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Phylogeny and Expression of Axonemal and Cytoplasmic Dynein Genes in Sea Urchins

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Transcripts ~14.5 kilobases in length from 14 different genes that encode for dynein heavy chains have been identified in poly(A)+ RNA from sea urchin embryos. Analysis of the changes in level of these dynein transcripts in response to deciliation, together with their sequence relatedness, suggests that 11 or more of these genes encode dynein isoforms that participate in regeneration of external cilia on the embryo, whereas the single gene whose deduced sequence closely resembles that of cytoplasmic dynein in other organisms appears not to be involved in this regeneration. The four consensus motifs for phosphate binding found previously in the β heavy chain of sea urchin dynein are present in all five additional isoforms for which extended sequences have been obtained, suggesting that these sites play a significant role in dynein function. Sequence analysis of a ~400 amino acid region encompassing the putative hydrolytic ATP-binding site shows that the dynein genes fall into at least six distinct classes. Most of these classes in sea urchin have a high degree of sequence identity with one of the dynein heavy chain genes identified in *Drosophila*, indicating that the radiation of the dynein gene family into the present classes occurred at an early stage in the evolution of eukaryotes. Evolutionary changes in cytoplasmic dynein have been more constrained than those in the axonemal dyneins.

INTRODUCTION

Dyneins are energy-transducing motor proteins of exceptionally high molecular mass ($1.2–2.0 \times 10^6$ Da) responsible for many forms of microtubule-based cell motility. Two major classes of dynein, axonemal and cytoplasmic, have been identified by previous work (reviewed in Witman, 1992). The axonemal dyneins constitute the outer and inner arms on the doublet microtubules of ciliary and flagellar axonemes, where they generate the localized sliding motion between doublets that underlies the oscillatory beating of these organelles (Gibbons, 1981). Cytoplasmic dynein occurs in both ciliated and nonciliated tissues of higher animals (Paschal *et al.*, 1987; Neely *et al.*, 1990; Mikami *et al.*, 1993), as well as in lower animals and plants, including those, such as *Caenorhabditis*, *Dictyostelium*, and yeast, that do not possess cilia or flagella at any stage of their life cycle (Lye *et al.*, 1987; Koonce *et al.*, 1992; Eshel *et al.*, 1993).

A variety of evidence indicates that cytoplasmic dynein is involved in retrograde transport in nerve axons (Paschal *et al.*, 1987), positioning of the Golgi apparatus (Corthésy-Theulaz *et al.*, 1992), and transport of feeding vesicles in *Paramecium* (Schroeder *et al.*, 1990). The localization of dynein in kinetochores during cell division suggests that it also plays a role in chromosome movements on the mitotic spindle (Pfarr *et al.*, 1990; Steuer *et al.*, 1990), although dynein does not appear to be essential for viability in yeast (Eshel *et al.*, 1993).

Both axonemal and cytoplasmic dyneins are multisubunit proteins containing two or three heavy chain polypeptide subunits of molecular mass >500 000 Da, as well as five to eight subunits of smaller size. The dynein molecule is seen by electron microscopy to consist of two or three globular heads joined by flexible tails to a common base (Porter and Johnson, 1989). Each of these head/tail units is formed of one of the dynein heavy chains, whereas most of the smaller subunits are located in the common base (Sale *et al.*, 1985; King and Witman, 1990). The individual heavy chain subunits each possess ATPase activity, and in favorable cases

they can be isolated in a form that retains the capability for ATP-dependent translocation and rotation of microtubules in an in vitro system (Sale and Fox, 1988; Vale and Toyoshima, 1988; Kagami and Kamiya, 1992). Low concentrations of vanadate inhibit dynein ATPase activity and motility by binding tightly to the γ-phosphate position of the ATP-binding site on the heavy chain (Porter and Johnson, 1989). Both the site of peptide scission by vanadate-mediated photolysis and the incorporation position of photoaffinity-labeled ATP-analogue indicate that the hydrolytic ATP-binding site is located near the middle of the heavy chain (Gibbons *et al.*, 1987). The predicted amino acid sequences of the heavy chains of axonemal and cytoplasmic dynein contain the P-loop consensus motifs for four nucleotide binding sites (GXXXXGKT/S/Q) (Walker *et al.*, 1982; Saraste *et al.*, 1990) spaced ~35 000 Da apart near the middle of the heavy chain. The P-loop closest to the N-terminus has been identified from the position of vanadate-mediated photolysis as the probable hydrolytic ATP-binding site (Gibbons *et al.*, 1991a; Ogawa, 1991). The region around this P-loop has the most tightly conserved amino acid sequence of the heavy chain among dyneins from different organisms (Koonce *et al.*, 1992; Mikami *et al.*, 1993).

A variety of evidence indicates that the heavy chains of dynein occur in multiple isoenzymic forms. In sea urchin sperm flagella, at least seven heavy chain isoforms can be distinguished on the basis of their electrophoretic migration and their sensitivity to vanadate-mediated photocleavage; two of these correspond to the α and β heavy chains that form the outer arms on the doublet microtubules, and the others are believed to constitute the inner arms (Gibbons *et al.*, 1976; Gibbons and Gibbons, 1987). In *Chlamydomonas* flagella, morphological, biochemical, and genetic procedures have demonstrated three species of dynein heavy chain in the outer arms and a minimum of six species of heavy chain in the inner arms (Goodenough *et al.*, 1987; Kamiya *et al.*, 1989; Piperno *et al.*, 1990; Witman, 1992). Although the different isoforms of axonemal dynein have similar enzymatic properties, their low level of immunological cross-reactivity (Piperno, 1984; King and Witman, 1988) has led to the prediction that they are products of a conserved multigene family rather than the result of posttranslational modification or alternative RNA splicing (Gibbons, 1988). Cytoplasmic dyneins, on the other hand, are simpler and contain two identical or nearly identical heavy chains (Neely *et al.*, 1990). Molecular genetic analysis suggests that organisms as diverse as *Dictyostelium* (Koonce *et al.*, 1992), rat (Mikami *et al.*, 1993), sea urchin (Gibbons *et al.*, 1992), *Drosophila* (Rasmusson *et al.*, 1994), and yeast (Eshel *et al.*, 1993) contain only a single gene encoding a cytoplasmic dynein heavy chain.

Recently published sequence analyses of the motor domains of myosin and kinesin have indicated that these families of motor proteins also fall into multiple classes that may correspond to specializations of their functions (Endow and Hatsumi, 1991; Goldstein, 1991; Cheney *et al.*, 1993; Goodson and Spudich, 1993). In this paper, we identify a family of genes encoding dynein heavy chains in sea urchin and present evidence distinguishing the axonemal and cytoplasmic isoforms. Phylogenetic analysis of their predicted amino acid sequences compared with those of *Drosophila* dyneins given in the accompanying paper (Rasmusson *et al.*, 1994) indicates that the motor domains of the dynein heavy chain family fall into six or seven distinct classes that are conserved among disparate organisms.

MATERIALS AND METHODS

Identification and Sequencing of cDNAs Encoding Dynein Heavy Chains

Degenerate oligonucleotide primers were designed upon the amino acid sequence of the sea urchin β heavy chain located on the P-loop of the hydrolytic ATP-binding site and at distances upstream and downstream from it that corresponded to the locations of the conserved regions of myosin sequence (Gibbons *et al.*, 1992). A similar procedure has been used previously to identify members of the kinesin family (Endow and Hatsumi, 1991; Stewart *et al.*, 1991). These primers were used in the polymerase chain reaction to amplify the single strand cDNA obtained by reverse transcription of sea urchin embryonic poly(A)+ RNA (RT-PCR). The different primer combinations that yielded a strong amplified product band of approximately the anticipated size were selected for subsequent work. The sequence of the sense-strand oligonucleotide primers was 5′-(GGGGGAATTCATCACICCI[TC]TIACIGA[TC][AC]G)-3′, on the basis of the amino acid sequence ITPLTDR (residues 1826–33 of the β heavy chain) (Gibbons *et al.*, 1991a), and 5′-(CCCCGGATCCTGCTGG[TCAG]AC[TCAG]GG[TCAG]AA[GA]AC)-3′, on the basis of the amino acid sequence PAGTGKT (residues 1853–59); these primers will be designated S1 and S2, respectively. The sequence of the antisense primer was 5′-(GGGGGAATTCCCIGG[AG]TTCATIGT[ATG]AT[AG]AA)-3′ on the basis of the amino acid sequence FITMNPG (residues 1951–57); this primer will be designated A1.

