Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7928–7932, September 1993
Cell Biology

The primary structure of rat brain (cytoplasmic) dynein heavy chain, a cytoplasmic motor enzyme

(neuronal cytoskeleton/axonal transport/cell motility)

ZHIZENG ZHANG*, YOSUKE TANAKA*, SHIGENORI NONAKA*, HIROYUKI AIZAWA*, HIROSHI KAWASAKI†, TAKAO NAKATA*, AND NOBUTAKA HIROKAWA*‡

*Department of Anatomy and Cell Biology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113, Japan; and †Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Hon-komagome, Bunkyo-ku, Tokyo, 113, Japan

Communicated by Keith Porter, May 17, 1993

ABSTRACT **Overlapping cDNA clones encoding the heavy chain of rat brain cytoplasmic dynein have been isolated. The isolated cDNA clones contain an open reading frame of 13,932 bp encoding 4644 aa (M_r, 532,213). The deduced protein sequence of the heavy chain of rat brain dynein shows significant similarity to sea urchin flagellar β-dynein (27.0% identical) and to *Dictyostelium* cytoplasmic dynein (53.5% identical) throughout the entire sequence. The heavy chain of rat brain (cytoplasmic) dynein contains four putative nucleotide-binding consensus sequences [$GX_4GK(T/S)$] in the central one-third region that are highly similar to those of sea urchin and *Dictyostelium* dyneins. The N-terminal one-third of the heavy chain of rat brain (cytoplasmic) dynein shows high similarity (43.8% identical) to that of *Dictyostelium* cytoplasmic dynein but poor similarity (19.4% identical) to that of sea urchin flagellar dynein. These results suggested that the C-terminal two-thirds of the dynein molecule is conserved and plays an essential role in microtubule-dependent motility activity, whereas the N-terminal regions are different between cytoplasmic and flagellar dyneins.**

The nerve cell develops a polarized morphology composed of highly branched dendrites, a long axon, and synapses. Because of the lack of protein synthesis machinery in the axon, proteins in the axon and synapses must be transported down the axon after being synthesized in the cell body. Many proteins are transported bidirectionally in membranous organelles of various kinds by fast axonal flow (200–400 mm/day), whereas other proteins such as cytoskeletal components are conveyed by slow axonal flow (0.5–2 mm/day). Electron microscopic studies of the neuronal cytoskeleton *in vivo* have suggested that microtubules and crossbridges between microtubules and membranous organelles form the structural basis for fast flow (1–3). Recently, two microtubule-activated ATPases, kinesin and brain dynein [cytoplasmic dynein, microtubule-associated protein (MAP) 1C], were identified as candidates for anterograde and retrograde molecular motors, respectively (4–7). In fact, kinesin is primarily associated with anterogradely moving membranous organelles in the axon, strongly supporting the hypothesis that kinesin is really an anterograde motor *in vivo* (8). Brain dynein is associated with both anterogradely and retrogradely moving membranous organelles (9), consistent with the suggestion that brain dynein is transported to the nerve terminal by fast flow and subsequently functions as a retrograde transport motor *in vivo*. Molecular genetic and ultrastructural approaches have dissected the molecular structure and functional domains of kinesin motors (10–12). However, detailed analysis of the primary structure of brain dynein has not yet been accomplished. To further elucidate the function of brain dynein and to clarify how brain dynein performs the transport of organelles *in vivo*, molecular biological studies will provide essential information. Thus as a first step toward these goals, we decided to clone and sequence overlapping cDNAs that encode the entire heavy chain of rat brain dynein.§

MATERIALS AND METHODS

Determination of Partial Amino Acid Sequences of Rat Cytoplasmic Dynein Heavy Chain. Cytoplasmic dynein was prepared from livers of 6-week-old rats by the method of Collins and Vallee (13). After electrophoresis using a 6% polyacrylamide gel, a piece of the gel containing dynein heavy chain was excised and homogenized in digestion solution [100 mM Tris, pH 9.0/0.1% SDS/lysylendopeptidase (3 μg/ml)]. After incubation for 8 hr at 37°C, the digestion solution containing peptides of dynein heavy chain was collected and the peptides were separated on a Vydac C_{18} reverse-phase column with DEAE-5PW column (Tosoh, Tokyo) as a precolumn. The separated peptides were sequenced with a gas-phase amino acid sequencer (model 470A protein sequencer, Applied Biosystems) as described (14). The four amino acid sequences of cytoplasmic dynein heavy chain fragments obtained were EGTEAXEAAMK (sequence 1), XEVETHK (sequence 2), XXELEEQQMHLNVGLRK (sequence 3), and RVEPLRNELQK (sequence 4) (see Fig. 2).

