Abstract. The unidirectional movements of the microtubule-associated motors, dyneins, and kinesins, provide an important mechanism for the positioning of cellular organelles and molecules. An intriguing possibility is that this mechanism may underlie the directed transport and asymmetric positioning of morphogens that influence the development of multicellular embryos. In this report, we characterize the *Drosophila* gene, *Dhc64C*, that encodes a cytoplasmic dynein heavy chain polypeptide. The primary structure of the *Drosophila* cytoplasmic dynein heavy chain polypeptide has been determined by the isolation and sequence analysis of overlapping cDNA clones. *Drosophila* cytoplasmic dynein is highly similar in sequence and structure to cytoplasmic dynein isoforms reported for other organisms. The *Dhc64C* dynein transcript is differentially expressed during development with the highest levels being detected in the ovaries of adult females. Within the developing egg chambers of the ovary, the dynein gene is predominantly transcribed in the nurse cell complex. In contrast, the encoded dynein motor protein displays a striking accumulation in the single cell that will develop as the oocyte. The temporal and spatial pattern of dynein accumulation in the oocyte is remarkably similar to that of several maternal effect gene products that are essential for oocyte differentiation and axis specification. This distribution and its disruption by specific maternal effect mutations lends support to recent models suggesting that microtubule motors participate in the transport of these morphogens from the nurse cell cytoplasm to the oocyte.
cytoplasmic motors that associate with microtubules are likely candidates to participate in the transport and localization of cytoplasmic components to and within the oocyte. In this paper, we report the molecular cloning of the complete 14.3-kb transcript encoding the cytoplasmic dynein heavy chain in Drosophila. The dynein transcript is expressed at high levels throughout development and is enriched in ovaries. In the developing egg chamber, the dynein gene is expressed in nurse cells, while the encoded dynein heavy chain is differentially localized to the presumptive oocyte. This localization initiates at an early stage in the differentiation of the pro-oocyte and coincides with the establishment of a polar array of microtubules emanating from the presumptive oocyte. Dynein localization requires the function of genes in both the germline and somatic follicle cells. Our results suggest a model in which the minus-end dynein motor "walks" into the pro-oocyte along a polarized track of microtubules. We discuss the implications of this localization in terms of a dynein-based mechanism for the transport of informational molecules to the differentiating oocyte.

Materials and Methods

Isolation of cDNA and Genomic Clones

A λ ZAP cDNA expression library (Stratagene, La Jolla, CA), constructed by random priming from Drosophila 2-14 h embryo mRNA, was screened with a rabbit polyclonal antibody raised against the Drosophila heavy chain polypeptide (Hays et al., 1994). The antibody was affinity-purified against each of two dynein polypeptide fragments produced from the cDNA sequence in both directions by hybridization screens of the genomic and genomic clones. Two genomic DNA libraries, a SEMBL3A and a cosmid library, were confirmed by restriction analysis and sequence comparison using cDNA probes and subsequently purified. Genomic clones. The DNA was digested with RNaseH, and the first strand cDNAs were sized to the 3' end with dCTP using terminal deoxynucleotidyl transferase. First strand cDNAs were amplified sequentially, using a 5' RACE anchor primer and nested antisense primers from within clone ZL2 (primer 1, nucleotides 1598 to 1617; primer 2, nucleotides 1476 to 1494). The PCR cDNA RACE products were subsequently amplified by the PCR with primers designed from the sequence of cDNA and genomic subclones. The PCR products were cloned into pBluescript II, resulting in the subclones pTR13 and pLR152 (see Fig. 1). The primers used to recover the subclone pTR13 were derived from known cDNA sequence. The 5' sense and the 3' antisense primers corresponded to nucleotides 539 to 558 and 1396 to 1416, respectively. The sense primer for pLR152 was designed from genomic sequence corresponding to nucleotides 88 to 107, while the antisense primer was based on known cDNA sequence, nucleotides 565 to 584. The primers were selected in such a way that the genomic DNA regions amplified by these primers harbor introns, and, therefore, the PCR products could be easily confirmed to be derived from mRNA and not from contaminating genomic DNA.

The PCR reaction conditions for pTR13 and pLR152 were as follows: 5 μL of PCR reaction product was amplified in a 50-μl reaction containing 50 mM KC1, 10 mM Tris-HCl pH 9.0, 0.18% Triton X-100, 1.5 mM MgCl2, 0.2 mM dNTP, 0.2 μM of each primer, and 0.3 U of Taq polymerase. cDNA was denatured for 2 min at 95°C; and then the reaction was run for 30 cycles of 50°C for 2 min, 72°C for 3 min, and 94°C for 1 min. The extension was completed at 72°C for 10 min.

DNA Sequencing and Analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase 2.0 polymerase (USB, Cleveland, OH) and plasmid vector primers. Both strands of the complete cDNA contained within overlapping clones, as well as selected genomic subclones, were sequenced using nested serial deletions generated by exonuclease III and S1 nuclease according to the method of Henikoff (1987). The template was a double-stranded plasmid prepared as described by Medberry et al. (1990). Sequencing reactions were electrophoresed on 6% denaturing polyacrylamide gels. The sequence data was assembled and analyzed using Intelligenetics and UWCGC software on a Sun Microsystems 2/120 computer, and the MacVector Sequence Analysis Software package (International Biotechnologies, Inc., New Haven, CT). Predictions of coiled-coil domains were based on the statistical significance of the preferential distribution of different amino acids in each position of the heptad repeat (Lupas et al., 1991). A window size of 28 residues was used and scores greater than 1.3 were taken to indicate a high probability of coiled-coil conformation (Lupas et al., 1991).

 Primer Extension and RNase Protection

Primer extension was performed as described by Ausubel et al. (1987). Fifteen micrograms of total embryonic RNA were annealed to 5 ng of 32P-end-labeled primer and reverse transcribed by M-MLV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). The antisense oligonucleotide primer was a 20mer, 5'-GGTTGGAATGACACGGTTCA-3' (nucleotides 209 to 228 in Fig. 3). RNase protection was performed as described by Melton et al. (1984). The template was a genomic DNA subclone extending from nucleotides -923 to +330. The plasmid was linearized at a unique HindIII site downstream from the T3 phage promoter. 32P-labeled antisense RNA was synthesized with T3 RNA polymerase in vitro (Stratagene). Fifteen micrograms of total embryonic RNA were annealed overnight at 45°C with labeled antisense RNA in the presence of 80% formamide, and then digested with a mixture of RNaseA and RNaseT1. The size of the products of both primer extension and RNase protection were determined on 6% acrylamide sequencing gels using genomic DNA as a size marker.

In Situ Hybridization

Digoxigenin-labeled probes were hybridized to whole-mount preparations.
of ovaries according to the procedure described by Tautz and Pfeifle (1989) with minor modifications. Ovaries were dissected in EBR saline (130 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM Hepes pH 6.9) and fixed for 20-25 min in 200-µl PP (4% paraformaldehyde in PBS), 20-µl DMSO, 600-µl heptane, and then washed several times in PBT (PBS/0.1% Tween-20). Proteinase K treatment (30 min at 50 mg/ml in PBT) was stopped by incubation of 2 ml/glycine and remainder washes in PBT. Ovaries were refixed in PP and then washed in PBT for 30 min with four changes. They were incubated 15 min in a 1:1 mixture of PBT and hybridization solution (HS), 10 min at room temperature in HS, then 1-2 h at 48°C in HS. Hybridization was for 20-30 h at 48°C using 0.5 mg/ml labeled cDNA probe in HS containing 10% dextran sulfate. Posthybridization washes were extended to 45-55 h at 48°C. Ovaries were moved stepwise into PBT at room temperature, then washed 5-10 min in room temperature PBT. They were incubated for 90 min with alkaline phosphatase-conjugated anti-digoxigenin antibody (previously preadsorbed against fixed ovaries) diluted 1:3,000 in PBT. After several brief rinses in PBT, the ovaries were washed overnight in PBT at 4°C. They were washed three times in alkaline phosphatase buffer at room temperature before staining with nitro blue tetrazolium (NBT) plus 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Color development was stopped with PBT washes, and ovaries were stored at 4°C in PBT until mounting in Immumount (Shandon, Pittsburgh, PA).