An amplified DNA band of high intensity and approximately the anticipated length was obtained by using the antisense-strand primer A1, together with sense-strand primer S1. Subsequent subcloning of the amplified cDNA into M13mp18 and sequencing led to identification of nucleotide sequences appearing to correspond to six new dynein genes (designated DYH for dynein heavy chain plus a number and a letter, see RESULTS). The deduced amino acid sequences of these transcripts indicated the presence of a seven-residue-long, completely conserved region, the CFDEFNR consensus motif, located downstream from the P-loop at residues 1902–08 (Gibbons *et al.*, 1991a) (see Figure 1). A new antisense strand primer, 5′-(GGGCGAATTC[TC][AG]TT[AG]AA[TC]TC[AG]TC[AG]AA[AG]CA)-3′, was therefore designed upon this amino acid sequence and designated primer A2. A second round of searching using RT-PCR with the A2 primer together with the S2 primer yielded transcripts of an additional six dynein genes, in some of which the FITMNPGY sequence used for the initial set of primers was subsequently shown to be incompletely conserved.

Poly(A)+ RNA (1–2 μg), prepared either from blastula stage (18 h) sea urchin embryos subjected to one deciliation followed by reciliation for 100 min, or from sea urchin testis was reverse transcribed in 40 μl reaction mixture containing ~100 pmol of primer A1, 2 μl Superscript (Bethesda Research Laboratories, Gaithersburg, MD), 0.5 mM each dNTP, 10 mM dithiothreitol, and the manufacturer's buffer (50 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 8.3, 75 mM

$MgCl_2$). After filter purification of the first strand RT product, the equivalent of ~0.5–1 μl of the original reaction was amplified in 100 μl of solution containing ~200 pmol of each of the degenerate primers, 1 μl Taq polymerase (Promega, Madison, WI) and the manufacturer's buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100). PCR was performed for 50 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min plus a 5-s increment each cycle. A parallel reaction with RNA omitted was performed to verify the absence of significant DNA contamination. In some experiments, the PCR amplification was performed in quadruplicate, and the amplified samples were pooled before cloning to minimize the tendency of PCR to amplify randomly certain sequences. The PCR product was purified by electrophoresis on agarose. The desired DNA band was excised, digested with the appropriate restriction enzymes, and ligated into M13mp18. The cloned single-stranded forms were recovered from the supernatants of minicultures and stored at 4°C. Sequencing by the dideoxy termination method was performed on double-stranded template obtained by PCR amplification of the supernatant with primers based on the M13 sequence flanking the insert, supplemented with specific sequencing primers as necessary to obtain a complete reading of both strands. Sequences were determined of at least two independently amplified and cloned samples in each case.

To extend the regions of initial sequence, we used our leapfrog PCR procedure with a random primed λgt11 library of cDNA from sea urchin embryos and a pair of nested specific oligonucleotide primers based upon the known sequence of each transcript (Gibbons *et al.*, 1991b). The sequenceable overlap region was used to verify that the amplified DNA was derived from the same dynein gene as the original fragment. This independent examination of the sequence in the region of the degenerate primers revealed that the original RT-PCR amplification could occur with as many as five base mismatches in this 23-base region. Although some pairs of transcripts are 90% identical at the amino acid level and 76% identical at the nucleotide level, careful choice of position for the specific primers made it possible to avoid "falling off" onto any undesired dynein of known sequence during the process of sequence extension. However, in two instances, transcripts of previously undiscovered dynein genes were amplified serendipitously.

Embryo Culture and Deciliation

Eggs from five females of the Hawaiian sea urchin *Tripneustes gratilla* were fertilized with sperm from a single male and cultured in Millipore-filtered sea water pH 8.5 at 23°C with constant stirring and aeration. A singly deciliated/reciliated sample was obtained by hypertonic sea water treatment (Stephens, 1977) of a portion of the embryos at 27 h postfertilization, followed by a 115-min period of reciliation before isolation of RNA (at ~29 h). A doubly deciliated/reciliated sample was obtained by subjecting another portion of the singly deciliated embryos to a second hypertonic sea water treatment at 29 h followed by a 115-min period of reciliation before RNA isolation at 31 h. The loss and regrowth of motile cilia were verified by light microscopy at each step. Because the manipulations needed to complete two cycles of deciliation and regrowth occupy ~4 h, during which time there are significant developmental changes in abundance of dynein and other transcripts, a base line of transcript abundance was established by processing undeciliated samples of the same embryos at 23, 27, 31, 35, 43, and 47 h after fertilization, encompassing the late blastula to late prism stages.

For confirmation of experimental results, another series of RNA samples was prepared from a separate batch of embryos that were subjected to three cycles of deciliation/reciliation.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from the sea urchin embryos by the guanidinium thiocyanate method of Chomczynski and Sacchi (1987) as modified by Puissant and Houdebine (1990). Selection of poly(A)+ RNA was performed by passage through a prepacked column of oligo(dT) cellulose (Pharmacia, Piscataway, NJ). Poly(A)+ RNA (6 μg/lane) was electrophoresed on 1% agarose formaldehyde gels and transferred overnight to nitrocellulose.

Isolation of Genomic DNA and Southern Blot Analysis

Genomic DNA was prepared from nine individual males of *T. gratilla* collected from a single location in Kaneohe Bay, Oahu. Sperm from each male were washed separately into 0.54 M KCl and treated with lysis buffer (0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.5% Sarkosyl) containing RNase A and proteinase K at 55°C. After the DNA had been purified by extraction twice with phenol/chloroform, it was recovered by precipitation with ethanol. Restriction enzyme digests were prepared by standard procedures (Sambrook *et al.*, 1989).

The digested DNA from each individual was electrophoresed on a 0.7% agarose gel in Tris-borate-EDTA medium, and transferred onto Gene-Image nylon membrane (United States Biochemical, Cleveland, OH). Seven blots were stripped and reused up to two times to obtain data for the 15 genes.

Preparation of Probes

Probes were selected from regions of the various transcripts that do not contain an *Eco*RI restriction site. The probes for DYH1a, DYH2, DYH3a, DYH5c, DYH6, and DYH7b were PCR-amplified cDNA spanning the 380–430-base pair (bp) region corresponding to amino acid sequences VITPLDR and FITMNPGY, whereas for DYH3b it spanned the 300-bp region from PAGTGKT to FITMNPGY (see above and Gibbons *et al.*, 1991a). Probes for DYH1b, DYH4, DYH5a, DYH5b, and DYH7a were 500–1000-bp extensions of the VITPLDR-FITMNPGY region in the 3′ direction (Gibbons *et al.*, 1991b), as was the probe of DYH7c used for Southern blotting. Probes for DYH7c (Northern blots) and DYH7d were 400–600-bp extensions in the 5′ direction. Probe for DYH3c was the 165-bp PAGTGKT-CFDEFNR region. The tubulin probe was obtained by PCR amplification from a cDNA library (Gibbons *et al.*, 1991b) with degenerate primers to amplify DNA encoding residues 293–418 of β-tubulin. This region is highly conserved among animal tubulins (Cleveland and Sullivan, 1985). The actin probe was a 1.7-kilobase (kb) *Bam*H1 restriction fragment of the sea urchin Tg616 actin gene kindly supplied by Dr. Tom Humphreys' laboratory, University of Hawaii (Fregien *et al.*, 1983). Radiolabelled probes were prepared with [α-^{32}P]dCTP using random hexamer primers and appropriate cDNA as template. Hybridization of RNA blots was performed overnight at 42°C in 50% formamide. Hybridization of DNA blots was performed at 68°C in the absence of formamide. Blots were washed to a final stringency of 0.2× SSPE (36 mM NaCl, 2 mM NaH_2PO_4, 0.2 mM EDTA pH 7.7), 0.1% sodium dodecyl sulfate (SDS) at 65°C. Autoradiograms were prepared on preflashed X-ray film and quantified by densitometry. Potential cross-hybridization was tested for all permutations of overlapping probes by dot blot analysis; it did not exceed 2%, except for DYH5a, which showed about 5% hybridization with DYH4 and with DYH7a.