Construction of cDNA Libraries. Total RNA was extracted from 5-day-old rat brains by the guanidinium isothiocyanate/cesium chloride method (15). Poly(A)$^+$ RNA was purified with oligo(dT)-cellulose column chromatography. Four cDNA libraries were constructed from poly(A)$^+$ RNA by using a You-Prime cDNA synthesis kit (Pharmacia) with $(dT)_{12-18}$ primer, primer B [5′-A(G/A)NCCNAC(A/G)TTNA(A/G)(A/G)TG-3′; degenerate complementary sequence corresponding to the amino acid sequence HLNVGL of sequence 3], primer C (5′-GTCATAATGGCTTTG-3′; complementary sequence corresponding to aa 2080–2085 in Fig. 2), and primer D (5′-CGTATGTGTTCCACA-3′; complementary sequence corresponding to aa 1461–1466 in Fig. 2). cDNAs were inserted into phage vector λgt10 (Stratagene) by T4 DNA ligase (Toyobo, Osaka) and then packed into bacteriophage particles using the packaging extract Gigapack II Gold (Stratagene) and grown on *Escherichia coli* C600Hfl.

PCR. PCR amplification was performed in 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.001% gelatin/200 μM dATP/200 μM dCTP/200 μM dGTP/200 μM dTTP/2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus)/

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: MAP, microtubule-associated protein.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D13896).

each PCR primer at 2 μM according to the following schedule: 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min with a 3-sec extension per cycle for 30 cycles. The PCR primers used were a sense oligonucleotide primer A [5′-GA(T/C)AA(T/C)GA(G/A)(T/C)TNGA(G/A)GA-3′, corresponding to the N-terminal sequence of sequence 3] and an antisense oligonucleotide primer B [5′-A(G/A)(G/A/T/C)CCNAC(A/G)TTNA(A/G)(A/G)TG-3′, corresponding to the C-terminal sequence of sequence 3]. An aliquot of the PCR solution using rat brain cDNA as a template was analyzed by electrophoresis with a 6% polyacrylamide gel. The amplified DNAs of 45 bp were eluted from the gel, treated with the Klenow fragment of DNA polymerase I (Toyobo), and then phosphorylated with T4 polynucleotide kinase (Toyobo). The products were then cloned into the *Hin*cII site of pUC18 (Takara Shuzo, Kyoto), which had been dephosphorylated by using bacterial alkaline phosphatase (Takara Shuzo) after digestion with *Hin*cII. The nucleotide sequence of the insert was completely consistent with the amino acid sequence of sequence 3. This insert was used as a starting probe for screening the rat brain cDNA libraries.

Isolation of cDNA Clones. cDNA clones were isolated from the above-mentioned rat brain libraries. ^{32}P-labeled probes were generated with a Multiprime DNA labeling system (United States Biochemical). The isolated recombinant phage DNA inserts were subcloned into plasmid vector pBluescript SK(+). Both strands of the cDNA inserts were sequenced by using the dideoxynucleotide chain-termination method (16).

Southern and Northern Blot Analyses. Rat genomic DNA was extracted from rat liver as described by Maniatis *et al.* (15). The Southern blot was probed with ^{32}P-labeled DNA in a hybridization solution containing 50% (vol/vol) formamide, 6× standard saline citrate (SSC), 5× Denhardt's solution, 0.1% SDS, and carrier DNA (100 μg/ml) at 42°C for 12 hr and then washed several times in 0.1× SSC/0.1% SDS at 65°C. Total RNA and poly(A)$^+$ RNA were prepared from rat tissues as described above. RNA was quantified by measuring the A_{260}, and the integrity of RNA was checked by staining after agarose gel electrophoresis. The RNA samples were denatured and electrophoresed in agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a nylon filter (Hybond-N$^+$; Amersham). Probes were labeled with a Multiprime DNA labeling system. Hybridization was carried out at 65°C for 8 hr in a rapid hybridization buffer (Amersham). The filters were washed in 0.1 × SSC/0.1% SDS at 65°C for 30 min and exposed to x-ray film (Fuji). A β-actin probe from the 3′ noncoding region (800 bp) was used to monitor the total amount of RNA in each lane.