Probes were prepared using a Boehringer Mannheim "Genius" kit according to the supplier's instructions (Boehringer Mannheim Corp., Indianapolis, IN), except that final primer concentrations in the labeling reaction were increased to 1 mg/ml. okar CDNA was kindly provided by Ruth Lehmann (Ephrussi et al., 1991). In situ hybridization to larval polytene chromosome squashes was carried out as previously described (Rasmussen et al., 1994). DNA probes were labeled with digoxigenin-dUTP using a Genius kit (Boehringer Mannheim) according to the manufacturer's directions.

**Southern and RNA Blot Analyses**

Genomic DNA for Southern analysis was prepared as previously described (Rasmussen et al., 1994) from an isogenized *Drosophila* stock, iso-1 (Tamkun et al., 1991). Seven micrograms of DNA were digested with restriction enzymes, fractionated on a 0.5% agarose gel and transferred to a Zeta-Probe nylon membrane (BioRad Labs.) by standard methods. Membranes were UV-cross-linked and dried in a vacuum oven at 80°C for 1.5 h. DNA probes were gel-purified and labeled with [32P]dATP using random hexamer primers (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) according to standard methods (Vogelstein and Gillespie, 1979). Hybridizations were carried out overnight at 42°C in 50% formamide, 10% dextran sulfate, 4× SSC (600 mM NaCl, 130 mM sodium citrate), 5× Denhardt's solution (0.02% each of BSA, Ficoll, and Polyvinylpyrrolidone), 100 µg/ml sheared denatured salmon sperm DNA, and 20 mM K2HPO4. Final washes (3× 20 min) were at high stringency, in 0.2× SSC, 0.1% SDS at 65°C. Isolation of total RNA from Oregon-R flies and subsequent blot analyses were performed as previously described (Rasmussen et al., 1994). Ovaries and testes were dissected in *Drosophila* EBR saline, flash frozen, and stored at −80°C. Staged embryos were flash frozen, stored at −80°C, and ground with a mortar and pestle immediately before RNA extraction. Twenty-five micrograms of total RNA were run on 0.75% agarose-formaldehyde denaturing gels. The hybridization and washing conditions were the same as for Southern blots.

**β-Galactosidase Reporter Construction and Expression**

An 14-kb BamHI fragment was isolated from the genomic subclone pCH81 and was inserted into the BamHI site of the *P*-element vector pcasSlgα (Thummel et al., 1988). The construct (600 µg/ml) was co-injected with 50 µg/ml helper plasmid pv257w (Thummel et al., 1988) into embryos of the *Drosophila* strain Df[1-2w-] (Lindsley and Zimm, 1992). Chromosomal locations of injected and reporter were determined by segregation from balancer chromosomes. β-Galactosidase activity during oogenesis was assayed by the protocol of Chen et al. (1992). Stained tissues were viewed under a Zeiss Axioskop 10 microscope using differential interference contrast optics. Images were captured on an IRIS Indigo Video board installed on a Silicon Graphics R3000 Indigo workstation. Hardcopy image prints were produced on a Tektronix Phaser IISDx dye sublimation printer.

**Antibody Production and Purification**

Polyclonal antibodies were elicited in rats against fusion proteins produced in *E. coli*. The dyein cDNA fragments ZAP6, 822, 828 and 3100 (Fig. 8A) were cloned into the expression vector pETs (Novagen, Inc., Madison, WI) and expressed in the bacterial strain BL21 (Novagen, Inc.; Studier et al., 1990). Cells were lysed by sonication and the insoluble inclusion bodies containing the fusion protein were pelleted by centrifugation. The inclusion bodies were washed three times in PBS (150 mM NaCl, 16 mM NαHPO4, 4 mM Na2HPO4, pH 7.3) containing 1% Triton X-100, 1% deoxycholate, and 0.1% SDS. They were solubilized in PBS containing 1% SDS, and fractionation on SDS-polyacrylamide gels (Laemmli, 1970). Gel slices corresponding to the fusion proteins were excised, washed in PBS to remove SDS, emulsified with an equal volume of complete Freund's adjuvant, and injected into rats. Subsequent boosts were done in incomplete Freund's adjuvant at 2-wk intervals. All injections were subcutaneous in a volume of 0.1-0.25 ml. The polyclonal sera obtained were separately affinity-purified against their corresponding peptides expressed as glutathione transferase fusion proteins in the appropriate pGEX vector (Smith and Johnson, 1988).

A 20-residue peptide, LGGSPGPGAGTGKTESVKAL, was synthesized at Mayo Clinic, Microchemistry facility (Rochester, MN) and used to immunize rabbits (Calico Biologicals, Reamstown, PA.). The peptide antibody (PEP-I) and the ZAP 8 antibody, were purified over an affinity column prepared with a ZAP8 fusion protein expressed in the vector pGEXI (Smith and Johnson, 1988) in the bacterial strain HB101. The affinity column was generated by coupling the fusion protein to Actigel (Sterogene Biocarps Inc., Arcadia, CA) resin according to the manufacturer's instructions. The antibody raised against the pET-3100 antigen was purified similarly against the pGEX-3100 fusion protein. The ZAP8 derived antibody was affinity purified according to the blot affinity method of Olmsted (1981). The affinity purified antibodies were stored at −20°C in 50% PBS/50% glycerol containing 1 mg/ml BSA.

**Ovary MAP Preparation**

Ovaries from well fed 3-d-old females were dissected in EBR, placed on ice until 100-µl ovaries were obtained, quick frozen in liquid nitrogen, and stored at −80°C until use. Ovaries were rinsed three times in PMEG buffer (100 mM Pipes, pH 6.9; 5 mM MgOAc4H2O; 5 mM EGTA; 0.1 mM EDTA; 0.5 mM DTT; 0.9 M glycerol) and homogenized on ice in 1.5 vol PMEG plus protease inhibitors (10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.1 mg/ml each of soybean trypsin inhibitor, α-tosyl-L-arginine methylsterile, and benzamidine). Cytoplasmic dynein was isolated from ovary homogenates as described previously for *Drosophila* embry homogenates (Hays et al., 1994).

**Immunoblots and Immunolocalization**

SDS-PAGE and immunoblotting were carried out as described by Laemmli (1970) and Towbin et al. (1979). Proteins were electrophoresed on 0.75-mm slab gels and blotted onto nitrocellulose. For the analysis of ovary MAP preparations, gels were prepared with a bis/acrylamide ratio of 1:100 (Porter and Johnson, 1983). Alkaline phosphatase–conjugated antibodies were diluted in PBS containing 0.05% Tween-20 and 5% BSA. Blots were developed with NBT plus BCIP (Sigma Chem. Co.) in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl2, 100 mM Tris, pH 9.5). Alternatively, blots were incubated with horseradish peroxidase–conjugated secondary antibodies (Amersham Corp., Arlington Heights, IL) and developed with the non-radioactive autoradiographic ECL method according to the suppliers' instructions (Amersham Corp.).