Sequence Analysis

Multiple alignments of the deduced amino acid sequences were generated by the computer program Clustal V with the default parameters (Higgins *et al.*, 1992). The relatedness of the aligned sequences was analysed with the following programs from the PHYLIP package, version 3.5c, kindly given us by Dr. Joe Felsenstein, University of Washington, Seattle. A pairwise distance matrix for the sequences in the alignment was calculated with the program PROTDIST by using the PAM001 amino acid substitution matrix (Dayhoff *et al.*, 1978) for scoring. The branching topology was constructed with the program FITCH, which produces an unrooted tree and permits different rates of change. This program seeks the tree that minimizes the difference between the distances calculated from the tree topology and the observed pairwise distances. The robustness of the tree topology was

tested by performing a maximum parsimony analysis with the program PROTPARS on a set of 100 randomly resampled alignments generated from the original by the bootstrap option of the program SEQBOOT and then averaging the resultant trees with the program CONSENSE (Felsenstein, 1988).

RESULTS

Identification of Dynein Heavy Chain Transcripts

We have identified 14 distinct transcripts that encode dynein-like sequences in poly(A)+ RNA isolated from sea urchin embryos; one of these (dynein heavy chain 2, DYH2) is the dynein β heavy chain that was previously sequenced in its entirety (Gibbons *et al.*, 1991a). The partial deduced amino acid sequences of these transcripts are shown aligned in Figure 1. The region shown is ~400 residues long for most of the transcripts and extends to ~1200 residues for a subset of six transcripts. The P-loop of the putative hydrolytic ATP-binding site of dynein ATPase, designated P1, is located at residues 0–7 of this region, corresponding to residues 1852–1859 of the complete β chain sequence. The characteristic eight-residue sequence motif of the P1 loop, GPAGTGKT, is completely conserved in all transcripts. The three additional P-loop motifs first described in the midregion of the β chain of ciliary dynein (Gibbons *et al.*, 1991a; Ogawa, 1991) are present at residues 319, 706, and 1098 of the alignment and are designated as P2, P3, and P4, respectively (Figure 1). In addition to the similarity in deduced amino acid sequence, cDNA probes from the transcripts hybridize at high stringency to a ~14-kb band on blots of poly(A)+ RNA from sea urchin embryos (see below). This transcript size is sufficient to encode the high molecular mass polypeptides, of 450–550 kDa, that are characteristic of dynein ATPases. On the basis of these data and the other sequence similarities described below, we conclude that the transcripts encode polypeptides having sufficient similarity to previously sequenced dynein heavy chains that they are most reasonably considered a single family.

A fifteenth transcript (DYH3c), which has been found only in poly(A)+ RNA from testis, is identified as a possible testis-specific dynein heavy chain transcript on the basis of its sequence similarity.

Analysis of Sequence Relatedness

Casual examination of these dynein sequences indicates that many of them fall into clusters within each of which the members resemble each other much more closely than they do those of any of the other clusters. Although the full significance of these relationships remains to be determined, it is desirable to adopt a tentative nomenclature for the dynein heavy chain transcripts that is indicative of their sequence relatedness. Phylogenetic relationships among proteins can be analyzed either from their distance as scored by the number and type of amino acid replacements or by the method of maximum parsimony, which determines relatedness, so as to minimize the number of nonsilent mutations required to derive the observed array of sequences from a single unknown ancestral sequence (Felsenstein, 1988). Figure 2 shows a tree of the distance between dynein heavy chains in sea urchin, derived from the predicted amino acid sequences of the 13 transcripts for which sequence is available over most or all of the ~400-residue length. Other trees, based either upon the more extended length of ~1200 amino acids available for six transcripts or calculated using the maximum parsimony procedure (Felsenstein, 1988), display essentially the same topology. The degree of sequence similarity in the hydrolytic ATP-binding region near the middle of the dynein heavy chain (residues 1812–2164 of the predicted 4466 residue β heavy chain) is likely to reflect the phylogeny of the dynein genes, although it is possible that differences in their N-terminal and C-terminal domains may represent specializations in their function that are currently unknown. The terminology used assigns a number to identify each cluster of closely related transcripts, together with a letter to designate the individual members within a cluster. DYH2 is the β heavy chain of the axonemal dynein that comprises the outer arms of cilia and sperm flagella. The sequence of DYH1a resembles that of the cytoplasmic dynein in the slime mold *Dictyostelium discoideum* (Koonce *et al.*, 1992) and rat (Mikami *et al.*, 1993) much more closely than do any of the other sequences, and it is accordingly believed to encode the principal cytoplasmic dynein in sea urchins (see below). The other sequences are numbered approximately according to their relatedness to this cytoplasmic form.

In most cases, the sequences within each cluster constitute robust groups that are strongly supported by the data and occurred in ≥99% of the trees obtained by random resampling of the original alignment (Figure 2). An exception is the cluster containing DYH5a, 5b, and 5c, which occurred in only ~70% of the resampled trees, but which has been maintained as a single cluster for simplicity until more information becomes available. The interrelatedness of the clusters, on the other hand, is generally less strongly supported by the data, and identical branches occurred in only 32–89% of the resampled trees.

Conserved Sequence Regions

The predicted sequence of the dynein isoforms in the vicinity of their putative hydrolytic ATP-binding site (residues −40 to 200) shows a relatively high level of overall conservation, with 29% of the residues being fully conserved and 48% partially conserved, whereas in the region downstream from this (residues 201–1159) only 7% of the residues are fully conserved and 38% partially conserved. Superimposed on this overall distribution of conserved residues, there is a patchy pattern