RESULTS AND DISCUSSION

Amino Acid Sequence Analysis of Rat Cytoplasmic Dynein Heavy Chain. We tried to determine the N-terminal amino acid sequence of dynein heavy chain, but no amino acid was released by the Edman degradation reaction, suggesting that the N terminus was blocked. Several peptides, which were produced by lysyl-endopeptidase treatment of rat brain dynein heavy chain, were purified by reverse-phase column chromatography (Vydac C_{18}) and sequenced: sequence 1, EGTEAXEAAMK; sequence 2, XEVETHK; sequence 3, XXELEEQQMHLNVGLRK; sequence 4, RVEPLRNELTK.

Isolation and Identification of cDNA Clones for Rat Brain (Cytoplasmic) Dynein Heavy Chain. To amplify a cDNA fragment of brain dynein heavy chain, we synthesized primer A and primer B corresponding to the N and C termini of sequence 3. A PCR using rat brain cDNA as a template amplified a single 45-bp cDNA fragment. This fragment was sequenced and found to encode sequence 3 (Fig. 1*A*). Using this PCR fragment of dynein cDNA as a probe, we screened

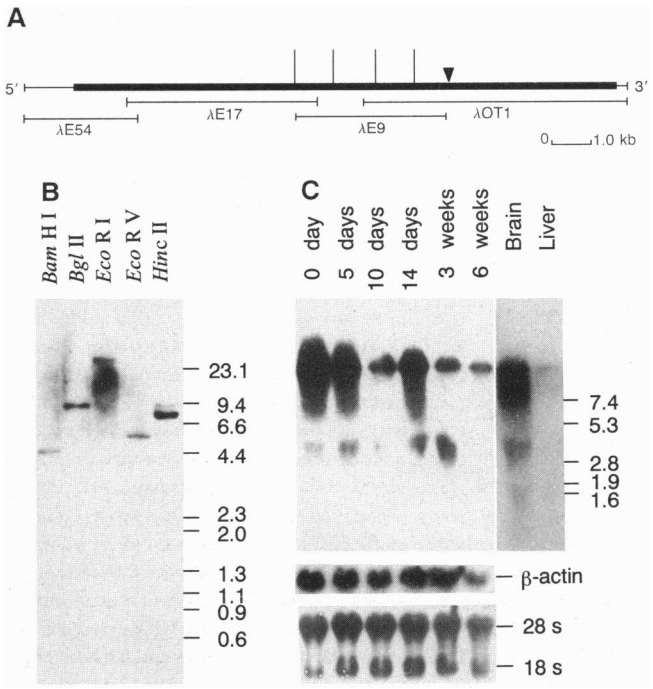

FIG. 1. Overlapping cDNA clones for the heavy chain of rat brain dynein and Southern and Northern blot analyses. (*A*) An open reading frame (thick line) flanked by 5′ and 3′ noncoding sequences (thin lines) is shown at the top. Regions included in cDNA clones are shown below. The position of a PCR fragment (GAGTTGGAGGAGCAGCAGATGCACCTGAACGTTGGGCTCCGAAAA) corresponding to amino acid sequence 3 (XXELEEQQMHLNVGLRK) is indicated by an arrowhead. Positions of four putative ATP-binding sites are indicated by vertical lines. (*B*) Southern blot analysis of rat genomic DNA. The restriction enzyme used is shown above each lane. An ≈300-bp fragment from λOT1 insert was used as the probe. (*C*) Northern blot analysis for the heavy chain of rat brain dynein. Total RNA (20 μg) from rats at different ages (0 day, 5 days, 10 days, 14 days, 3 weeks, and 6 weeks) was loaded and probed with a λE54 clone. Bands of β-actin and rRNAs stained with methylene blue are shown below each lane. The lanes marked Brain and Liver are from total RNA of 6-week-old rat. Markers are in kb.