For immunofluorescence microscopy, ovaries were dissected from 2-4-d-old females in EBR and fixed for 5 min in 100-µl deviellinizing buffer/600-µl heptane using the method of Cooley et al. (1992). Deviellinizing buffer was 1 vol buffer B:1 volume 36% formaldehyde:4 vol H2O. Buffer B contains 100 mM K2HPO4/KH2PO4, pH 6.8, 450 mM KCl, 150 mM NaCl, and 20 mM MgCl2. After fixation, ovaries were rinsed three times with PBS, rinsed briefly three times with PBS/0.1% Triton X-100 (PBT), and then washed 1 h in PBT at RT on a rotating wheel (Cole Palmer, Inc., Chicago, IL). Ovaries were then dissected into individual ovarioles, washed for 1 h in PBT, and then blocked in PBT containing 1% BSA (Block buffer) at RT. Primary antibody incubations were carried out overnight at 4°C. The ovarioles were then washed for 2 h at RT with eight changes of Block buffer, and incubated with secondary antibody for 1.5–2 h at RT. Ovarioles were again washed with Block 2 h with at least eight changes, rinsed 3× in PBT, and washed for 5 min in PBT, and mounted as a solution of 10% PBS, 90% glycerol containing 1 mg/ml p-phenylenediamine (Sigma Chem. Co.). All antibodies were diluted in Block buffer. Affinity-purified anti-dynein heavy chain antibodies were used at a dilution of 1:50. Fluores-
cein-conjugated goat-anti-rabbit and goat-anti-rat (Boehringer Mannheim Corp.) secondary antibodies were preabsorbed against 100-μl embryos overnight at 4°C in a dilution of 1:100. Preabsorbed secondary antibodies were used at a final dilution of 1:100 in the presence of 5% normal goat serum. Ovarioles (egg chambers) were examined on either a Zeiss Axioskop 10 microscope with epifluorescence illumination using plan-neofluar 16 ×0.5, 40 ×0.75 lenses or a 63 ×1.4 oil planapochromat lens, or a Nikon diaphot microscope with an MRC-600 confocal imaging system (Bio-Rad Labs.), using a 60 ×1.4 oil planapochromat lens. The wild-type Drosophila strain OregonR was used in all localization experiments unless otherwise noted. The alleles BicDp1165, BicDp26, egp6, capi, and spire were provided by Dr. Trudi Schüpbach (Princeton University, NJ) and are described in Suter et al. (1989), Maneusz and Schüpbach (1989), and Schüpbach and Wieschaus (1991). The deficiency Df(2L)TWI19 that removes the BicD gene was obtained from Ruth Steward and is described in Steward et al. (1987). Dynein localization was examined in BicD/D(2L)TWI19 females that were recovered from the cross BicD/CyO × Df(2L)TWI19/CyO. The stauD3 allele was provided by Dr. Paul MacDonald (Stanford University, CA), and is described in St. Johnston and Nusslein-Volhard (1993). The role of Notch during oogenesis is discussed in Ruohola et al. (1991) and Xu et al. (1992). An Afffinity-purified, COOH terminus specific, dynein heavy chain antibody (see Materials and Methods) identified two positive clones in immunological screens of a Drosophila embryo cDNA expression library. The putative dynein clones were plaque-purified and their cDNA inserts were used as probes for RNA blots to determine the size of the RNA transcript from which they were derived. SDSPAGE analysis of the intact and UV-cleaved dynein polypeptide from Drosophila embryos predicts a heavy chain of >435 kD (Hays et al., 1994). Only one of the putative dynein cDNA clones, designated MS3, hybridized to an RNA transcript of sufficient size, 13-15 kb, to encode the dynein heavy chain. The 2.1-kb insert from MS3 is derived from position 10,945 bp to 13,115 bp near the 3' end of the dynein heavy chain gene (Fig. 1).

To isolate the full-length coding sequence of the dynein heavy chain, the MS3 insert was used to initiate a walk in cDNA libraries to isolate overlapping clones that extended toward both the 5' and 3' ends of the dynein transcript. Fig. 1 shows the final map of the overlapping cDNA clones spanning ~15 kb which were isolated, restriction mapped, and sequenced. Several independent clones representing the 3' end of the transcript were isolated. Sequence analysis indicated that each was derived from the same transcript and included a long polyadenine tail that initiated at the same position in the sequence of each clone. One of these clones, designated PL4, is shown (Fig. 1). Overlapping cDNA clones extending toward the 5' end of the transcript were obtained from a randomly primed λZAP cDNA library (Stratagene). The first nine clones from PL4 to ZAP22 each contain at least 190 bp of overlapping sequence and span 13.5 kb.}

### Results

#### Isolation of the Full-Length Dynein cDNA

The molecular characterization of the Drosophila cytoplasmic dynein heavy chain gene was initiated by isolating cDNA clones encoding the full-length dynein polypeptide. An affinity-purified, COOH terminus specific, dynein heavy chain antibody (see Materials and Methods) identified two positive clones in immunological screens of a Drosophila embryo cDNA expression library. The putative dynein clones were plaque-purified and their cDNA inserts were used as probes for RNA blots to determine the size of the RNA transcript from which they were derived. SDS-PAGE analysis of the intact and UV-cleaved dynein polypeptide from Drosophila embryos predicts a heavy chain of >435 kD (Hays et al., 1994). Only one of the putative dynein cDNA clones, designated MS3, hybridized to an RNA transcript of sufficient size, 13-15 kb, to encode the dynein heavy chain. The 2.1-kb insert from MS3 is derived from position 10,945 bp to 13,115 bp near the 3' end of the dynein heavy chain gene (Fig. 1).

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kb of open reading frame (ORF) in the dynein transcript. cDNA clones extending further 5' to the end of the dynein transcript were recovered using a genomic probe to screen the cDNA library, and by priming the synthesis of new cDNA from embryonic mRNA (see Materials and Methods). The 5' coding sequence determined from PCR-derived cDNA clones was verified in the sequence of genomic clones.

Since the 5' most cDNA sequence did not contain stop codons in all three reading frames, primer extension and RNase protection experiments were conducted to determine the transcription start site and to confirm that the isolated cDNA clones contained the entire dynein transcript. Two extension products were revealed that correspond to start sites at positions 1 and 92, respectively (Fig. 2 A). To show that these two sites reflect the bonafide ends of transcripts and not premature termination due to secondary structure, RNase protection experiments were conducted. The protection of a genomic subclone extending from nucleotides -923 to 330 was analyzed. The sizes of the two fragments protected are in close agreement with the start sites indicated by primer extension (Fig. 2 B). A 582-bp genomic DNA fragment corresponding to the position of -341 bp to -921 bp, a position not expected to be transcribed, was used as a negative control and produced no protected fragment (data not shown). From these results, we conclude that the entire heavy chain transcript is cloned and that two sites are used to initiate the cytoplasmic dynein heavy chain transcript.

The first ATG codon in both transcripts initiates the same long ORF. If this ATG represents the translation start site in both transcripts, then the only difference between the two transcripts is in the length of the 5' untranslated leader sequences. One transcript is 14,381 nucleotides in length (starting at base pair 1) and the other transcript initiates 92 bp downstream. The 5' untranslated leaders are 162 and 70 nucleotides long, respectively. No TATA box was found by examination of the upstream sequence. The sequence flanking the first ATG (position 163 bp) differs from the consensus sequence (C/A, AA N/c AUG) for translation initiation in Drosophila (Cavener, 1987). Additional ATG codons in the dynein ORF lie downstream and could also represent translation start sites. Like the first ATG, the second inframe ATG at position 499 bp in the cDNA does not show a consensus flanking sequence for translational initiation. However, the third inframe ATG at position 695 bp is flanked by a consensus sequence for translation initiation.