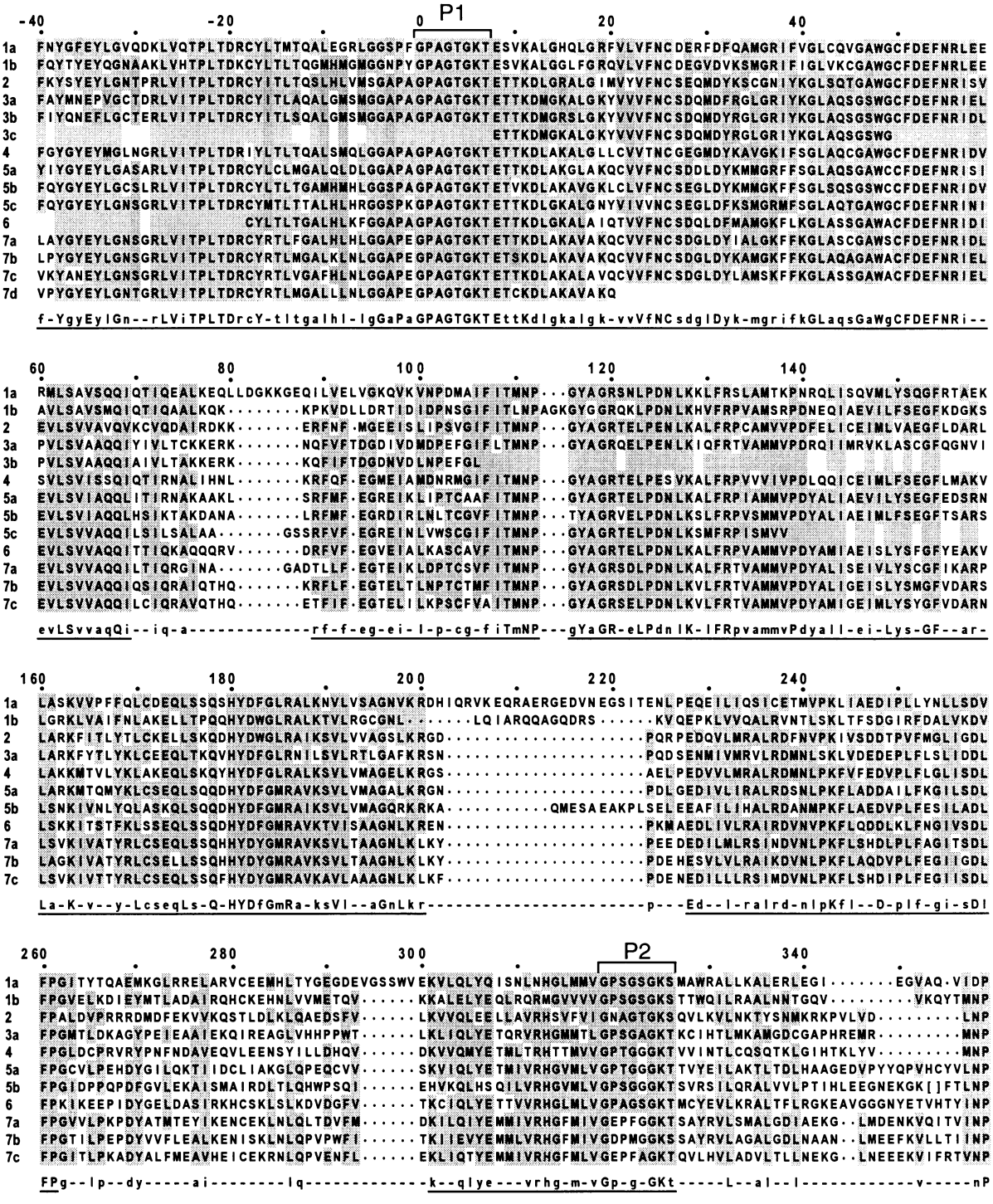

Figure 1. Alignment of the predicted amino acid sequences of 15 dynein heavy chain genes that are expressed in sea urchin embryo and testis. This ~1200-residue alignment represents the midregion of the dynein heavy chains and constitutes ~25% of their full lengths. The single letter amino acid code is used. The P-loop consensus motif of the probable hydrolytic ATP-binding site is designated as P1. The three additional P-loop motifs closer to the carboxy-terminus of the heavy chain are designated successively as P2, P3, and P4. ·, dots indicate gaps introduced to obtain optimum alignment. The left column shows the nomenclature used for transcripts as derived from the analysis in Figure 2 (see text). The numbering above the sequences indicates amino acid position relative to an arbitrary zero at the first glycine of the P1 loop motif, which is absolute position 1852 for DYH2, the β heavy chain (Gibbons *et al.*, 1991a). Positions in the alignment are shaded where at least one-half of the residues are identical or conservative replacements. In a few instances conservative replacements have been left unshaded

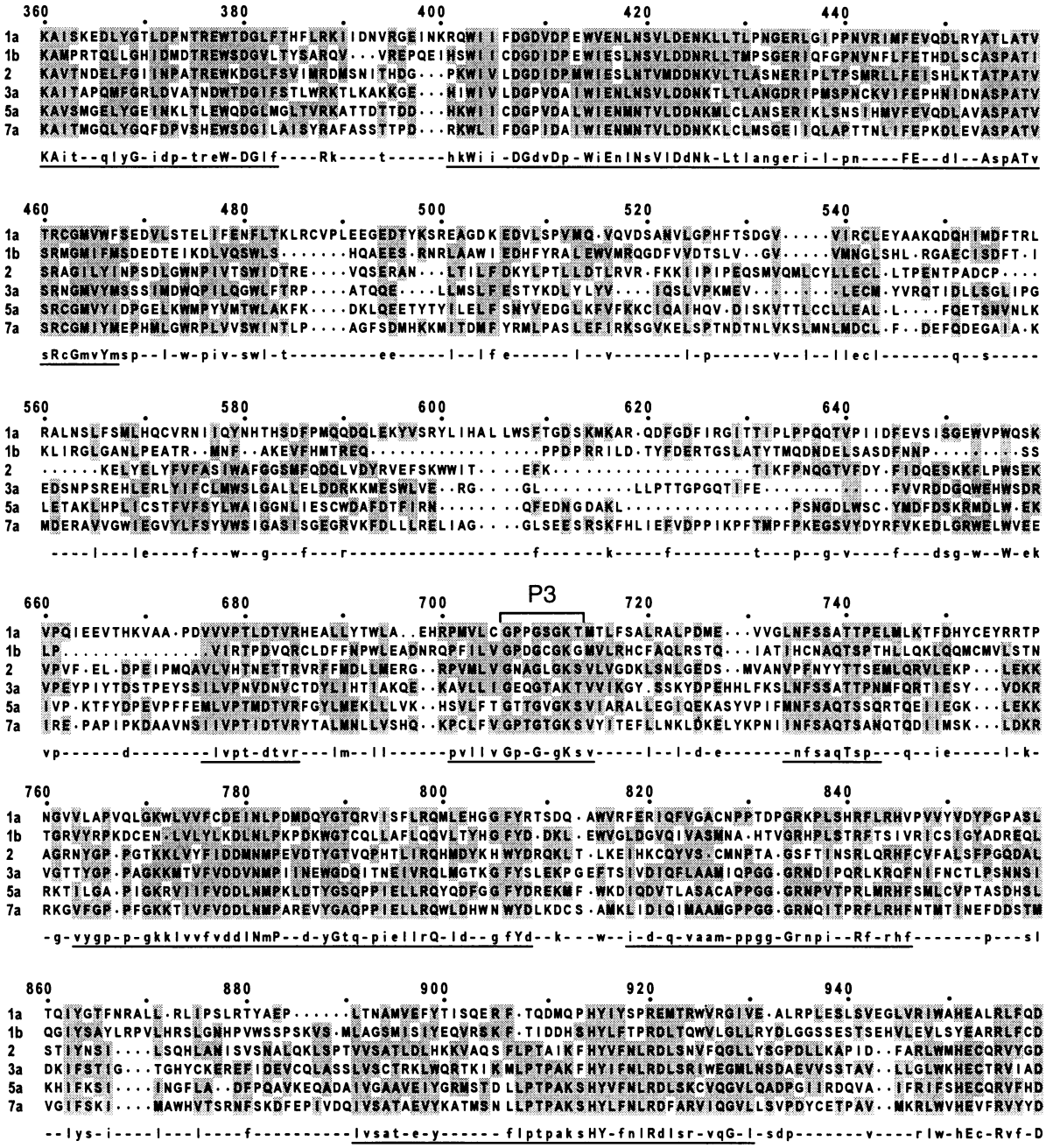

Figure 1. (Continued) for increased emphasis. In the consensus line below the alignment, uppercase letters indicate positions of complete amino acid conservation; lowercase letters indicate positions at which a residue is conserved in at least one-half of the sequences; ---, nonconserved positions; the underlining indicates stretches of sequence with moderate to high levels of conservation. Sequence extending ~1200 residues is shown for transcripts DYH1a, DYH1b, DYH2, DYH3a, DYH5a, and DYH7a. Sequence extending ~400 residues is shown for DYH4, DYH5b, DYH6, DYH7b, and DYH7c. The available sequences of DYH3b, DYH3c, DYH5c, and DYH7d are shorter, and regions for which no sequence is available are shown blank. Sequence in the region of degenerate primers is shown only where it has been independently confirmed. The sequences were aligned by the computer program ClustalV using default parameters (Higgins *et al.*, 1992), followed by minor manual adjustment. The nucleotide sequences corresponding to these amino acid sequences have been deposited in the EMBL/GenBank/DDBJ database under accession numbers: DYH1a, Z21941; DYH1b, U03969; DYH2, X59603; DYH3a, U03970; DYH3b, U03971; DYH3c, U03972; DYH4, U03973; DYH5a, U03977; DYH5b, U03974; DYH5c, U03976; DYH6, U03975; DYH7a, U03978; DYH7b, U03979; DYH7c, U03980; DYH7d, U03981.