3 × 10^5 independent cDNA clones of a primer-B-directed λgt10 library, and clone λE9 was obtained. Since this clone still lacked the 5′ part of the coding sequence, the primer-C-directed cDNA library was screened using the λE9 insert as a probe, and clone λE17 was obtained (Fig. 1*A*). This clone also lacked the 5′ part of the coding sequence, and therefore, the primer-D-directed cDNA library was screened using the λE17 insert as a probe, and clone λE54 was obtained. Since these clones still lacked the 3′ part of the coding sequence, we screened an oligo(dT)-primed cDNA library by using λE9 insert as a probe and obtained clone λOT1. λOT1 contains two in-frame stop codons, a putative polyadenylylation signal, and a long poly(A)$^+$ tail. Northern blot analysis of rat brain RNA using each insert of the four clones revealed a single transcribed product of 16 kbp, suggesting that these clones are from a single transcript of the heavy chain of rat brain dynein (data not shown). Nucleotide sequence analysis revealed the presence of a single open reading frame of 13,932 nt that encodes 4644 aa (M_r, 532,213) (Fig. 2). The initial methionine codon of this open reading frame followed two in-frame termination codons in the 5′ noncoding region of 1340 bp (data not shown). The deduced amino acid sequence contained all of the above-mentioned peptide sequences (sequences 1 to 4) of rat cytoplasmic dynein heavy chain (Fig. 2). So we concluded that we had cloned the complete cDNA of the heavy chain of rat brain dynein.