At the 3' end of the transcript, a 298 nucleotide noncoding sequence follows a TAA stop codon (position 14,080 bp) and shows an enrichment (74%) in AT dinucleotides. The consensus sequence (AAATAAA) for polyadenylation (Proudfoot, 1989) does not appear in the 3' noncoding sequence. However, eighteen nucleotides upstream from the polyadenylate tail there is a sequence of AAATA which has been found in the 3' noncoding sequence of other Drosophila genes (Berleth et al., 1988; Lehner and O'Farrell, 1989; Lantz et al., 1992) and may serve as a polyadenylation signal.

**Structural Similarities among Dynein Heavy Chains**

The nucleotide sequence and deduced amino acid sequence of the long ORF predicts a dynein heavy chain polypeptide containing 4,639 amino acid residues with a molecular mass of 530 kD and a pl of 5.9 (Fig. 3). The predicted amino acid sequence of the Drosophila dynein heavy chain was compared to the sequences for dynein heavy chains from sea urchin (Gibbons, et al., 1991; Ogawa, 1991), rat (Mikami et al., 1993; Zhang et al., 1993), slime mold (Koonce et al., 1992), and yeast (Li et al., 1993; Eshel et al., 1993). In each case, a high degree of sequence similarity over long stretches of the dynein polypeptides is apparent using a Pustell matrix comparison (Fig. 4 A). The Drosophila sequence was also compared to itself to reveal potential internal duplications. Other than the main diagonal resulting from self identity, no off-set diagonals were detected.

The Drosophila cytoplasmic dynein heavy chain is most similar to the predicted rat cytoplasmic dynein heavy chain polypeptide (Fig. 4 A and Table I; Mikami et al., 1993; Zhang et al., 1993). Overall the two sequences share 84% similarity and 72% identity at the amino acid level. As indicated in Fig. 4 A and Table I, the homology is distributed across the entire length of the polypeptides. By comparison, the overall similarity between the Drosophila dynein se-
Figure 3. Complete sequence of the cytoplasmic dynein heavy chain mRNA and protein. Nucleic acid sequence of cDNA and 5' flanking genomic DNA, and the deduced amino acid sequence is shown. Two vertical arrows indicate transcription initiation sites for the heavy chain mRNA. The first start site is numbered as position 1. The termination codon is indicated by an asterisk. Four phosphate-binding loop motives are underlined. The sequence of a 20-amino acid synthetic peptide used to elicit a rabbit antibody (PEP-I) is framed.
Figure 3.

sequence and the Dictyostelium (Koonce et al., 1992), Aspergillus (Xiang et al., 1994), yeast (Li et al., 1993; Eshel et al., 1993), sea urchin (Gibbons, et al., 1991; Ogawa, 1991), and Chlamydomonas dynein (Mitchell and Brown, 1994; Witman et al., 1994) polypeptides is substantially lower. As shown by the matrix comparisons, this reduced similarity is most evident over the NH2-terminal one third of the polypeptides. Further comparisons between the sequences were made using NH2-terminal (residues 1-1500), Central (residues 1500-3000), and COOH-terminal (residues 3000-4639) segments from the Drosophila sequence. Each segment was aligned to the other published dynein sequences to determine the % identity and % similarity in each of the segments (Table I). The sequence comparisons demonstrate that the NH2-terminal one third of the dynein polypeptides is most divergent, and that a central domain is the most highly conserved in all cases (Fig. 4 A and Table I). Within the more conserved central domain (residues 1500 to 3000; see Table I), the percentage of identity between the Drosophila and other published dynein heavy chain polypeptides is: rat (79%), Aspergillus (62%), Dictyostelium (61%), yeast (61%), sea urchin (39%), Chlamydomonas α (31%), Chlamydomonas β (33%), and Chlamydomonas γ (33%).

The conserved central domain encompasses a cluster of four phosphate-binding loop (P-loop) motifs with the consensus sequence of GXXGXGKT/S (Fig. 4 B). The positions of these four P-loops in the central domain of the dynein polypeptide, the spacing of the sites relative to one another, as well as the amino acid residues that comprise each site, are highly similar in all reported dynein sequences (Fig. 4 B). The predicted sequence of the first P-loop (residues 1895-1902) in Drosophila is totally conserved among all dynein sequences. The sequence of the second P-loop (residues 2580-2587) is more divergent, and the third P-loop (residues 3150-3157) is totally conserved in rat, Drosophila, rat, Dictyostelium, Aspergillus, and yeast.

Table I. Dynein Heavy Chain Sequence Comparisons

| Drosophila Dhc64C |  |
|-------------------|---|
| **Full length**   |  |
| **Residues**      |  |
| **1-1500**        |  |
| **1500-3000**     |  |
| **3000-end**      |  |

| Cytoplasmic       |  |
|--------------------|---|
| Rat                | 72 (84) |
| Aspergillus        | 51 (69) |
| Dictyostelium      | 52 (70) |
| Yeast              | 30 (55) |

| Aoxenomel          |  |
|--------------------|---|
| Sea urchin         | 26 (51) |
| Chlamy. β          | 26 (50) |
| Chlamy. γ          | 26 (50) |
| Chlamy. α          | NA     |

Amino acid sequence comparisons of Drosophila cytoplasmic dynein with dynein heavy chains from other species. Numbers in bold indicate percent identity residues. Numbers in parentheses indicate percent similar residues. NA, not alignable. Alignments and comparisons were made using the UWGGC BISST program. The multiple sequence alignment was used for rat, Aspergillus, Dictyostelium, yeast, sea urchin and Chlamydomonas are Genbank sequences from respectively, Mikami et al. (1993); Koonce et al. (1992); Eshel et al. (1993); Gibbons et al. (1991); Mitchell and Brown (1994); and Witman et al. (1994).
Figure 4. Structural similarities among dynein heavy chain polypeptides. (A) Dot plots show protein sequence matrices between two dynein heavy chains. X-axes for all plots represent the Drosophila cytoplasmic dynein heavy chain. Y-axes are: rat brain cytoplasmic dynein (top left; Rattus norvegicus; Mikami et al., 1993; Zhang et al., 1993), slime mold cytoplasmic dynein (top right; Dicyostelium discoideum; Koonce et al., 1992; Eshel et al., 1993), yeast cytoplasmic dynein (bottom left; Saccharomyces cerevisiae; Eschel et al., 1993; Yeh et al., 1993), sea urchin flagellar ß axonemal dynein (bottom right; Tripneustes gratilla; Gibbons et al., 1991). Numbers indicate amino acid positions. The PAM250 scoring matrix (Pearson, 1990), a window size of 15 residues, and a minimum score of 60% were used for the analysis. Arrows identify positions of the four P-loop motifs. (B) Sequence alignment of amino acid residues surrounding the four conserved P-loop motifs in cytoplasmic and axonemal dynein heavy chains. The Drosophila sequence is shown on the upper line of the comparisons. Dots represent identical amino acids. A bar above each alignment indicates the position of the P-loop. Sequence identities within the P-loop are indicated by asterisks. Species source of dynein sequences is shown at left (dmdhc, Drosophila melanogaster cytoplasmic dynein heavy chain; rmdhc, Rattus norvegicus cytoplasmic dynein heavy chain; mmdhc, Mus musculus cytoplasmic dynein heavy chain; kmdhc, Koonce et al., 1992; edhc, Emericella (Aspergillus) nidulans cytoplasmic dynein heavy chain; xmdhc, Xiang et al., 1994; zmdhc, Zschokke et al., 1993; smdhc, Schleier et al., 1993).
Eight human cDNA sequences (accession numbers: T07853, T06228, T03672, T09530, T05469, T05294, M62078, and M85915) present in the collection of expressed sequence tags (EST; Adams et al., 1991, 1992) show significant similarity to the *Drosophila* dynein heavy chain sequence. Each of the EST clones shares at least 40% identical residues in overlaps of 54–109 amino acids. In addition to these EST clones, Vaisberg et al. (1993) have recently recovered a PCR-derived human dynein cDNA clone that contains the first P-loop motif. Besides the identification of other dynein sequences, our searches of the sequence databases revealed no other similarities of clear significance. Direct comparisons between the predicted *Drosophila* dynein heavy chain sequence and the predicted amino acid sequences of other known microtubule-associated proteins, including kinesin isoforms, MAP2, and tau, also failed to reveal any striking similarities.