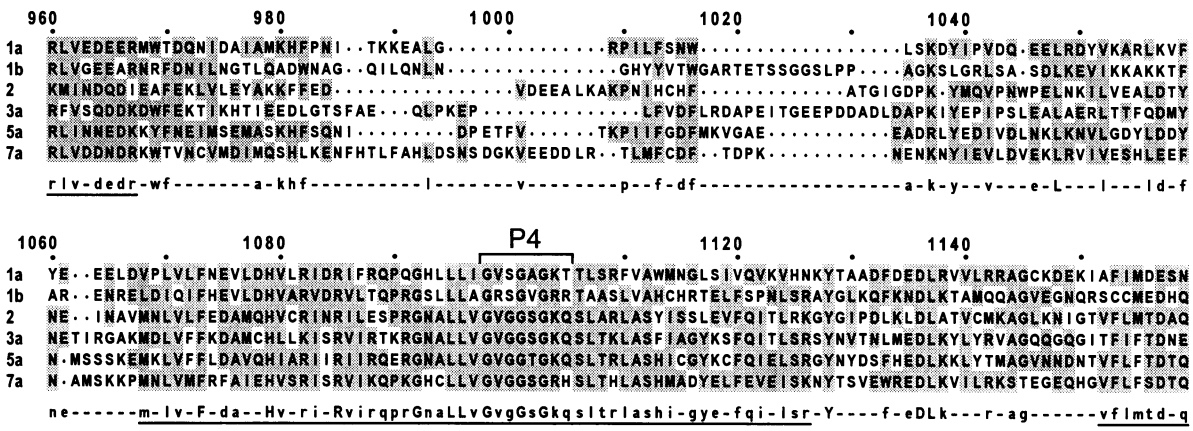

Figure 1. (Continued)

of conservation on a smaller scale, with short sections of moderately to highly conserved sequence (indicated by the underlining in the consensus sequence in Figure 1) being interspersed with sections of more variable sequence.

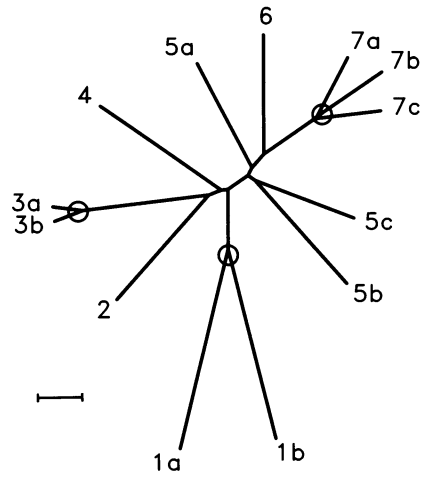

Figure 2. Phylogenetic tree of the dynein heavy chains in sea urchin. The tree was computed from pairwise distance matrix scores for the DYH sequences in Figure 1, utilizing positions −40 to 359 of the alignment. The length of each branch in the tree represents the percentage amino acid sequence divergence between the sequences at its two ends. Sequence divergence is taken as the observed number of amino acid differences, scaled by the PAM001 substitution matrix, and adjusted for multiple substitutions. The scale on the figure indicates the distance corresponding to 10% sequence divergence. The average percentage SD in branch length was 6.8%. The nodes (circled) defining the cluster of DYH1a and DYH1b, the cluster of DYH3a and DYH3b, and the cluster of DYH7a, DYH7b, and DYH7c, are strongly supported by the data, with an identical node being obtained in >99% of the resampled data sets subjected to parsimony analysis. Other nodes were identical in only 32–89% of the resampled sets, indicating that the order in which their branches diverged is uncertain. Because of the much shorter length available, DYH3c and DYH7d were not included in the analysis; over the available sequence, DYH3c is 98% identical to DYH3a, and DYH7d is 92% identical to DYH7b.

In addition to the P1 loop, which as noted above is absolutely conserved in all the dynein heavy chains, the generally conserved region between residues −40 and 200 contains four sections of notably high conservation. The most striking of these is the CFDEFNR motif located at residues 50–56, which is present in all the sea urchin dynein isoforms as well as in published dyneins of other species and thus may be a useful diagnostic signature for dynein heavy chains. The others with a slightly lesser level of conservation are the RLVITPLTDRCY sequence at residues −28 to −17, which is conserved in at least nine of the isoforms, the FITMNPGYAGR sequence at residues 107–120, conserved in at least eight isoforms, and the HYD(F,W,Y)G(L,M)RA sequence at residues 180–187. In addition to these sections, there are fairly numerous isolated residues that are conserved, resulting altogether in 29% of the residues in this region being completely conserved in all the isoforms.

Although the region of the heavy chain between residues 201 and 1158 is generally less conserved, the three P-loop motifs originally described in the β chain sequence are predicted to be present in all the isoforms. The P2 loops fit the usual P-loop definition of [G,A]XXXXGK[S,T] (Walker *et al.*, 1982; Saraste *et al.*, 1990) with most of the undefined residues of the motif being glycine, proline, and serine but with more bulky residues, including methionine and phenylalanine, being present in DYH7a, b, and c. The P3 loops mostly fit the same definition, but the undefined residues show more variation, and the second glycine of the pattern is replaced by alanine in DYH3a. In the P4 loops, the variation takes a different form with the undefined residues comprising mostly glycine, serine, and valine, whereas the final residue of the motif remains as threonine only in DYH1a and is substituted by glutamine in DYH2, 3a, and 5a, by arginine in DYH1b, and by histidine in DYH7a, while the usually invariant lysine is replaced by arginine in DYH1b and DYH7a. To extend

the definition of a P-loop motif to cover these variations, as well as adenylate kinase and the regulatory GTPases, the definition of a P-loop motif could be changed to [G,A]XXXX[G,A][K,R][T,S,G,Q,R,H].

The sequence immediately surrounding the P2, P3, and P4 loops is not particularly conserved, although that around the P4 loop (residues 1070–1126) is conserved better than that around P2 and P3. However, it is to be noted that the P2 and P3 loops each have two moderately conserved sections beginning ~50 and 100 residues downstream from the P-loop motif itself, at approximately the same distance as the highly conserved CFDEFNR and FITMNPGY sequences are located downstream from the P1 loop (Eshel *et al.*, 1993).

Within the more generally conserved region between residues −40 and 200, there is a single section of hypervariable sequence located approximately between residues 72 and 93. In this section even the more closely related transcripts, such as DYH7a, 7b, and 7c, show substantial differences. Some differences in length also occur here with DYH1a containing an eight-residue insertion and DYH5a and DYH5b containing a single inserted residue. DYH1b is unique in having a three-residue insertion within the otherwise nearly conserved FITMNPGYAGR sequence (Figure 1). In the generally less conserved region (residues 201–1158), sections of highly variable sequence are more numerous. Especially notable examples occur between residues 341 and 357, 484 and 675, and 986 and 1036. Within these the sequences of the isoforms show several insertions and deletions.

Identification of Transcripts as Separate Genes

Because our preparations of poly(A)+ RNA were derived from several sea urchins in a wild population, it was necessary to examine whether the dynein transcripts that we identified all derive from distinct genes or whether some represent multiple alleles at a single locus. For this purpose, cDNA derived from each transcript was used to probe a blot of *Eco*RI-digested genomic DNA from each of nine individual male sea urchins. The resultant patterns of hybridization showed that the fifteen transcripts identified were present in all the nine sea urchins examined (Figure 3). In DNA samples from most individuals, a given probe hybridized either to a single restriction fragment or to two different size fragments of lesser intensity. Hybridization to two such restriction fragments of nearly equal intensity is taken to reflect restriction length polymorphism. The minimum number of restriction length alleles in the population examined is 6, 5, 3, 8, 6, 3, 4, 4, 4, 6, 5, 4, 4, 5, and 8 for DYH transcripts 1a, 1b, 2, 3a, 3b, 3c, 4, 5a, 5b, 5c, 6, 7a, 7b, 7c, and 7d, respectively; this level of polymorphism is comparable to that reported previously for other genetic loci in sea urchins (Britten *et al.*, 1978; Simmen *et al.*, 1986). This observed pattern of hybridization indicates that all the DYH transcripts identified derive from distinct, single-copy genes that are presumably located at separate loci. The probes 1b, 3b, 4, 7b, and 7d identified an additional one to four more weakly hybridizing restriction fragments that did not match any other hybridizing fragments in size. These weakly hybridizing fragments may be indicative of additional single-copy genes not yet identified, or they may be the result of internal *Eco*RI sites in introns located within the coding region represented by the probes.

