribution of Dynein Transcripts

Total RNA was prepared from 0–12-h embryos, adult females, and adult males by guanidinium isothiocyanate extraction and LiCl precipitation (Laughon *et al.*, 1986). The RNA was reverse transcribed and subsequently amplified by the PCR (Kawasaki *et al.*, 1988). Before RT, total RNA was treated with RQ1 RNase-free DNAse (Promega), extracted with phenol:chloroform (1:1), and recovered by ethanol precipitation. One microgram of the DNAse-treated total RNA was then converted into cDNA by avian myeloblastosis virus reverse transcriptase MP (Life Sciences, St. Petersburg, FL) according to supplier's instructions. The RT reactions contained 400 U/ml reverse transcriptase and 100 nM primer 4 (see above) in a total volume of 20 μl. Control reactions to which no reverse transcriptase was added were performed in parallel and carried through the identical amplification and blotting procedures described below.

The first strand cDNA products were then used as templates for amplification in the presence of the degenerate sense primer 2 and an appropriate sequence-specific antisense primer that was nested to lie 5′ to primer 4. The seven nondegenerate, sequence-specific, antisense PCR primers corresponding to each of the *Drosophila* dynein sequences are shown below written 5′ to 3′, and their positions in

each of the seven sequences are indicated in Figure 2. *Dhc64C:* GCG-CAC-TTG-TTT-GCC-CAC-CAA; *Dhc62B:* T-CAG-CTC-CGT-ACC-TTC-GAA-CAT-GA; *Dhc16F:* GCA-GGA-ACG-ATT-GAT-CTT-AAT-CTC; *Dhc93AB:* AC-GCA-ACT-GAT-CAT-CTC-GCC-CAT-G; *DhcYh3:* GT-GCG-CAA-AGC-AAT-ATG-CTC-T; *Dhc98D:* GCC-GCA-TTT-GGG-ATC-TAG-ATG; and *Dhc36C:* GG-ATC-CAG-TGT-CAA-AGT-GGT-G.

The amplification reactions contained 2 μl reverse transcription reaction, 0.2 mM each dNTP, 300 nM primer 2 (1152-fold degenerate), 250 nM sequence-specific (i.e., nondegenerate) primer, 1× reaction buffer and 0.025 U/μl Taq DNA polymerase (Promega) in a total volume of 50 μl. Reactions underwent 40 cycles of 55°C for 1 min, 72°C for 1 min, and 95°C for 1 min. The RT-PCR reaction products were treated with mung bean nuclease to digest single-stranded products that resulted from the disparity in the degeneracy of paired oligonucleotide primers. After nuclease digestion, RT-PCR products were electrophoresed in 1% agarose/2% NuSieve gels and blotted to Magnagraph (Micron Separations, Westboro, MA) nylon membrane. Southern blotting and hybridization procedures were as described for genomic Southern analysis with the exceptions that digoxigenin-labeled DNA probes were used (see below) and posthybridization final washes were in 0.1× SSC/0.1% SDS.

Preparation of Probes

Probes for genomic Southern and Northern blot analyses were prepared from pGEX-1 plasmid subclones (see above) containing each of the seven dynein sequences. Plasmid DNA was isolated by standard procedures (Sambrook *et al.*, 1989) and digested with *Eco*RI to release the appropriate dynein insert to be used as a probe. The dynein inserts were gel purified and radiolabeled with ^{32}P-dATP using random hexamer primers according to standard methods (Vogelstein and Gillespie, 1979). Equal amounts of each probe template were radiolabeled and purified on Sephadex G-50 (Sigma, St. Louis, MO) spin columns. Equal counts of radiolabeled probes were used for hybridization.

Probes for in situ hybridization to polytene chromosomes and for the Southern blot analysis of the RT-PCR products were generated by PCR amplification. Individual dynein sequences cloned into the *Eco*RV site of the pBluescript SK-plasmid were amplified using the T3 and T7 primers that flanked the insertion site. The amplified DNA fragment containing the dynein sequence and ~150 bp of vector sequence were ethanol precipitated and labeled by random priming using digoxigenin-dUTP according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis IN).

The specificity of each probe was examined in control Southern blots (Figure 5B). Each dynein sequence was separately amplified from the corresponding subclone using degenerate primer 2 paired with the appropriate sequence-specific primer. The seven PCR products were treated with mung bean nuclease and ethanol precipitated. Approximately 20 ng of each were loaded into separate lanes on 1% agarose/2% Nusieve (FMC) gels and size fractionated. Seven replicate gels containing each of the seven PCR products were prepared and blotted to Magnagraph nylon membrane. Each blot was then separately hybridized with a probe derived from one of the seven different sequences, as described in the preceding paragraph. Hybridization and wash conditions were identical to those that were used in Southern blot analyses of the RT-PCR products.