**Genomic Organization of the Cytoplasmic Dynein Heavy Chain Transcription Unit**

Genomic DNA blot experiments show that the cytoplasmic dynein heavy chain is encoded by a single copy gene (Fig. 5 A). In addition, in situ hybridization to polytene chromosomes from larval salivary glands also reveals a single cytological map position for the cytoplasmic dynein gene (Fig. 5 B). Labeled fragments from the inserts of the MS3, ZAP4, and ZAP8 cDNA clones each hybridized to a single site located at position 64C on the third chromosome (Fig. 5 B). During the course of this work, partial sequences of seven dynein-related heavy chain genes were identified in *Drosophila* genomic DNA by PCR strategies (Rassmusson et al., 1994). This family of dynein heavy chain genes includes genes that appear to encode several axonemal dynein isoforms, as well as the cytoplasmic dynein isoform. One of the partial (~400 bp) dynein clones recovered by PCR also maps to position 64C. The complete cDNA sequence reported here encompasses the partial sequence of the dynein gene previously designated *Dhc64C* according to its cytological map position.

To recover a functional transcription unit of the dynein heavy chain gene, we have analyzed genomic clones encompassing the dynein coding sequence (Fig. 1 B). A 320-bp restriction fragment from the 5' end of the MS3 cDNA clone was used to probe a λEMBL3A library (Tamkun et al., 1992). Three genomic clones were isolated and their restriction enzyme sites were mapped. As shown in Fig. 1 B, the phage clone EMBL#4 contains the 3' end of the dynein heavy chain transcript and extends ~14 kb towards the 5' end of the gene. We subsequently screened a cosmid library (Tamkun et al., 1992) with a 2.0-kb HindIII insert from the genomic subclone EM12 (Fig. 1 B). Two of ten positive clones, pCS3 and pCS14, included the entire coding sequence of the dynein heavy chain gene. The clone pCS3 extends ~5 kb upstream from the predicted translational start site and includes a portion of each of two additional transcription units located 5' and 3' to the dynein gene. The positions of selected restriction enzyme sites in both the phage and cosmid genomic clones are shown in Fig. 1 B. The sequences of both ends of contiguous subclones of genomic DNA fragments spanning the dynein gene were determined. Both these end-run sequences and the restriction maps of the genomic clones matched with the overlapping cDNA clones, with the exception of one HindIII restriction site polymorphism toward the 3' end of the cDNA and the altered restriction map of genomic clones towards the 5' end of the gene due to the presence of a relatively large intron. The HindIII restriction site polymorphism and the boundaries of the large 5' intron

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**Figure 5.** Copy number and chromosomal location of the *Drosophila* cytoplasmic dynein gene. (A) Genomic DNA blots. DNA was digested with restriction enzymes as indicated (E, EcoRI; P, PstI). (Panel 1) DNA was probed with a 0.4-kb internal DNA fragment that spans bp 5770–6177, and includes the first P-loop motif. (Panel 2) DNA was hybridized to a ~1-kb BamHI–HindIII fragment at the 3' end of the dynein gene (see Fig. 1). Neither probe contained internal restriction sites for EcoRI or PstI, and both probes hybridized to a single, unique restriction fragment. Molecular size markers are shown at left. (B) In situ localization of the dynein gene on polytene chromosomes. The insert of the ZAP4 cDNA was labeled using digoxigenin-dUTP and hybridized to polytene squashes. Detection of the hybridized probe was by anti-digoxigenin coupled to alkaline phosphatase. Inset shows an enlargement of the tip of chromosome 3L and the location of the heavy chain gene at position 64C is indicated by an arrow. (C) RNA blots probed with genomic DNA extending 5' and 3' outside the dynein gene. Two additional transcription units were identified. (Lane 1) A probe extending 9 kb upstream recognizes a 3-kb transcript in RNA from ovaries; and all other tissues examined (data not shown). (Lane 2) A probe extending into the 3' region beyond the dynein gene hybridizes with a 1.3-kb transcript which is only detected in RNA from testes.
were confirmed by sequence analyses and comparison of genomic and cDNA clones. The restriction map of the cDNA differs from the genomic map in the 5' region that is included in the subclones pCH71 and pCH81 (Fig. 1). The 3.6 kb of genomic DNA contained in these subclones was sequenced completely and comparison to the cDNA sequence revealed the presence of a ~1-kb intron and a smaller intron of 62 bp.

As another verification of the limits of the dynein transcript, RNA from various tissues was probed with genomic DNA extending 5' and 3' from the gene. We identified two transcription units that flank the 5' and 3' ends of the dynein heavy chain gene (Fig. 1 B). RNA blot analysis shows that the Drosophila gene located 5' to dynein encodes a 3-kb transcript that is present in all tissues examined (Fig. 5 C). No additional 5' transcripts were detected on RNA blots with a probe that extends 9 kb upstream of the dynein gene (Fig. 5 C). Adjacent to the 3' end of the dynein coding sequence, we identified a testes-specific transcript. RNA blots were probed with the genomic subclone EM13, which contains the last 34 bp of the dynein cDNA sequence and ~1.3 kb of additional downstream sequence. A 1.3-kb transcript was detected only in total RNA prepared from testes.

Two cDNA clones containing a portion of the 5' flanking transcript were isolated with a probe derived from pCH81. Both cDNA clones initiate at position -171 and are transcribed from the DNA strand opposite to that of the cytoplasmic dynein gene. Analysis of the sequence derived from pCH81 and the two cDNA clones predicts an ORF that begins at position -305 bp and extends through position -921 bp. The partial sequence determined for the ORF predicts 200-amino acid residues that share 72% similarity and 59% identity with leucine-tRNA ligase precursor in fungi (Chow et al., 1989), yeast (Herbert et al., 1988) and E. coli (Hartlein and Madern, 1987).

Our analyses suggest that a genomic fragment extending from position ~924 bp upstream of the dynein coding sequence to position ~15,681 bp that lies ~100 bp downstream of the cDNA transcript is likely to contain a complete and functional genomic transcription unit for the cytoplasmic dynein gene.

**The Cytoplasmic Dynein Transcript Is Abundantly Expressed in Ovaries**

Expression of the Dhc64C dynein gene was examined throughout development (Fig. 6). RNA blots were probed with subcloned genomic and cDNA fragments that span the entire dynein gene. All cDNA probes derived from the dynein heavy chain coding sequence recognized a transcript of ~14.5 kb in all stages and tissues examined. The cytoplasmic dynein heavy chain gene is differentially expressed during development, with the highest levels of transcripts observed in ovaries and 0–2-h embryos. The level of the dynein transcript falls dramatically in RNA prepared from 2–4-h embryos, and then increases in later staged embryos. The decrease in dynein transcript at 2–4 h is likely due to the turnover of maternally supplied transcript before the initiation of zygotic transcription. The cytoplasmic dynein transcript is also readily detected in larval and pupal stages, as well as in adult tissues including heads and testes. A second transcript of slightly larger size is also detected in relatively low levels in adult ovary, larval, and pupal RNA (Fig. 6). Probes derived from the cDNA clones pTR13, ZAP8, and PL4 lie, respectively, at the 5' end, middle, and 3' end of the Dhc64C gene. Each of these probes detects both transcripts on RNA blots. Since our probes are specific to Dhc64C on genomic DNA blots, the two transcripts may result from the alternate splicing of exons. Alternatively the larger transcript may represent an immature mRNA that retains unspliced introns. A probe derived from the 1-kb intron at the 5' end does not hybridize to either transcript on RNA blots (data not shown). However, we have not mapped the complete intron/exon organization of the genomic clones and therefore cannot exclude other introns that could be present in a larger immature dynein mRNA.