Changes in Gene Expression in Response to Deciliation

Changes in the steady-state concentration of mRNAs after deciliation can help to distinguish between genes encoding axonemal and cytoplasmic products (Lefevre and Rosenbaum, 1986; Gong and Brandhorst, 1987; Harlow and Nemer, 1987). In our work, preliminary experiments using cDNA from the β dynein heavy chain (DYH2) to probe poly(A)+ RNA from normal and deciliated embryos at different stages of development suggested that one or two cycles of deciliation of gastrula stage embryos produced an adequate boost in mRNA levels. Hybridization of actin and tubulin probes to blots of this series of poly(A)+ RNA samples and densitometry of the resultant radiograms illustrates that these genes show a clear difference in the effect of deciliation on their level of mRNA (Figures 4 and 5), for although the level of actin expression in the once deciliated and twice deciliated samples is <30% changed from the corresponding control samples at 27 and 31 h, the level of tubulin mRNA is boosted 2.5- to 3-fold. The developmental increase in abundance of actin transcripts in the nondeciliated embryos agrees with the report of Fregien *et al.* (1983) of a large increase between the gastrula and pluteus stages.

To determine whether the dynein transcripts can be segregated into classes that resemble the expression patterns of either actin or tubulin, blots of this series of poly(A)+ RNA were probed with cDNA from 13 of our embryo transcripts (Figures 4 and 5). A threefold or greater increase in the abundance of DYH2, DYH3a, DYH3b, DYH4, DYH5a, DYH7a, DYH7b, and DYH7c transcripts, as well as a somewhat lesser increase for DYH1b, DYH5b, and DYH5c, was observed in the twice deciliated sample (Figure 5). Thus, in almost all cases, the magnitude of the increases equalled or exceeded the average 2.5-fold increase observed for transcripts of tubulin. By contrast, the abundance of transcripts of DYH1a showed no significant change in response to deciliation; this pattern resembles that of actin. DYH6, which shows no boost in Figure 5, showed a 1.6- to 2.0-fold boost in other similar preparations of RNA.

DYH3c, which was obtained from testis RNA, did not hybridize with any band in the embryo poly(A+) RNA and was not included in this experiment.

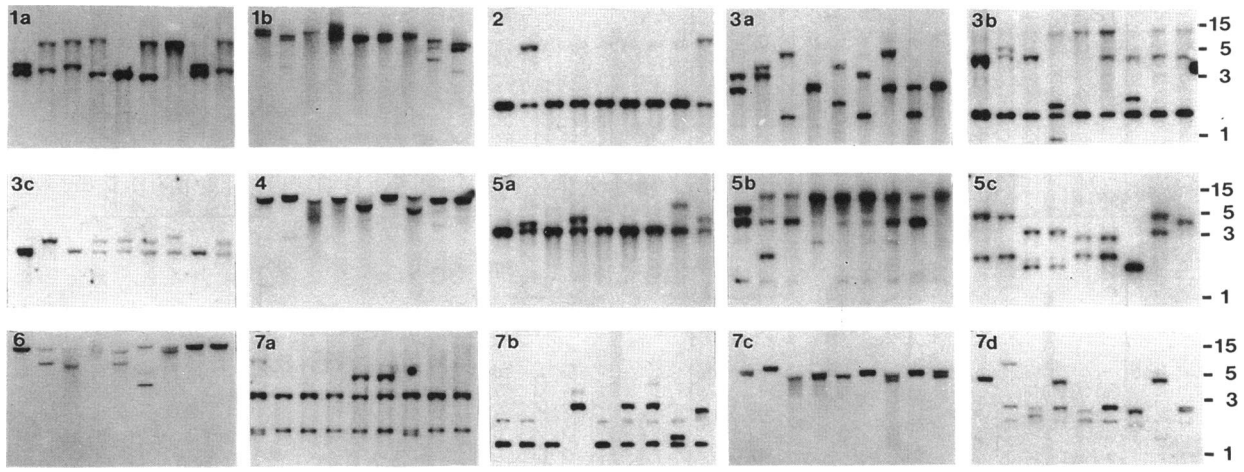

Figure 3. Analysis of restriction fragment length polymorphism in the DYH genes corresponding to the sequences shown in Figure 1. Each panel shows a blot probed with cDNA from one of the 15 transcripts as indicated. The nine lanes of each blot contain 2–4 μg *Eco*RI-digested genomic DNA from sperm of each of nine individual sea urchins. Molecular sizes in kilobases are indicated at the right. The restriction fragment sizes range from 700 bp to ~15 kb, with a median of 3.3 kb. None of the probes contains an internal *Eco*RI site. The uniqueness of the pattern observed with each probe indicates that there was no significant cross-hybridization. (The faint bands in panel 5b are a residue from the previous probing of this blot with DYH7b).

DISCUSSION

The results presented indicate that sea urchins possess a family of at least 15 dynein heavy chain genes, one of which encodes the β axonemal dynein present in both embryo cilia and sperm flagella that was sequenced previously (Gibbons *et al.*, 1991a; Ogawa, 1991). The 12 newly identified genes for which predicted amino acid sequence is available over most or all of a ~400 residue region encompassing the putative hydrolytic ATP-binding site have a >41% sequence identity to the β axonemal dynein, as well as to the corresponding region of the cytoplasmic dynein of *Dictyostelium* (Koonce *et al.*, 1992). The predicted sequences of the dynein gene family show a completely conserved nucleotide-binding motif, GPAGTGKT, that forms the P-loop of the putative hydrolytic ATP-binding site (Gibbons *et al.*, 1991a). Probes prepared from all 13 of the dynein genes tested hybridize at high stringency to a single ~14-kb band on blots of poly(A)+ RNA from sea urchin embryos, indicating that the transcripts of the genes are an appropriate length to encode for polypeptides of the characteristically high molecular weight (450–550 kDa) of dynein heavy chains.

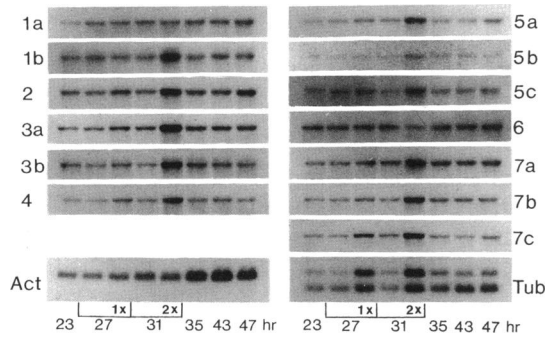

Figure 4. Autoradiograms showing changes in abundance of DYH, actin, and tubulin transcripts in response to deciliation of sea urchin embryos. Blots of poly(A)+ RNA (6 μg/lane) from embryos harvested at various times after fertilization were probed with ^{32}P-labelled cDNA derived from the DYH transcripts identified in top left corner, with similar probes for actin (Act) and tubulin (Tub) being used for comparison. All DYH probes hybridized to a single band of ~14 kb; probe for Tub hybridized to two bands of 5.6 and 4.3 kb; probe for Act hybridized to a single band of 3.8 kb. Only the region of the blots containing bands hybridizing to the probes is shown. 1× and 2× indicate lanes with RNA from embryos that had been deciliated and allowed to reciliate, either once or twice. The other lanes contain RNA from the control series of undeciliated embryos harvested at the indicated times after fertilization with the 27- and 31-h points serving as controls for the two deciliated samples. Relative intensities can be compared only for a single probe; the specific activities and exposure times differ for different probes. DYH7d was not included in this experiment.