RESULTS

Comparison of Seven Drosophila Dynein Sequences

Seven unique dynein-related sequences were identified in *Drosophila* genomic DNA by PCR strategies (see MATERIALS AND METHODS). The deduced amino acid sequences of the seven partial dynein clones are shown in Figure 2. The corresponding amino acid sequence of

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SUDYH2    CYITLTQSLHLVMSGAPAGPAGTGKTETTKDLGRALGIMVY
DDCDYNHC  CYLTLTQALESRMGGNPFGPAGTGKTETVKALGSQLGRFVL
Dhc64C    CYLTMTQALESRLGGSPFGPAGTGKTESVKALGNQLGRFVL
Dhc62B    CYRTLVGAYQLHLNGAPEGPAGTGKTETTKDLAKALAVQCK
Dhc16F    CYLCLMGAFQMDLGGAPAGPAGTGKTETTKDLAKALAKQCV
Dhc93AB   CYITLTQSLHLVMGGAPAGPAGTGKTETTKDLGRAIGISVY
DhcYh3    CYITLTQSLHLVMGGAPAGPAGTGKTETTKDLGRALGMMVY
Dhc98D    IYLTITQALLMNLGGAPAGPAGTGKTETVKDLAKAMGLLCV
Dhc36C    CYRTLFAALNLHLGGAPEGPAGTGKTETTKDLAKAVAKQCV
           *             * * *********  * *

SUDYH2    VFNCSEQMDYKSCGNIYKGLSQTGAWGCFDEFNRISVEVLS
DDCDYNHC  VFCCDEGFDLQAMSRIFVGLCQCGAWGCFDEFNRLEERILS
Dhc64C    VFNCDETFDFQAMGRIFVGLCQVGAWGCFDEFNRLEERMLS
Dhc62B    VFNCSDGLDYKAMGKFFKGLASCGAWACFDEFNRIELEVLS
Dhc16F    VFNCSDGLDYKMMGRFFSGLAQCGAWCCFDEFNRIDIEVLS
Dhc93AB   VFNCSEQMDYQSCGNIYKGLAQTGAWGCFDEFNRITVEVLS
DhcYh3    VFNCSEQMDYKSIGDIHKGLAQTGAWGCFDEFNRISVEVLS
Dhc98D    VTNCGEGMDYRAVGTILSGLVQCGAWGCFDEFNRIDISVLS
Dhc36C    VFNCSDGLDYLALGKFFKGLASCGAWSCFDEFNRIDLEVLS
          *  *   *          **   *** *******     **

SUDYH2    VVAVQVKCVQDAIR....DKKERFNFM..GEEISLIPSVG
DDCDYNHC  AVSQQIQTIQVALK...ENSKEVELLG..GKNISLHQDMG
Dhc64C    ACSQQIQTIQEALKYEMDSNKESITVELVGKQVRVSPDMA
Dhc62B    VVAQQILLIIQAVR....SNATKFMFE..GTELTLNPACY
Dhc16F    VIAQQLITIRTAKA....MRVKRFIFE..GREIKINRSCC
Dhc93AB   VVAVQVKSVQDAIR....DKKDKFNFM..GEMISCVPTVG
DhcYh3    VVAVQVKCIQDAIK....SKKQTFSFL..GEHIALRTTVG
Dhc98D    VISTQLQTIRNGLI....RKLDRFVFE..GVEIHLDPKCG
Dhc36C    VVAQQILTIQRGIN....SGSPTLVFE..GTTLTLDPTCA
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Figure 2. Deduced amino acid sequences (in single letter notation) of the seven *Drosophila* dynein clones (Dhc). The corresponding regions of the sea urchin β heavy chain of axonemal dynein (SUDYH2) (Gibbons *et al.*, 1991) and the *Dictyostelium* heavy chain of cytoplasmic dynein (DDCDYNHC) (Koonce *et al.*, 1992) are shown for comparison. *, identical amino acid residues found in all nine sequences. ···, gaps introduced for alignment using the "pileup" algorithm (GCG sequence analysis program, Madison, Wisconsin) and manual inspection. ____, sequences for which sequence-specific primers were designed for RT-PCR; ____, the locations of introns in the *Dhc16F* and *Dhc98D* genomic sequences. The Genbank accession numbers for the seven *Drosophila* dynein sequences are: L25122 (*Dhc64C*), L23196 (*Dhc62B*), L23197 (*Dhc16F*), L23198 (*Dhc93AB*), L23199 (*DhcYh3*), L23200 (*Dhc98D*), and L23201 (*Dhc36C*).