The abundant expression of cytoplasmic dynein in ovaries suggests a role for the microtubule motor in oogenic transport. To examine this possibility, we have compared the spatial distribution of the dynein transcript with that of the encoded heavy chain polypeptide. To detect dynein transcript, ovaries were separately hybridized with digoxigenin-labeled DNA probes from several regions across the dynein coding sequence. All probes revealed a similar pattern of dynein transcript distribution. Low levels of the transcript are detected in the germarium and early stage egg chambers, while an elevation in the amount of dynein transcript in the nurse cell complex is apparent in later stage 8–10 egg chambers (Fig. 7 A). No enrichment of the transcript in the oocyte is observed, indicating that the dynein transcript is not accu-

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**Figure 6.** Developmental profile of the dynein mRNA. RNA blot contains total RNA isolated from ovaries; embryos (0–2, 2–4, 4–8, 8–12, and 12–24 h); larvae (mixed instars); pupae; testes; adult heads. The blot was hybridized to a radiolabeled probe from the region surrounding P-loop. The same expression profile was observed with probes derived from the 5' and 3' ends of the dynein cDNA. To control for equivalent loading and the integrity of the RNA the blot was later hybridized to a probe, RP49, derived from a ribosomal protein gene (lower panel; Vaslet et al., 1980). RNA size markers are indicated in kb at right side.
bridized with either a dHynein transcript are apparent in the nurse cell complex cytoplasm of the early stage egg chambers. Elevated levels of the dynein transcript are detected throughout the cytoplasm of the early stage egg chambers. Elevated levels of the dynein transcript are apparent in the nurse cell complex (nc) in late egg chambers. No enrichment of the dynein transcript in the presumptive oocyte is observed. In contrast, the control oskar probe (B) shows the posterior localization of the oskar transcript in the oocyte. (C) Reporter construct. A DNA fragment carrying the dynein promoter was inserted into the BamHI site of the P-element vector pCaSpeR βgal. The transcription start site of the larger dynein transcript is marked as number 1. The hatched box in the insert represents the 5' untranslated region of the dynein gene and in the open box in the insert represents the region encoding the first 13 aa of the dynein polypeptide. The first ATG in the dynein gene is indicated. (D) Expression of reporter construct. A single ovariole from a transformed female stained with X-gal. Intense staining is observed in the nurse cell nuclei. (nc, nurse cell complex; o, oocyte).

The Cytoplasmic Dynein Motor Encoded by the Dhc64C Transcript Accumulates in the Pro-oocyte

To examine the distribution of the cytoplasmic dynein heavy chain polypeptide during oogenesis, we generated polyclonal antisera against three distinct dynein epitopes contained within the non-overlapping cDNA subclones pET822, pET828, and pET3100 (Fig. 8, A and B, lanes 1–3; and Materials and Methods). The antisera were subsequently affinity-purified and shown to be specific for the dynein domain against which they were raised (Fig. 8 B, lanes 4–12). Furthermore, each antisera can detect the enrichment of the dynein heavy chain as a microtubule-associated protein. As shown in Fig. 8 C, the heavy chain polypeptide prepared from ovaries is enriched in microtubules assembled in the absence of ATP, and can be eluted from microtubule pellets in the presence of 10 mM MgATP. We have previously demonstrated that cytoplasmic dynein can be purified from embryos in this same manner and have directly demonstrated its minus-end motor activity in vitro (Hays et al., 1994). These results suggest that the Dhc64C dynein heavy chain in ovaries is also present in a functional dynein motor complex.

In striking contrast to the expression and distribution of the cytoplasmic dynein transcript in nurse cells, the encoded dynein heavy chain polypeptide is differentially localized to the oocyte. Each of the dynein antibodies was used to probe whole mount preparations of Drosophila ovarioles. As illustrated with the PEPI antiserum in Fig. 9, each of the affinity-purified antibodies detects the differential accumulation of cytoplasmic dynein in the presumptive oocyte during oogenesis. Initially, dynein is uniformly distributed in the anterior and mitotically active regions 1 and 2a of the germarium (Fig. 9 A; bracket). The elevated level of staining of a single cell in the 16-cell cyst is first detected in region 2b of the germarium (arrowhead), just anterior of where the egg chambers bud off from the germarium. As egg chamber development and migration down the ovariole proceeds, the cytoplasmic dynein antigen remains differentially localized to the presumptive oocyte (Fig. 9, A and B). Then, before the egg chamber cytoplasmic domain of stage 10 egg chambers (Fig. 9 D). Dynein is also detected at a low and uniform intensity in the follicle cells surrounding the egg chambers. At later stages this follicle cell dynein appears to concentrate to the apical region of each follicle cell that surrounds the oocyte (Fig. 9 D). This apical pattern of dynein distribution
Figure 8. Antibodies specific for distinct dynein heavy chain epitopes. (A) A map of the position of peptides used in the production of dynein heavy chain antibodies is shown. (B) The antisera PEP1, 828, and 3100 define three distinct, non-overlapping epitopes in the Drosophila cytoplasmic dynein heavy chain and specifically recognize the pET5-822, pET5-828, and pET5-3100 bacterially expressed fusion proteins, respectively. Four replicate samples of pET5-822 (lanes 1, 4, 7, and 10), pET5-828 (lanes 2, 5, 8, and 11), and pET5-3100 (lanes 3, 6, 9, and 12) were run on 7.5% SDS-polyacrylamide gels. One set of the gel fractionated samples was stained with Coomassie blue (lanes 1-3). Molecular mass markers are shown at left (lane M). The remaining three replicate gels were blotted to nitrocellulose and separately probed with antisera PEP1 (lanes 4-6), antisera 828 (lanes 7-9), or antisera 3100 (lanes 10-13). Approximately 1 µg of fusion protein was loaded in each lane. (C) Cytoplasmic dynein is enriched in microtubules prepared from ovary homogenates. Duplicate sets of identical samples from the purification steps in a typical preparation of microtubules that extends from the MTOC and encompasses the oocyte nucleus. During stage 9, the oocyte has grown to occupy approximately a third of the egg chamber volume (Fig. 9 C). At this stage, we detect an accumulation of dynein antigen at the posterior end of the oocyte, which is relatively lower level than the Drosophila ovary extracts were fractionated on separate 7.5% SDS-polyacrylamide gels. (Lanes 1-8) One gel was stained in Coomassie blue. (Lanes 9-12) The replicate gel was blotted to nitrocellulose and probed with affinity-purified PEP-1 antibody. Approximately 1 µg of protein from each purification step was loaded onto a 7.5% polyacrylamide gel. (Lane 1) Ovary homogenate; (lane 2) high speed clarification pellet; (lane 3) high speed supernatant; (lane 4) taxol-assembled microtubule pellet; (lane 5) microtubule-depleted supernatant; (lane 6) MgATP extracted tubulin pellet; (lane 7) MgATP extracted microtubule associated protein supernatant; (lane 8) wash of taxol-assembled microtubule pellet before MgATP extraction. Lanes labeled M were loaded with molecular mass standards. A high molecular mass band present in the taxol microtubule pellet (lane 4 and 4') and enriched in the ATP eluted microtubule supernatant (lane 7 and 7') is specifically recognized by the PEP-1 dynein peptide antibody.