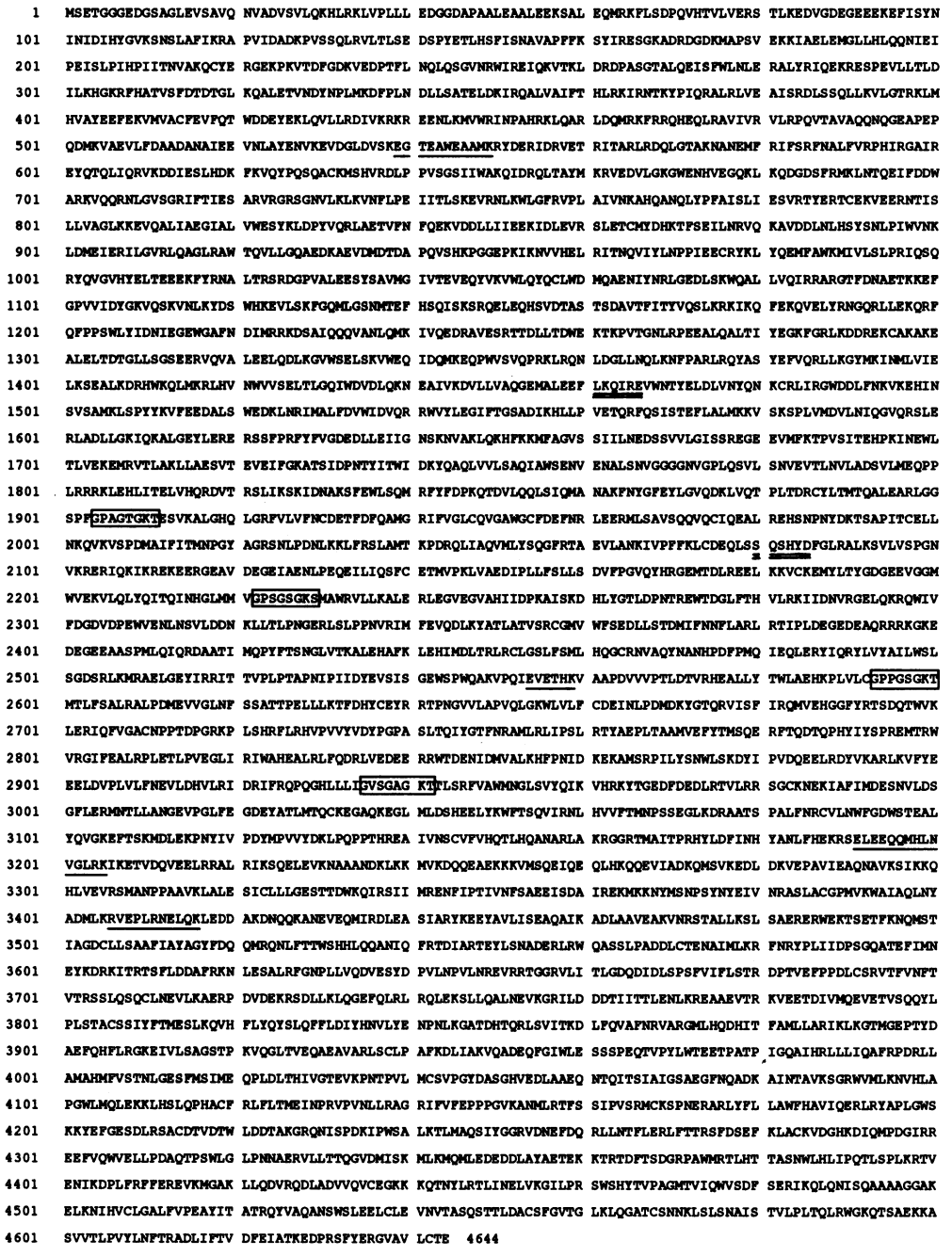

FIG. 2. Deduced amino acid sequence of rat brain dynein heavy chain. Four P-loop consensus sequences of ATP-binding domain are boxed. The four peptide sequences of dynein heavy chain obtained are underlined. The amino acid sequences of primer C and primer D are double underlined.

Southern and Northern Blot Analyses. Genomic Southern blots probed with a fragment (≈300 bp) from clone λOT1 suggest that the rat genome contains a single copy of the brain dynein gene (Fig. 1*B*). Northern blot analysis revealed that the transcript of rat brain dynein heavy chain was ≈16 kbp (Fig. 1*C*). The heavy chain transcript was expressed in brain and liver, but the expression in liver was much lower than in brain. We also studied the expression levels of dynein heavy chain transcript in brain during postnatal development (Fig. 1*C*). There were high levels of expression until postnatal day 5 that decreased by a factor of 10 on postnatal day 10. This level was maintained at 3 weeks and 6 weeks, but there was a transient increase at postnatal day 14. This increment of brain dynein transcript is consistent with the maturation of axons and dendrites, especially the transition of the expression of MAPs (early MAPs and late MAPs) (17). In rat brains, synaptogenesis begins at postnatal day 14 (18). Thus these data may imply some relationships with the functions of brain cytoplasmic dynein.

Characteristics of the Amino Acid Sequence of Rat Brain Dynein Heavy Chain. Rat brain dynein heavy chain contains four P-loop consensus sequences of the ATP-binding domain [$GX_4GK(T/S)$] in the central part of the polypeptide, similar to the heavy chains of flagellar β-dynein and *Dictyostelium* cytoplasmic dynein. In contrast to flagellar dynein (19, 20), and like *Dictyostelium* cytoplasmic dynein (21), rat brain dynein does not contain the fifth consensus site near its N terminus. This may reflect some functional differences between flagellar and cytoplasmic dyneins.

Diagonal dot matrix comparisons of sequence similarity demonstrated that rat brain cytoplasmic dynein is similar to

both *Dictyostelium* and sea urchin dyneins in its primary structure (Fig. 3). However, the GCG program BESTFIT with a gap weight of 3.0 and a length weight of 0.1 demonstrated that the two cytoplasmic dynein sequences are 54% identical over their entire length, whereas sea urchin flagellar dynein showed the relatively lower identity of 28% to *Dictyostelium* and 27% to rat brain dynein, respectively. Sea urchin and rat belong to the Animalia and are evolutionarily much more closely related to one another than to *Dictyostelium*, which belongs to Protoctista. So, higher similarity of the primary structures between rat and *Dictyostelium* dynein heavy chains suggests that they are not counterparts of sea urchin flagellar dynein and that cytoplasmic dyneins are functionally distinct from flagellar dyneins.