the β heavy chain of axonemal dynein from sea urchin embryo (Gibbons *et al.*, 1991) and the sequence of a *Dictyostelium* cytoplasmic dynein heavy chain (Koonce *et al.*, 1992) are included for comparison. All seven *Drosophila* sequences contain a consensus phosphate-binding (P-Loop) sequence, GPAGTGKT, that is identical to that found in sea urchin (Gibbons *et al.*, 1991; Ogawa, 1991), *Dictyostelium* (Koonce *et al.*, 1992), rat (Mikami *et al.*, 1993), and yeast dynein heavy chains (Eshel *et al.*, 1993; Li *et al.*, 1993). This conserved P-loop lies within the putative hydrolytic ATP-binding domain of the dynein ATPases (Gibbons *et al.*, 1991). In addition to the conserved P-loop motif, the seven *Drosophila* clones also share several adjacent short stretches of highly conserved amino acids with the dynein sequences from other organisms (see Figure 2). Over the ~120 amino acid residues sequenced, each *Drosophila* se-

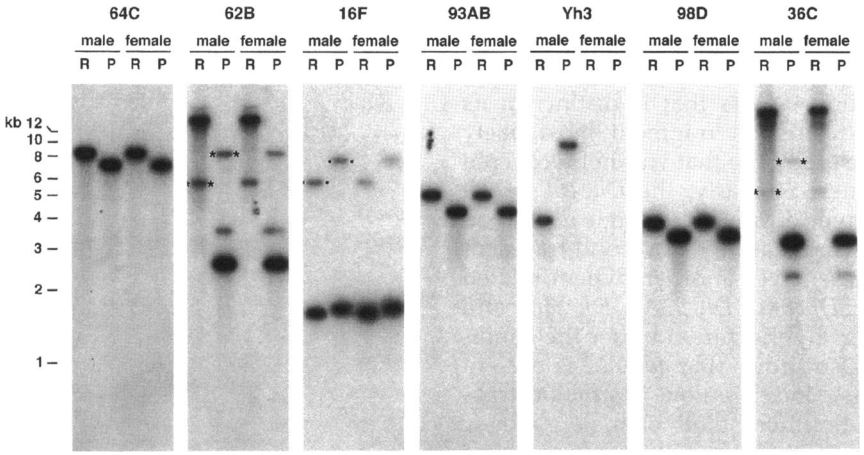

Figure 3. Southern blot analysis of the seven *Drosophila* dyneins showing that each gene is present once in the genome. Equal amounts of male and female genomic DNA were digested with *Eco*RI (R) and with *Pst*I (P), enzymes that do not cut within the dynein sequences used as probes. Replicate samples were blotted and hybridized with the single dynein probe indicated at the top of each panel. Each probe strongly hybridizes to a unique single genomic fragment in each restriction digest. The blot hybridized with probe for *Dhc62B* shows weak cross-hybridization with the restriction fragment of *Dhc36C*. Conversely, the blot hybridized with the probe for *Dhc36C* shows weak cross-hybridization with the restriction fragment for *Dhc62B*. Both probes also weakly hybridize to a third restriction fragment of unique mobility (indicated by *) that is not recognized by the other dynein probes. Similarly, the blot hybridized with the *Dhc16F* probe reveals weak cross-hybridization to another novel restriction fragment (indicated by •) not identified by any of the other probes. Molecular size markers in kb are shown at the left.

quence shows at least 45% amino acid identity with the corresponding region of the sea urchin β heavy chain sequence and the cytoplasmic dynein sequences of *Dictyostelium* and rat brain (Koonce *et al.*, 1992; Mikami *et al.*, 1993). These results suggest that the seven *Drosophila* sequences are derived from a family of genes that encode dynein heavy chain polypeptides. We have designated the corresponding putative dynein heavy chain genes (*Dhc*) according to their physical map positions, as determined by in situ hybridization to polytene chromosomes (e.g., *Dhc16F;* see below).

One of the *Drosophila* dynein heavy chain genes identified, *Dhc64C*, appears to encode a cytoplasmic dynein isoform. Within the sequenced domain reported here, *Dhc64C* is 46% identical to the corresponding β heavy chain sequence of sea urchin axonemal dynein, but 73% identical to *Dictyostelium* cytoplasmic dynein (Koonce *et al.*, 1992). In contrast, the sequences of both the *Dhc93AB* and *DhcYh3* partial clones show >84% amino acid identity to the sea urchin β heavy chain sequence, but >50% amino acid identity to *Dictyostelium* cytoplasmic dynein. The other four *Drosophila* dynein-related sequences are between 46 to 54% identical to both the sea urchin β axonemal dynein and the *Dictyostelium* cytoplasmic dynein isoform. Additional analyses of the relationships between *Drosophila* and sea urchin partial dynein sequences are presented in the companion paper (Gibbons *et al.*, 1994).