Maternal Effect Mutations Disrupt the Localization of Cytoplasmic Dynein

To examine the genetic requirements for dynein localization to and within the oocyte, we have asked whether the distribution of dynein is altered by several maternal effect mutations (Fig. 10). The gene products of the maternal effect loci Bicaudal-D (Bic-D) and egalitarian (egl) are the two earlist gene products known to be required for the specification of an oocyte within the syncytial 16-cell cyst. Loss of function mutations in either of these two genes disrupt oocyte differentiation and give rise to egg chambers in which each of the 16 cells develop as polyploid nurse cells (Schüpbach and Wieschaus, 1991). The differential localization of cytoplasmic dynein to the pro-oocyte appears to coincide with oocyte specification, suggesting that the Bic-D and egl products might be required for the accumulation of cytoplasmic dynein in the pro-oocyte. As shown in Fig. 10 A, eglW150 mutant ovaries show no accumulation of dynein in the oocyte. Dynein staining is uniformly distributed in the cytoplasm in the anterior most regions of the germarium, but rarely becomes localized to a single cell within the syncytial 16-cell cyst (compare Figs. 9 A and 10 A). Occasionally, we have observed a slightly elevated accumulation of dynein in a cell at the posterior of the center of the cyst. In the later stage egg chambers, dynein staining is uniformly distributed throughout the 16 nurse cells. The localization of the dynein protein in sibling flies that are heterozygous for
Asymmetric localization of dynein during oogenesis. Ovaries from 3-d-old females were dissected, processed for immunocytochemistry, and probed with the affinity-purified PEP-1 antibody. (A) The dynein polypeptide is detected uniformly throughout regions 1-2a of germarium (bracket). Dynein becomes concentrated in the presumptive oocyte as early as region 2b in the germarium (small arrowhead). As the egg chamber proceeds down the ovariole, the dynein antigen remains highly enriched in the developing oocyte relative to the nurse cells and gradually becomes concentrated to the posterior end of the oocyte (large arrowhead). In early egg chambers, a focus of staining is observed on the posterior side of the oocyte nucleus (see inset). (B) Early and late stage 9 (C) egg chambers showing the transition between the uniform (B) and posterior (C) accumulation of dynein in the growing oocyte. (D). In the stage 10 egg chamber the posterior focus of dynein staining is reduced, while a rim of dynein staining becomes apparent in the apical region of the follicle cells. The level of dynein staining observed in the nurse cell cytoplasm of both stage 9 and 10 egg chambers appears increased relative to earlier stages. The PEP-1 antibody, the antibodies elicited against the bacterial fusion proteins encoded by nonoverlapping cDNAs (3100, 828) and the ZAP8 cDNA all show the same pattern of dynein localization in ovaries. fc, follicle cells; nc, nurse cells; o, oocyte; magnification in A, B, and C are identical. Bar shown in C is 50 μm. Bar shown in D is 100 μm.

The posterior concentration of dynein during stage 9 of oogenesis is similar to the concentration of the pole plasm components staufen protein and oskar RNA. This suggests that the cytoplasmic dynein motor may play a role in the assembly of the pole plasm during stages 8 and 9 of oogenesis. To address this possibility, we localized dynein in ovaries from flies homozygous for maternally inherited mutations, cappuccino (capu) and spire. These genes act upstream of staufen and are required for the localization of staufen protein and oskar RNA to the posterior pole of the oocyte. In ovaries from capu mutant flies, the pattern of dynein localization is unaffected until stage 9 of oogenesis (Fig. 10 C and D). As shown for ovaries derived from capu mutant flies, the enrichment of dynein at the posterior pole of the oocyte in stage 9 egg chambers is never achieved, and a uniform level of dynein is detected throughout the nurse cell and oocyte cytoplasm. Yet before stage 9, the initial accumulation of dynein to the presumptive oocyte is indistinguishable from that observed in wild-type ovaries (Fig. 10 C). The identical result was observed in oocytes from spire mutant flies (data not shown). Control flies heterozygous for either the capu or spire mutations show a dynein distribution in oogenesis that is completely wild type. We have also examined the localization of dynein in ovaries from flies carrying a mutation in staufen protein. In contrast to the capu and spire mu-
Figure 10. The effects of egl, BicD, capu, and N mutations on dynein localization in oogenesis. (A–E) Confocal images of ovarioles and egg chambers from ovaries dissected and processed for immunocytochemistry with the affinity-purified PEP1 antibody. The orientation of all egg chambers is with the anterior end to the left. (A) Ovarioles from an egl^W350/egl^W126 female. Dynein does not accumulate in a single cell in the syncytial 16 cell cyst and remains uniformly distributed throughout all 16 nurse cells in the egg chamber. (B) Ovarioles from a BicD^Df(2L)TW119 female. A transient accumulation of dynein in the pro-oocyte is observed through stage 2-3 egg chambers. In contrast to wild-type egg chambers, the perinuclear focus of dynein staining in the pro-oocyte is located anterior to the oocyte nucleus (see inset). (C) Germarium and egg chambers from a capu^ts1/capu^ts1 female. Dynein accumulates in the oocyte through stage 8 of oogenesis as in wild-type ovaries. The same result is observed with ovaries from the spire^ts1 mutant (not shown). (D) A stage 9 egg chamber from a capu^ts1/capu^ts1 female. Dynein is not enriched at the oocyte posterior, but is uniformly distributed throughout the nurse cell and oocyte cytoplasm. Like capu^ts1, the spire^ts1 mutant specifically disrupts the posterior localization of dynein in the stage 9 egg chamber. (E) A stage 9 egg chamber from a N^ts1/ N^ts1 female incubated at the restrictive temperature of 32°C for 30 h. Dynein is not enriched at the oocyte posterior, but is mislocalized to a spot in the center of the oocyte. A slight accumulation of dynein is detected at the anterior of the oocyte and in the cortical region of the oocyte extending along the anterior-posterior axis. Control N^ts1/+ siblings from the N^ts1/FM7 stock incubated at the restrictive temperature show normal dynein distribution throughout oogenesis. Magnification in A and B, and in D and E, are identical. Bars: (A and C) 100 μm; (D) 50 μm.

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tations, flies homozygous for staufen^D3 showed no effect on accumulation of dynein in the oocyte, or on the enrichment of dynein at the oocyte posterior (data not shown). These results suggest that dynein is not a component of the pole plasm, but that like cappuccino and spire, dynein might participate in the assembly of the pole plasm. To test this hypothesis further, we localized dynein in ovaries from females homozygous for a temperature-sensitive allele of Notch, N^ts1. Notch is required in the posterior follicle cells for the specification of polarity along the anterior-posterior axis in the oocyte (Ruocho et al., 1991). In N^ts1 females incubated at the restrictive temperature, the anterior determinant bicoid (bcd) RNA is localized to both the anterior and posterior of the oocyte, while oskar RNA and staufen protein are mislocalized to a spot in the center of the oocyte (Ruocho et al., 1991). As shown in Fig. 10, E, we observe a similar misplacement of cytoplasmic dynein to the center of oocytes derived from N^ts1/N^ts1 flies incubated at the restrictive temperature of 32°C for 30 h. This mislocalization of dynein is restricted to stage 9 egg chambers, with no discernable
change in the accumulation of dynein during early stages of oogenesis. In heterozygous N\\textsuperscript{w}/+ flies incubated at the restrictive temperature, cytoplasmic dynein distribution appears normal throughout oogenesis.