The N-terminal one-third (aa 1–1500) of rat brain dynein heavy chain showed high similarity (44% identical) to that of *Dictyostelium* cytoplasmic dynein but low similarity (19% identical) to that of sea urchin flagellar β-dynein (Fig. 3). This suggests that the N-terminal region may play an important role in the specific interaction of cytoplasmic or axonemal dynein with other intracellular components such as vesicles, kinetochores, or microtubules by ATP-insensitive manner, and so on.

The central region (aa 1500–3000) of rat brain dynein showed high similarity to the central regions of *Dictyostelium* and sea urchin dyneins. This region contains four putative ATP-binding motifs—i.e., P-loop consensus sequences of ATP-binding domain. Fig. 4*A* shows direct comparisons of the amino acid sequences around the four P-loop consensus sequences. Among the four putative motifs, the first consensus sequence of GPAGTGKT is completely conserved in the three dynein molecules, and the similarity at the surrounding region of this motif is highest compared with the other three. This suggests that the region of the first P-loop consensus sequence is the strongest candidate as a hydrolytic ATP-binding site of dynein molecules. The structural similarity among the ATP-binding regions of the three dyneins, including surrounding regions of ATP-binding motifs, also indicates that these regions may be derived from a common ancestor.

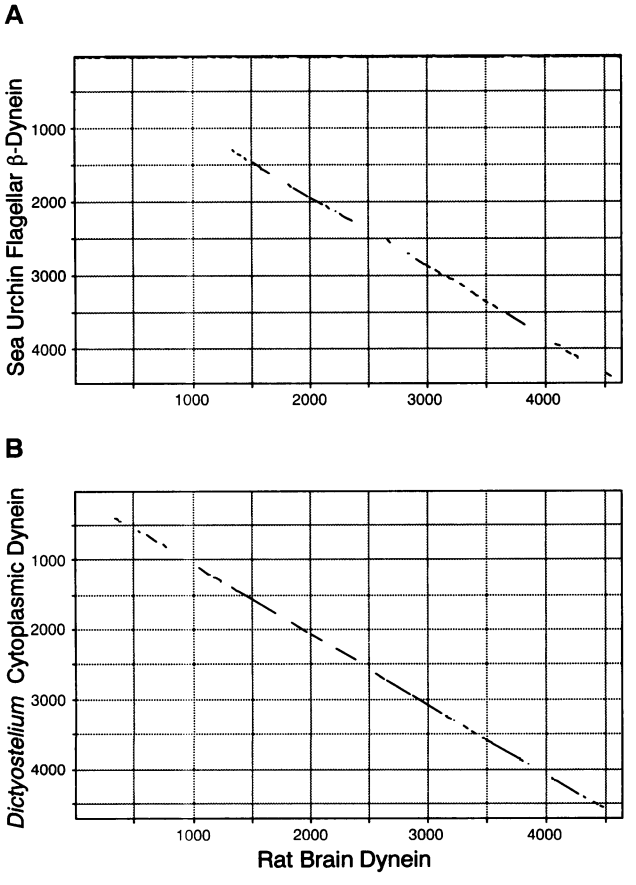

FIG. 3. Diagonal dot matrix comparisons. (*A*) Rat brain dynein heavy chain and sea urchin flagellar dynein (19, 20). (*B*) Rat brain dynein heavy chain and *Dictyostelium* cytoplasmic dynein (18). Comparison was performed on MacVector DNA analysis software using a window size of 50 and stringency of 30% for flagellar dynein and 60% for *Dictyostelium* cytoplasmic dynein.