The partial genomic sequences recovered for *Dhc16F* and *Dhc98D* appear to contain short introns. To verify the presence of introns in these genomic sequences and to identify the splice sites, the corresponding domains of both genes were amplified from a mixture of adult male and female total RNA using sequence-specific oligonucleotides (see MATERIALS AND METHODS and below); the resulting PCR products were cloned and sequenced. Compared to the cDNA clones, the genomic clones of *Dhc16F* and *Dhc98D* contain introns of 62 bp and 53 bp, respectively, near their 3′ ends (see Figure 2).

Copy Number and Chromosomal Location of Drosophila Dyneins

The copy number of each *Drosophila* dynein sequence was examined by Southern blot analysis. Genomic DNA was separately digested with the enzymes *Eco*RI and *Pst*I. Cleavage sites for these restriction enzyme sites are not represented within the dynein probes used for hybridization. The results, presented in Figure 3, show that each of the seven *Drosophila* dynein sequence probes predominantly hybridizes to a single, unique restriction fragment, indicating that each sequence derives from a gene that is present once in the genome. In the cases of *Dhc62B, Dhc16F,* and *Dhc36C,* the probes also show a relatively weak hybridization to additional restriction fragments. In particular, the *Dhc62B* probe appears to weakly hybridize to the restriction fragments to which the *Dhc36C* probe strongly hybridizes. Conversely, the *Dhc36C* probe appears to weakly hybridize

to the restriction fragments of *Dhc62B*. In addition to this cross-hybridization between *Dhc36C* and *Dhc62B*, both probes weakly recognize a third restriction fragment in both restriction digests that is distinct in its mobility. This third restriction fragment most likely represents a dynein-related gene that was not recovered among our PCR clones. Similarly, the *Dhc16F* probe shows predominant hybridization to a single restriction fragment but weak hybridization to a second fragment that is likely to represent yet another dynein-related gene. The probes for *Dhc64C*, *Dhc93AB*, *DhcYh3*, and *Dhc98D* show no cross-hybridization under the conditions used. Figure 3 also shows that *DhcYh3* is present on the *Y* chromosome, since a unique restriction fragment is observed in Southern blots of male genomic DNA and no fragment is seen in genomic DNA prepared from females.

In situ hybridization to salivary gland polytene chromosomes was used to determine the cytological locations of the seven *Drosophila* dynein heavy chain genes. For each dynein sequence except *DhcYh3*, a single unique site of hybridization is detected. The cross-hybridization observed for the *Dhc62B*, *Dhc16F*, and *Dhc36C* probes by Southern blot analysis did not result in the detection of additional sites of hybridization in polytene chromosomes. Four of the sequences are present on the third chromosome: *Dhc62B* and *Dhc64C* on the left arm and *Dhc93AB* and *Dhc98D* on the right arm. *Dhc16F* is on the X chromosome, and *Dhc36C* is on the left arm of the second chromosome. As expected, no site of hybridization was detected for *DhcYh3* because the *Y* chromosome is heterochromatic and therefore not polytenized to the extent of euchromatin. However, *DhcYh3* has been mapped to the distal end of the long arm of the *Y* chromosome by Southern analysis using a series of *Y* chromosome deficiencies and *X-Y* translocations (Gepner and Hays, 1993). This gene is designated *DhcYh3* because of its location in heterochromatic region 3 of the *Y* chromosome.

Expression of Drosophila Dynein Genes

We have analyzed the expression of the seven *Drosophila* dynein genes by standard Northern blot analysis. As shown in Figure 4A, probes for six of the identified dynein sequences hybridize to large (~12–14 kilobase [kb]) transcripts in RNA preparations from *Drosophila* testes. The probe for the seventh gene, *Dhc93AB*, failed to detect a large transcript in testes RNA, even upon extended exposures. If the *Dhc93AB* transcript is expressed in testes, it is at levels greatly reduced relative to the other dynein transcripts. The presence of six *Drosophila* dynein transcripts in testes RNA is consistent with the biochemical and genetic data indicating the functional significance of multiple dynein heavy chains in the assembly and motility of the flagellar axonemes