Our finding that the sequence surrounding the P1 loop is much more tightly conserved among sea urchin dynein isoforms than that surrounding the other three P-loops supports the previous indication, on the basis of the position of vanadate-mediated photolysis (Gibbons *et al.*, 1991a), that the P1 loop constitutes part of the hydrolyic ATP-binding site of dynein ATPase. The retention of the P2, P3, and P4 motifs in all the isoforms, in spite of the overall sequence divergence in this region of the heavy chain being such that only ~7% of the total residues are conserved, is consistent with their being required for a significant role in dynein function. However, their imperfect sequence conservation implies that their roles are less demanding than the ATP hy-

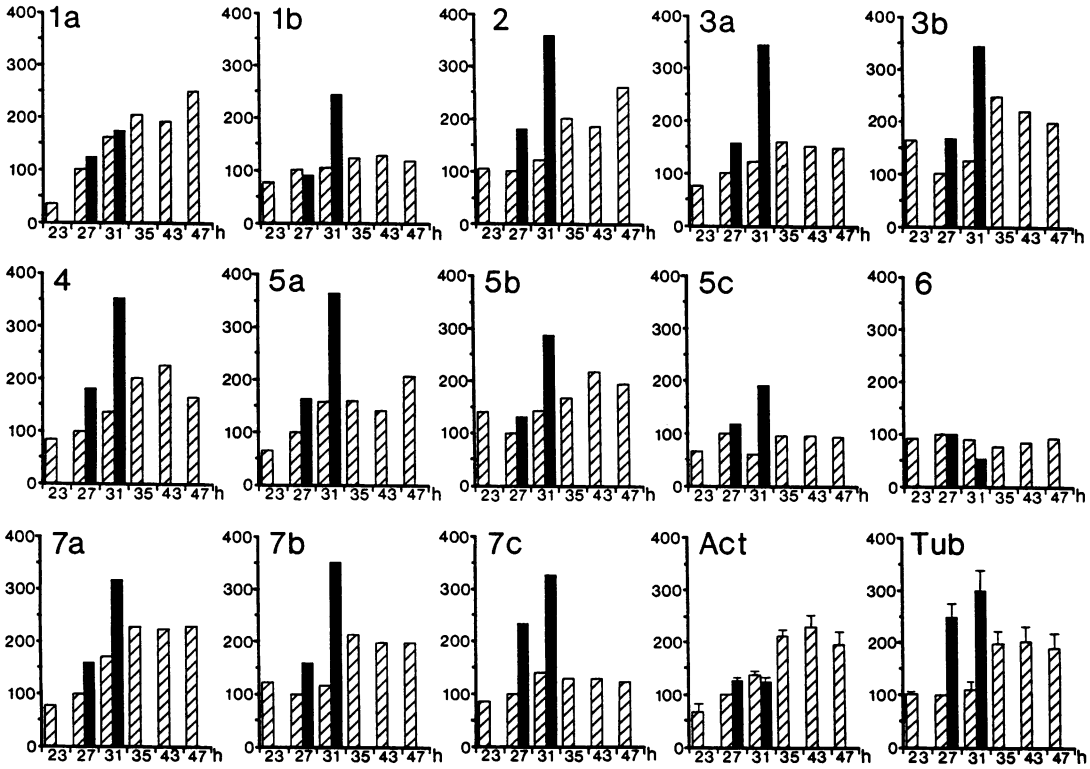

Figure 5. Quantitation of changes in abundance of DYH transcripts shown in Figure 4. Autoradiograms were scanned densitometrically. ▨, intensity of ^{32}P-cDNA hybridization to poly(A)+ RNA from undeciliated embryos at indicated times after fertilization; ■, hybridization to poly(A)+ RNA from reciliating embryos after one deciliation/reciliation (27 h) and after two deciliation/reciliations (31 h). In each panel, the abundance of hybridizing RNA is expressed as a percentage of that in the 27 h undeciliated embryos. For tubulin both hybridized bands were scanned as one (Cleveland and Sullivan, 1985). The data for actin and tubulin are the average of four and six blots, respectively, with the error bars indicating SD. Probing of a second set of RNA samples, from a three times deciliated preparation, confirmed these results, showing <10% change in the level of DYH1a transcript abundance in response to deciliation, and an increase of 55–168% for all the other genes except DYH4, which showed a 35% increase. The increase of tubulin transcript in this second preparation was 83%, and the actin transcript level showed a nominal decrease of 4%.

drolysis and mechanochemical energy transduction associated with the P1 loop. Possible roles include binding of regulatory cations or phosphate. Alternatively, these secondary P-loops may be vestigial structures in the axonemal dynein and serve a function only in cytoplasmic dynein where they are better conserved.

The similarity in overall size and in the sequence of the putative ATP-binding region does not in itself preclude the existence of substantial functional differences within the other domains of the dynein heavy chains. Although the number of amino acids used in this analysis provides a statistically valid sampling of dynein peptides, it is possible that by focussing on one region of the putative motor domain we may be underestimating the extent of dynein diversity. On the other hand, we have found that the full length of the β axonemal dynein from sea urchin, except for the N-terminal ~200 residues, can be aligned with 26% identity to the cytoplasmic dynein of *Dictyostelium* and that the region of homology comprises not only the mid- and C-terminal domains that are believed to contain the motor function of the protein but also includes at least three-quarters of the N-terminal domain of ~1000 residues that is believed to contain the structural, nonmotor functions (Gibbons *et al.*, 1991a; Mocz and Gibbons, 1993). The nearly identical size of the heavy chains in cytoplasmic and β axonemal dyneins, together with the relatively small size of their nonhomologous region, suggests that dynein genes have not undergone large-scale rearrangements during evolution. This contrasts with the extensive nonhomologous sequence and differences in domain size and position that are found among the different branches of the kinesin and myosin superfamilies of motor proteins (Goldstein, 1991; Endow and Titus, 1992; Cheney *et al.*, 1993; Goodson and Spudich, 1993).

The level of sequence divergence among the axonemal and cytoplasmic isoforms of sea urchin dynein is substantially higher than that among cytoplasmic dyneins from the most widely different eukaryotes. Thus, only 13% of the amino acids appear fully conserved in a 1200 residue alignment of six sea urchin isoforms

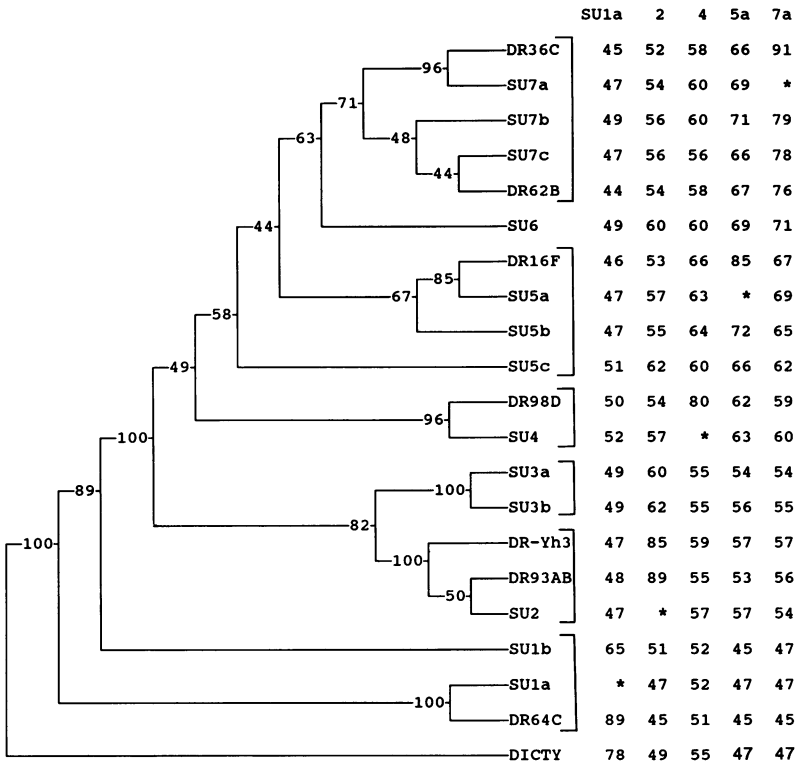

Figure 6. Phylogenetic tree of the predicted amino acid sequences in the ATP-binding region of dynein heavy chains in sea urchin, *Drosophila*, and *Dictyostelium*. The tree was obtained by maximum parsimony analysis (Felsenstein, 1988) of residues −18 to 105 of the sea urchin dyneins in Figure 1, together with the *Drosophila* dynein sequences in the preceding paper (Rasmusson *et al.*, 1994) and the corresponding sequence region of cytoplasmic dynein from *Dictyostelium* (Koonce *et al.*, 1992). The sea urchin dyneins are identified by SU prepended to the identifiers used in Figure 1, and the *Drosophila* dyneins by DR prepended to their cytological locations. The *Dictyostelium* dynein (Dicty) was arbitrarily taken as an outlier and used to root the tree. Numbers beside nodes show the percentage of boot-strapping trials in which an identical node was obtained and are thus a measure of the robustness of the data supporting that node. As an additional means of comparison, the percentage identity of each sequence to a representative member of five major classes of sea urchin dynein is shown at right.