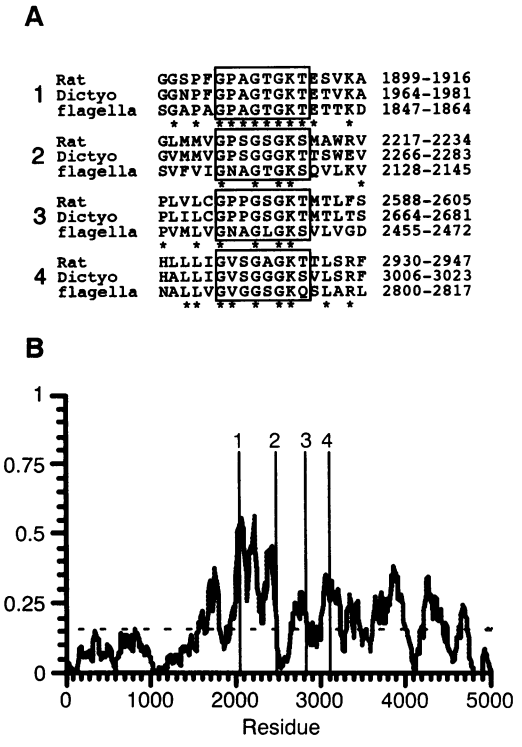

FIG. 4. (*A*) Comparison of four regions in and around the putative P-loop consensus sequences [$GX_4GK(T/S)$]. The amino acid sequences of three dynein heavy chains were aligned. (*B*) Similarity plot among rat, *Dictyostelium* (21), and sea urchin (19, 20) dynein heavy chains. The three dyneins were aligned and numbered by CLUSTALV (22). The identity index of each residue was scored as one when the residue was conserved in all three sequences and as zero otherwise. The moving average of 90 residues with Gaussian weight was plotted. Dotted line shows the mean value, and vertical lines 1–4 indicate the positions of P-loop consensus sequences. Axes: *x*, residue number with calculation of gaps; *y*, percentage of similarity.

Since the amino acid sequence of rat brain dynein heavy chain is very large, it is difficult to predict its secondary structure. Nonetheless, the Chou–Fasman method (23) predicted two large α-helices in the rat dynein sequence at aa 3171–3285 and aa 3409–3494. The first α-helical region does not show obvious periodicities of positively and negatively charged residues, except for a strong peak of hydrophobic residues at position d of 7-aa repeats (Fig. 5*B*). The second α-helical region showed a heptapeptide repeat pattern with an enrichment of hydrophobic amino acid residues at positions a and d and the periodicities of positively and negatively charged amino acids (Fig. 5*A*). This characterizes a coiled-coil structural conformation at the second α-helical region (24). *Dictyostelium* dynein was reported to form an α-helical coiled-coil conformation at the C-terminal region (21), and we also found that sea urchin flagellar β-dynein has a region forming α-helical coiled-coil structural conformation at the C-terminal region. Coiled-coil structure is a common motif in the formation of protein complexes. Thus, this may provide a structural basis to the previous observations that dynein

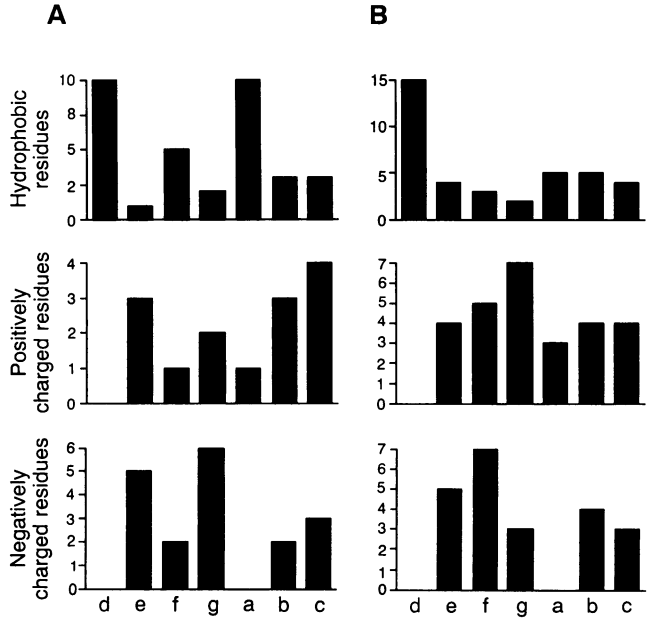

FIG. 5. Periodicities of hydrophobic, positively charged, and negatively charged amino acids in the two predicted α-helical regions of C-terminal region (24). (*A*) Region from aa 3408 to 3494. This region showed structural characteristics of an α-helical coiled-coil conformation. (*B*) Region between Pro-3171 and Pro-3285. This region showed little characteristics of a coiled-coil conformation. Bars d, e, f, g, a, b, and c are the position of each residue in the heptapeptide repeats.

molecules consist of multiple polypeptides including two heavy chains and several intermediate and light chains (25).