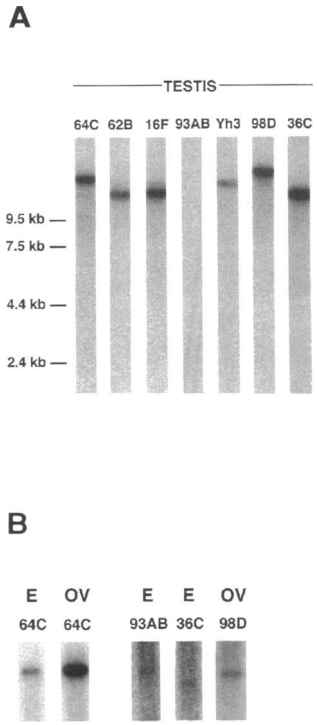

Figure 4. Northern blot analysis of dynein transcripts. (A) Total RNA prepared from testes was probed with each dynein sequence. A single RNA species of 12–14 kb is identified by each probe. The autoradiogram was exposed for 16 h. (B) Expression outside the testes. Northern blots of RNA prepared from ovaries (OV) and 12–24 h staged embryos (E) hybridized to probes for *Dhc64C*, *Dhc93AB*, *Dhc36C*, and *Dhc98D*. In each lane a single species of RNA, 12–14 kb in length, is recognized by each probe. The autoradiograms for *Dhc64C* were exposed 16 h, the autoradiogram for *Dhc98D* was exposed 66 h, and the autoradiograms for *Dhc93AB* and *Dhc36C* were exposed for 11 d.

(Goodenough *et al.*, 1987; Kamiya *et al.*, 1989; Piperno *et al.*, 1990; Witman, 1992).

The expression of dynein transcripts outside the testes was also examined. Northern blots were prepared using total RNA isolated from several sources including ovaries, staged embryos (0–2 h, 2–4 h, 4–8 h, 8–12 h, 12–24 h), larvae, pupae, whole adults, and adult heads. Sets of replicate RNA samples were fractionated on the same gel, blotted to membrane, and separately hybridized with each dynein probe. Our results show that *Dhc64C* is a predominant dynein transcript detected in embryos and ovaries (Figure 4B). As shown for *Dhc93AB*, *Dhc36C*, and *Dhc98D*, probes for each of the other dynein genes, except *DhcYh3*, detect small amounts of a large transcript in RNA prepared from one or more embryonic stages and/or ovaries. In each of these cases, the relative amount of expression was significantly less than that observed for *Dhc64C*, and less than that observed for each gene in testes. We detected no expression of *DhcYh3* in RNA from any nontestes tissue or developmental stage even with 11-d film exposures.

To confirm the Northern blot results, we assayed for the presence of dynein transcripts in RNA samples by using the more sensitive technique of RT-PCR (Kawasaki *et al.*, 1988). In these experiments, total RNA from 0–12-h embryos, adult females, and adult males was separately converted into first strand cDNA using primer 4 (Figure 1). Primer 4 is degenerate and will prime the synthesis of cDNA from all seven *Drosophila* dynein

sequences if they are expressed as RNA. To detect the presence of cDNA derived from any single one of the seven dynein genes, the resulting cDNA mixture was used as template for subsequent amplification using the 5′ degenerate primer 2 paired with the appropriate 3′ primer on the basis of the specific sequence of that particular dynein (see Materials and Methods and Figure 2). The amplification of a specific sequence that was originally present in an RNA preparation was then assayed by Southern blot hybridization using probes derived from each of the seven dynein sequences (Figure 5A).

Several precautions were taken in the RT-PCR amplification of RNA and the subsequent detection of specific dynein sequences in the amplified products. First, because even a minute amount of contaminating DNA in an RNA preparation can lead to significant amplified product, all RNA samples were thoroughly treated with DNAse before RT. In addition, to detect any contaminating DNA, control reactions were carried out in which no reverse transcriptase enzyme was added. These reactions did not yield any detectable product upon PCR amplification, confirming that DNA had been removed from the RNA preparations (see Figure 5A). This result demonstrates that the products amplified in the presence of reverse transcriptase are derived from RNA. A second set of controls was carried out to demonstrate that the hybridization probes used to assay the presence of each dynein sequence were highly specific. In these control experiments, each of the seven cloned *Drosophila* dynein sequences was PCR amplified using its corresponding sequence-specific primer and primer 2. The resulting products were fractionated on agarose gels and analyzed by Southern hybridization using probes for each of the seven dynein sequences. The results, shown in Figure 5B, show that under the identical hybridization and wash conditions used in the analyses of the RT-PCR reactions, the dynein probes are specific except for a weak cross-hybridization between the *Dhc36C* probe and the *Dhc62B* sequence. A third set of controls assessed the specificity of the amplification reactions. In these control experiments, each of the sequence-specific primers and primer 2 were used in amplification reactions containing each of the seven cloned *Drosophila* dynein sequences as template. No amplified product was observed by ethidium bromide–stained gels except when the template plasmid DNA contained the dynein sequence corresponding to the sequence-specific primer used in the reaction (Figure 5C).