(Figure 1), whereas 30% are fully conserved in an alignment of the same region of the cytoplasmic dyneins from sea urchin, *Dictyostelium* (Koonce *et al.*, 1992), rat (Mikami *et al.*, 1993), *Drosophila* (Li *et al.*, 1993), and yeast (Eshel *et al.*, 1993). The greater divergence among the different classes of axonemal dynein heavy chain may be partly because these classes originated very early in the evolution of eukaryotes, concurrent with the development of the 9 + 2 axonemal structure, and partly because there are fewer constraints upon the divergence of the axonemal dyneins than there are upon the cytoplasmic isoform. The importance of the latter factor is emphasized by the apparently more primeval character of cytoplasmic dynein, in which the sequences within the P2, P3, and P4 loops show a greater resemblance both to one another and to nucleotide-binding sites in other proteins than do the P-loops of the axonemal heavy chains. The sequences in the vicinity of the P1 and P3 loops of cytoplasmic dynein show a resemblance that possibly derives from their putative origin by gene replication (Eshel *et al.*, 1993), whereas no such resemblance has been detected among the axonemal heavy chains.

The number of 14 putative axonemal dynein genes identified in sea urchins exceeds the number of five to seven dynein isoforms resolved at the protein level in invertebrate sperm flagella by differences in their electrophoretic mobility, their locus of vanadate-mediated photocleavage (Gibbons and Gibbons, 1987; Stephens and Prior, 1992), or the nine distinct dynein heavy chains that have been identified in flagella of *Chlamydomonas* (Goodenough *et al.*, 1987; Kamiya *et al.*, 1989; Piperno *et al.*, 1990; Witman, 1992). Moreover, part of the observed protein diversity could reflect posttranslational modification or differential splicing rather than separate genes, although the low level of antigenic crossreactivity suggests that such changes are not a major factor (Piperno, 1984; King *et al.*, 1985; Ogawa *et al.*, 1990). Perhaps much of the seeming gene excess is accounted for by axonemal dyneins that occur in different types of embryo cilia with some of the almost identical sequences possibly corresponding to functionally equivalent polymorphisms. One gene, DYH3c, may encode a protein occurring in spermatids but not in embryonic cilia. Biochemical studies of cytoplasmic dyneins of the sea urchin egg have been complicated by the presence of an apparent axonemal precursor isoform (Foltz and Asai, 1988; Porter *et al.*, 1988; Pratt, 1989), but nonetheless suggest that the high molecular weight polypeptide of the HMr-3 fraction alone possesses the enzymatic properties characteristic of other cytoplasmic dyneins (Grissom *et al.*, 1992). This is compatible with our finding of a single cytoplasmic dynein gene, DYH1a.

Adequate interpretation of the sequence relatedness of the sea urchin dyneins requires that they be considered in relation to the sequences of dyneins of other species. Figure 6 presents a parsimony analysis that includes, in addition to our sea urchin sequences, the

available dynein sequences from *Drosophila* in the preceding paper (Rasmusson *et al.*, 1994) and from *Dictyostelium* (Koonce *et al.*, 1992). Each of the *Drosophila* dynein sequences falls clearly into one of the sea urchin dynein clusters that were identified in Figure 2. Moreover, the *Drosophila* sequence often resembles a particular sea urchin sequence in its cluster more closely than this sea urchin sequence resembles any of the other sea urchin sequences. Thus, the sea urchin DYH1a is 89% identical to the *Drosophila* Dhc64C and 78% identical to the cytoplasmic dynein of *Dictyostelium*, whereas it is only 65% identical to DYH1b, its closest relative among the other sea urchin dyneins. Similarly, the *Drosophila* Dhc98D, Dhc16F, and Dhc36C pair with DYH4, DYH5a, and DYH7a, respectively, whereas two others, Dhc93AB and DhcY-h3, closely resemble the sea urchin axonemal β chain DYH2. The robustness of these relationships is demonstrated by the fact that they occurred in as many as 85–100% of the parsimony trees obtained by bootstrap resampling. The occurrence in *Drosophila* of dynein heavy chains that closely resemble members of the DYH1a, 2, 4, 5, and 7 classes in sea urchin indicates that the duplication and divergence of these branches of the dynein gene family occurred at a stage of evolution substantially predating the divergence of insects and echinoderms.

In general, the dynein heavy chains that are most closely related in the sequence of their ATP-binding regions are likely to have similar functions in the cell. The close relationship of DYH1a to the cytoplasmic dynein of *Dictyostelium*, as well as to Dhc64C, that appears to be the principal cytoplasmic dynein of *Drosophila* (Rasmusson *et al.*, 1994), fits well with our data showing that the expression of DYH1a in sea urchin embryos is unaffected by multiple cycles of deciliation and reciliation. The fact that only a single gene encoding the major cytoplasmic dynein has been found in sea urchin, *Drosophila* (Rasmusson *et al.*, 1994), *Dictyostelium* (Koonce *et al.*, 1992), rat (Mikami *et al.*, 1993), and yeast (Eshel *et al.*, 1993) strongly suggests that the dimeric, double-headed, cytoplasmic dynein molecule observed by electron microscopy (Neely *et al.*, 1990) is a homodimer containing two identical heavy chains. Cytoplasmic and axonemal dyneins have conserved differences in sequence adjacent to the GPAGTGKT motif of the putative hydrolytic ATP-binding site that may account for their characteristic differences in nucleotide specificity and other enzymatic properties (Pallini *et al.*, 1983; Shpetner *et al.*, 1988; Koonce and McIntosh, 1990). For example, at position 12, as defined in Figure 1, the alanine in DYH1a, DYH1b, *Dictyostelium*, *Drosophila*, and rat is replaced by aspartate in all the axonemal sequences. At position 28 the aspartate in the cytoplasmic sequences is replaced by serine or glycine, and at positions 59–60 the glutamate-arginine in DYH1a, *Dictyostelium*, and rat are replaced by (hydrophobic)-(neutral or acidic) residues. Other conserved differences occur further removed from the P-loop motif.

The effect of deciliation on transcript abundance in sea urchin embryos suggests that at least 11 of the other dynein genes encode heavy chain isoforms that are either contained within the structure of axonemes or are otherwise involved in the process of ciliary regeneration. This interpretation is supported by the boost in the transcript level of DYH2, which encodes the β heavy chain of the axonemal dynein in embryo cilia and sperm flagella (Gibbons *et al.*, 1991a; Ogawa, 1991). Although the involvement of these isoforms with ciliary regeneration does not preclude their having additional, more general functions in the cytoplasm, the apparent involvement of such a high percentage of family members with a single organelle marks a notable difference from the families of kinesin and of myosin.

Although dynein heavy chains with similar sequences are likely to have similar functions, this is not necessarily the case. An example is DYH1b, whose predicted sequence most closely resembles that of the principal cytoplasmic dynein DYH1a, but whose expression becomes substantially enhanced after deciliation. One possibility is that DYH1b encodes a different cytoplasmic dynein that plays a role in the transport of materials required for ciliary regeneration, perhaps being related to the dynein associated with ciliary membranes in *Tetrahymena* and *Aequipecten* (Dentler *et al.*, 1980). Alternatively, this gene may encode a true axonemal dynein whose sequence resembles that of the cytoplasmic dyneins for reasons that remain obscure.

The extent to which sequence relatedness reflects functional specialization is a major question, not only for the dynein family, but also for the kinesins and myosins. Although there are indications of significant redundancy, it is clear that the integrity of many branches of these families has been conserved through considerable periods of evolution. The identification and classification of motor isoforms by sequence analysis and by patterns of expression will provide an essential framework within which to address functional questions by genetic and cell biological techniques.

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