The C-terminal two-thirds of the flagellar and two cytoplasmic dyneins is more similar than their N-terminal regions (Fig. 4*B*), suggesting that this region contains essential structural and mechanochemical domains of dynein.

We thank Dr. M. Koonce and Dr. J. R. McIntosh for informing us of the sequence of *Dictyostelium* cytoplasmic dynein in preprint. We thank Dr. R. Liem for various important suggestions and discussions. We also want to show special thanks to Drs. Y. Okada and R. Sato-Yoshitake for various kind suggestions and technical advice. Finally, Z.Z. thanks the Watanuki International Scholarship Foundation for its financial aids. This work was supported by a Special Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and a grant from RIKEN to N.H.

1. Hirokawa, N. (1982) *J. Cell Biol.* **94,** 129–142.
2. Miller, R. H. & Lasek, R. J. (1985) *J. Cell Biol.* **101,** 2181–2193.
3. Hirokawa, N. & Yorifuji, H. (1986) *Cell Motil. Cytoskel.* **6,** 458–468.
4. Brady, S. T. (1985) *Nature (London)* **317,** 73–75.
5. Vale, R. D., Schnapp, B. J., Mitchison, T., Steuer, E., Reese, T. S. & Sheetz, M. P. (1985) *Cell* **43,** 623–632.
6. Paschal, B. M. & Vallee, R. B. (1987) *Nature (London)* **330,** 181–183.
7. Lye, R. J., Porter, M. E., Scholey, J. M. & McIntosh, J. R. (1987) *Cell* **51,** 309–318.
8. Hirokawa, N., Sato-Yoshitake, R., Kobayashi, N., Pfister, K. K., Bloom, G. H. & Brady, S. T. (1991) *J. Cell Biol.* **114,** 295–302.
9. Hirokawa, N., Yoshida, T., Sato-Yoshitake, R. & Kawashima, T. (1990) *J. Cell Biol.* **111,** 1027–1037.
10. Hirokawa, N., Pfister, K. K., Yorifuji, H., Wagner, M. C., Brady, S. T. & Bloom, G. S. (1989) *Cell* **56,** 867–878.
11. Yang, J. T., Laymon, R. A. & Goldstein, L. S. B. (1989) *Cell* **56,** 879–889.
12. Scholey, J. M., Heuser, J., Yang, J. T. & Goldstein, L. S. B. (1989) *Nature (London)* **338,** 355–357.
13. Collins, C. A. & Vallee, R. B. (1989) *Cell Motil. Cytoskel.* **14,** 491–500.
14. Tarr, G. E. (1986) in *Methods of Protein Microcharacterization: A Practical Handbook,* ed. Shively, J. E. (Humana, Clifton, NJ), pp. 155–194.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Matus, A. (1988) *Annu. Rev. Neurosci.* **11,** 29–44.
18. Aghajanian, G. K. & Bloom, F. E. (1967) *Brain Res.* **6,** 716–727.
19. Gibbons, I. R., Gibbons, B. H., Mooz, G. & Asai, D. J. (1991) *Nature (London)* **352,** 640–643.
20. Ogawa, K. (1991) *Nature (London)* **352,** 643–645.
21. Koonce, M. P., Grissom, P. M. & McIntosh, J. R. (1992) *J. Cell Biol.* **119,** 1597–1604.
22. Higgins, D. G. & Sharp, P. M. (1989) *Comput. Appl. Biosci.* **5,** 151–153.
23. Chou, P. Y. & Fasman, G. D. (1974) *Biochemistry* **13,** 222–245.
24. McLachlan, A. D. & Karn, J. (1983) *J. Mol. Biol.* **164,** 605–626.
25. Vallee, R. B. & Shpetner, H. S. (1990) *Annu. Rev. Biochem.* **59,** 909–923.