The results of the RT-PCR experiments do not provide information on the relative abundance of the dynein transcripts because the use of degenerate primers in both

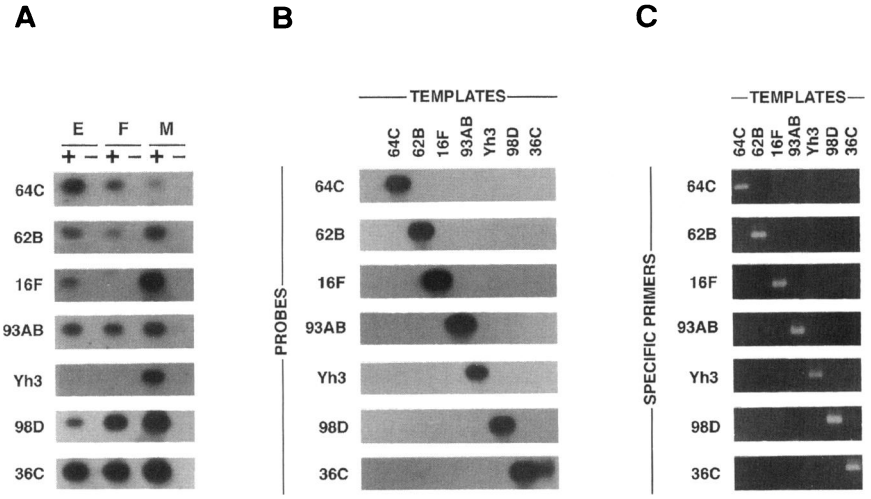

Figure 5. RT-PCR analysis of the expression of the seven *Drosophila* dynein genes. (A) Total RNA from embryos (E), adult females (F), and adult males (M) was reverse transcribed and then amplified by PCR (see MATERIALS AND METHODS). The resulting PCR products were analyzed by Southern hybridization using probes specific for each dynein gene. +, reactions to which reverse transcriptase was added; −, control reactions to which no reverse transcriptase was added. Volumes of PCR reactions loaded: *Dhc36C* = 0.2 μl; *Dhc62B, Dhc64C, Dhc93AB, and Dhc98D* = 1 μl; *Dhc16F and DhcYh3* = 10 μl. Exposure times: *Dhc64C and Dhc62B* = 12 h; *Dhc16F, Dhc93AB, and DhcYh3* = 4.5 h; *Dhc98D and Dhc36C* = 5 min. (B) Specificity of the individual probes was examined by Southern blot hybridization to blots containing all seven of the dynein sequences. The digoxigenin-labeled probe used for hybridization is shown to the left of each panel, and the positions of dynein sequences blotted to the nylon membrane are indicated at the top of the panel. Exposure times: *Dhc64C* and *Dhc62B* blots were exposed 2 min; *Dhc16F, Dhc93AB, DhcYh3,* and *Dhc98D* blots were exposed 5 min; *Dhc36C* blot was exposed 15 min. (C) 3′ primers based on the specific sequence of the individual dyneins preferentially amplify the appropriate corresponding insert from plasmid subclones. When paired with the 5′ degenerate primer 2 an amplified PCR product is apparent only when the 3′ primer corresponds to the template present in the reaction. PCR products were fractionated in 1% agarose/2% Nusieve gels and stained in ethidium bromide.

the RT and amplification steps might have favored the amplification of a particular sequence or set of sequences. However, the ability of the RT-PCR strategy to amplify and to assay the presence or absence of a specific dynein sequence is supported by the *DhcYh3* observations. We have shown that *DhcYh3* is located on the *Y* chromosome (Figure 3, see also Gepner and Hays, 1993) and therefore transcripts derived from this gene should only be present in males. As shown in Figure 5A, the *DhcYh3* probe detects a product only in the RT-PCR reactions that used adult male RNA as the starting template. The RT-PCR results show that all seven *Drosophila* dynein genes are transcribed and that each transcript is present in at least one of the RNA preparations that were examined (Figure 5A). The RT-PCR results are consistent with the Northern blot analyses of expression and provide additional evidence that, except for *DhcYh3*, the identified dynein genes are transcribed outside of males and therefore their expression is not restricted to the testes. However, as indicated by the Northern blot analyses, the level of expression outside the testes is greatly reduced except in the case of *Dhc64C*.

DISCUSSION

We have identified seven *Drosophila* dynein sequences that show >45% amino acid identity with the corresponding domain of the previously characterized dynein heavy chain polypeptides in sea urchin (Gibbons *et al.*, 1991; Ogawa *et al.*, 1991), *Dictyostelium* (Koonce *et al.*, 1992), rat brain (Mikami *et al.*, 1993), and yeast (Eshel *et al.*, 1993; Li *et al.*, 1993). The conserved domain includes an identical P-loop motif, GPAGTGKT, as well as several flanking stretches of predicted amino acid sequence identity. This amino acid conservation between species may reflect the predicted significance of this nucleotide binding domain in nucleotide hydrolysis and dynein function (Gibbons *et al.*, 1991).

In the accompanying paper (Gibbons *et al.*, 1994) evidence is presented for a similar family of dynein heavy chain genes in the sea urchin, *Tripneustes gratilla*. Fifteen distinct dynein heavy chain genes have been identified in sea urchin, and each has been shown to encode an ~14-kb transcript that is large enough to encode a dynein heavy chain polypeptide. Significantly, each of the seven *Drosophila* dynein sequences reported in this study is more similar in its predicted amino acid sequence to one of the sea urchin sequences than to any other *Drosophila* sequence. This relationship between the *Drosophila* and sea urchin dynein genes provides further evidence that the *Drosophila* sequences represent dynein heavy chain genes and provides an indication of how long the diversification of the dynein gene family has existed.

The fact that fifteen dynein heavy chain genes were identified in sea urchin (see Gibbons *et al.*, 1994) and only seven were identified in *Drosophila*, may reflect a genuine difference between the sea urchin and *Drosophila* dynein gene families. *Drosophila* may require fewer dynein motors, because except for the sperm flagella, no other motile axonemes or ciliated tissues have been observed (Demerec, 1950; Perry, 1968; Kiefer, 1973). On the other hand, additional *Drosophila* dynein genes may exist that were not recovered in our PCR screen. Southern blot analyses using each of the dynein clones as probes indicate that at least two additional dynein genes most likely exist but also suggest that the majority of the closely related dynein genes in *Drosophila* have been identified.

The expression analyses of the *Drosophila* dynein transcripts may provide insight into the potential functions of the encoded heavy chain polypeptides in axonemal and/or cytoplasmic motility. Unlike the ciliated blastula of sea urchins or the ciliated epithelia of olfactory and tracheal tissues in mammals, insects apparently do not possess motile cilia in their somatic tissues (Smith, 1968; Kiefer, 1973). Instead, motile axonemes containing characteristic dynein arms appear only in sperm flagella and therefore only in the testes. Therefore, expression of dynein transcripts exclusively in the testes may suggest axonemal function, whereas expression outside the testes would suggest a cytoplasmic function.

Our Northern blot analyses show that at least six of the *Drosophila* dynein genes are expressed in testes. Five of these genes, *Dhc16F, Dhc62B, Dhc98D, DhcYh3*, and *Dhc36C* are expressed at higher levels in testes than in the other tissues and developmental stages examined. This pattern of expression in testes suggests the encoded gene products participate in flagellar motility as axonemal dyneins. This interpretation is supported by the observation reported in the companion paper (Gibbons *et al.*, 1994) that the expression of the sea urchin dynein gene most closely related to each of these genes is upregulated in response to deciliation. However, we cannot rule out the possibility that some or all of these five transcripts encode dyneins that are components of a cytoplasmic transport process within the testes, as opposed to structural components of the axoneme. In particular, the transcript derived from *Dhc64C* is comparably abundant in RNA prepared from testes, ovaries, and embryos. These results suggest that the encoded *Dhc64C* dynein heavy chain is a motor that is involved in cytoplasmic microtubule-based transport processes. Significantly, the transcript of the closely related sea urchin dynein, *DYH1a*, is not observed to increase after deciliation (Gibbons *et al.*, 1994), suggesting that the expression of this cytoplasmic dynein isoform is apparently not required for the assembly of the axoneme.

Although *Dhc64C* is apparently the most abundant dynein transcript expressed in ovaries and embryos, it is not the only dynein transcript detected outside the testes. With the exception of *DhcYh3*, each of the seven

dynein transcripts is present in embryos or ovaries as detected by Northern blot and RT-PCR analyses. However, compared to *Dhc64C*, the relative amounts of embryonic and ovarian transcripts detected on Northern blots for the other dynein genes are greatly reduced. The functional significance of the low levels of dynein transcripts observed in embryos remains to be determined. For example, this level of expression could be because of functionally important transcripts that are restricted to a few specific cells in the embryo or alternatively may represent some basal level of expression in most cells that is of unknown functional significance. However, since there may be additional dynein genes not recovered in our analysis, we cannot exclude the possibility that at these long exposures, our probes crosshybridize with other dynein transcripts. The isolation of mutations in each dynein gene and the analyses of mutant phenotypes should help to resolve this issue.

The partial sequences reported for the seven dynein genes span a highly conserved portion of the putative hydrolytic ATP-binding site. Because of the functional significance of this domain, the comparison of the *Drosophila* sequences to dynein sequences from other species may also help reveal isoforms that serve similar functions. For example, the reported sequence for *Dhc64C* is 73% identical at the amino acid level to the corresponding region of the *Dictyostelium* cytoplasmic dynein (Koonce *et al.*, 1992) but only 46% identical to the corresponding region of the sea urchin axonemal β heavy chain polypeptide (Gibbons, 1991). Because *Dictyostelium* contains no cilia or flagella, the similarity between *Dhc64C* and the *Dictyostelium* sequence indicates that this *Drosophila* dynein isoform most likely represents a cytoplasmic dynein isoform. In this case, both the expression pattern information and the sequence comparisons support the prediction that *Dhc64C* encodes the major cytoplasmic dynein isoform in *Drosophila*.

Across the same region of sequence, both *DhcYh3* and *Dhc93AB* are >84% identical to the corresponding amino acid residues of the β heavy chain polypeptide of axonemal dynein in sea urchin, suggesting that these sequences represent axonemal dyneins. The mapping of *DhcYh3* to a region of the *Y* chromosome required for male fertility (Gepner and Hays, 1993), as well as the expression of *DhcYh3* in testes, provide additional evidence that this gene encodes an axonemal dynein with a role in flagellar motility. Surprisingly, although the sequence reported for *Dhc93AB* shows extensive amino acid identity (90%) to the corresponding region of sea urchin β axonemal dynein, transcripts derived from this gene are not observed in testes at levels comparable to the other family members. Instead, expression analyses suggest that the *Dhc93AB* gene is only transcribed at low levels, including expression in embryos where no motile axonemes are known to exist. The functional significance of this expression remains to be explored.

The four remaining sequences reported for the *Drosophila* dynein genes, *Dhc16F*, *Dhc36C*, *Dhc62B*, and *Dhc98D*, show similar levels of amino acid identity (46–54%) to both the sea urchin β heavy chain axonemal dynein sequence and all published cytoplasmic dynein sequences. For these genes, sequence comparisons within the small region in question are not useful predictors of cytoplasmic or axonemal function. However, as described above, transcripts for these four genes are more prevalent in testes than in embryos or the other tissues examined. On the basis of these observations it is likely that these genes encode additional axonemal dynein isoforms, but we cannot rule out their potential participation in other nonaxonemal or cytoplasmic functions in embryos.

In summary, we provide evidence for a family of dynein heavy chain genes in *D. melanogaster*. The seven identified *Drosophila* sequences share significant amino acid identity with one another, as well as with the sequences of other known dynein heavy chain polypeptides. All seven of the dynein sequences hybridize to transcripts of sufficient size to encode the characteristically large, ~500 kDa, dynein heavy chain polypeptides. Six of the dynein transcripts are abundant in testes, consistent with previous biochemical and genetic data that have shown that multiple dynein heavy chain polypeptides participate in axonemal motility (reviewed in Witman, 1992). One of the dynein genes that is expressed in testes, *DhcYh3*, is located on the *Y* chromosome within *kl-5*, a region that is required for male fertility (Brosseau, 1960; Hardy *et al.*, 1981; Goldstein *et al.*, 1982; Gepner and Hays, 1993). Another dynein gene identified in this study, *Dhc64C*, was previously recovered using expression cloning methods. The full length cDNA for *Dhc64C* has been cloned and sequenced (Li *et al.*, unpublished data) and shares extensive homology throughout its length with cytoplasmic dyneins characterized from other systems (Koonce *et al.*, 1992; Mikami *et al.*, 1992). Finally, each of the seven reported *Drosophila* dynein sequences is highly related to dynein sequences found in sea urchin (Gibbons *et al.*, 1994). These results and observations argue strongly that the sequences recovered by PCR in this study are derived from bona fide dynein heavy chain genes. Each of the seven *Drosophila* dynein genes is a single copy gene that has been designated according to its cytological map position on polytene chromosomes. Future efforts involving genetic and molecular studies should help to resolve the specific cytoplasmic and/or axonemal functions of the *Drosophila* dynein heavy chain genes.

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