**Discussion**

The cytoplasmic dynein heavy chain gene, Dhc64C, encodes a dynein heavy chain that is similar to cytoplasmic dyneins characterized from other organisms. The predicted *Drosophila* dynein protein sequence shares 72% and 52% amino acid identity with the cytoplasmic dynein heavy chains reported for rat brain and *Dictyostelium*, respectively. In contrast only 26% of the *Drosophila* dynein amino acid residues are identical to those found in either the sea urchin or *Chlamydomonas* β-heavy chain axonemal dyneins that function to power flagellar motility. The greater similarity of the *Drosophila* dynein sequence to cytoplasmic isoforms is also observed in the conservation of residues that surround each of the four clustered P-loop motifs (Fig. 4B). Interestingly, for all known cytoplasmic dynein sequences the amino acid residues of the first and third P-loops are totally conserved. In the case of the axonemal dyneins, the residues of the first and fourth P-loops are totally conserved. The conservation of amino acid residues in the first P-loop regions most likely reflects the participation of this domain in the binding and hydrolysis of ATP by the dynein motors. While the presence and positions of three additional phosphate-binding motifs in the central domain of the polypeptide is characteristic of all known dynein heavy chains, the contribution of these sites to motor function and/or structure remains to be determined.

In contrast to the P-loop regions and the central domain, the NH\textsubscript{2}-terminal ~1500 residues of the dynein heavy chains are the most divergent. As viewed by electron microscopy, the native structures of cytoplasmic and axonemal dynein heavy chains are quite similar and appear to consist of a globular head domain and a short slender elongated stalk domain (for reviews see McIntosh and Porter, 1989; Vallee, 1993; Schroer, 1994). The short stalk domain is believed to provide the attachment of the dynein motor to the associated cargo. In the case of axonemal dyneins the cargo is the A-tubule of the outer doublet microtubule, while for cytoplasmic dyneins the cargo may include a variety of membranous organelles such as Golgi and synaptic vesicles (Vallee and Shpetner, 1990; Vallee, 1993; Schroer, 1994). The recent analysis of a β-axonemal dynein mutation in *Chlamydomonas* has shown that the NH\textsubscript{2}-terminal domain of the dynein heavy chain is located within the stalk of the native dynein complex (Sakakibara et al., 1993). This work demonstrates that an NH\textsubscript{2}-terminal 160-KD portion of the heavy chain is capable of stable assembly into the axosome and is associated with a wild-type complement of intermediate and light chain polypeptides. Given these results, the sequence divergence in the NH\textsubscript{2}-terminal domains of dynein heavy chains may reflect the varied attached cargoes of the different dynein heavy chains. Whether the NH\textsubscript{2}-terminal sequence specifies and directly interacts with a cargo, or instead mediates the interaction through its association with intermediate and light chain polypeptides, is at present unknown.

In comparison to the *S. cerevisiae* dynein sequence, the *Drosophila* and all the other full-length dynein sequences appear to have extended COOH-termini. The predicted cytoplasmic dynein heavy chain polypeptides encoded by the *Drosophila*, rat, *Dictyostelium*, and *Aspergillus* sequences, and the axonemal dyneins encoded by the *Chlamydomonas* β and γ, and sea urchin β heavy chain sequences, are respectively 4639, 4644, 4725, 4344, 4568, 4483, and 4466 residues in length. The reported full-length yeast sequence is only 4092 residues in length (Eshel et al., 1993). Multiple sequence alignment of the dynein sequences indicates that much of this length difference is accounted for by termination of the yeast sequence ~300 residues earlier than the other heavy chain polypeptides. While the location of the COOH terminus in the folded polypeptide remains to be determined, it does not appear to be required for the nucleotide-insensitive binding of axonemal dynein and therefore may contribute exclusively to the globular head domain (Sakakibara et al., 1993). No extended stretches of amino acid identities are observed in comparisons of the last 300 residues of the longer sequences and similar to the NH\textsubscript{2}-termini these divergent COOH-termini may reflect heavy chain-specific functions. The cloning of the dynein heavy chains in a number of organisms will now permit the construction of truncated as well as chimeric dynein heavy chains. The reintroduction of these engineered hybrid dyneins using transformation methods should help to reveal the functional significance of the extended COOH-termini and other divergent and/or conserved domains.

The established globular head and flexible stem structural motifs of native axonemal and cytoplasmic dynein complexes are not mirrored by a bipartite secondary structure prediction. Dynein sequences do not predict an extended coiled-coil tail domain analogous to that observed for members of the myosin and kinesin superfamilies. β-sheets, β-turns, and α-helical domains are intermixed throughout the length of the heavy chain. The two longest α-helical domains are present in the COOH terminus of the *Drosophila* heavy chain polypeptide (residues 3158-3272 and residues 3397-3542). These domains appear to be conserved in their relative positions in both axonemal and cytoplasmic dynein isoforms (e.g., *Dictyostelium* residues 3253-3365 and residues 3492-3629; Koonce et al., 1992; sea urchin residues 2975-3223 and residues 3260-3314; Gibbons et al., 1991). In all cases they are predicted to form α helical coiled-coil domains and may participate in the folding of the globular domain of the heavy chain polypeptide or function in the binding of a separate component or regulator of the dynein complex. Recent work has described a "spring-loaded" mechanism for the conformational change of influenza hemagglutinin that is dependent on the dynamic interactions of coiled coils (Carr and Kim, 1993). Whether similar dynamics in the putative coiled-coil domains of the dynein globular head might contribute directly to motor function will require further investigation.

The *Drosophila* Dhc64C transcript is expressed throughout development and appears to encode the major cytoplasmic dynein present during oogenesis and embryogenesis (see also Rasmussen et al., 1994). This result suggests that the native *Drosophila* cytoplasmic dynein most likely contains a homodimer of the Dhc64C dynein heavy chain. Consistent with this interpretation, we previously showed that vanadate-mediated (V\textsubscript{3+}) cleavage of the *Drosophila* cytoplasmic dynein appears to occur at a single site in the heavy chain and
produces only two heavy chain polypeptide fragments (Hays et al., 1994). In comparison, similar cleavage of axonemal outer arm dynein produces multiple heavy chain fragments corresponding to the multiple distinct heavy chains known to be present in the outer arm complex (Mocz et al., 1988; King and Witman, 1987, 1988). Observations of a single major species of cytoplasmic dynein containing two identical dynein heavy chain polypeptides have been reported in rat brain, Dictyostelium, and rat testis (Mikami et al., 1993; Zhang et al., 1993; Koonce et al., 1992). However, in Drosophila we have detected minor levels of other dynein transcripts in embryos and ovaries, and we cannot rule out the possibility of additional dynein complexes containing other heavy chain polypeptides (Rasmussen et al., 1994).

The Dhc64C cytoplasmic dynein transcript is present in the nurse cell complex of the egg chamber throughout most stages of egg chamber maturation in the ovariole. In contrast, a striking differential localization of the dynein motor to the oocyte is observed with antibodies directed against three separate epitopes of the heavy chain polypeptide. Why does cytoplasmic dynein accumulate in the oocyte? One explanation is that the dynein motor is maternally supplied and stored in the oocyte for its later use in the syncytial embryo. The large size of the dynein heavy chain gene most likely precludes the synthesis of full length transcripts during the rapid nuclear division cycles of the syncytial blastoderm (Shermon and O'Farrell, 1991). The high level of the dynein transcript detected in 0–2-h embryos is therefore presumed to represent the maternal supply that is rapidly transferred into the oocyte before nurse cell breakdown. The subsequent loss of the dynein transcript in 2–4-h old embryos suggests that this maternal endowment is degraded, and then later (4–8-h embryos) replaced by the zygotic transcription of the dynein heavy chain gene. We showed previously that the dynein heavy chain polypeptide is present throughout the cytoplasm in the syncytial blastoderm and appears to associate with mitotic and interphase microtubules during the rapid nuclear division cycles, as well as other microtubule arrays (e.g., CNS axons) at later stages (Hays et al., 1994). In association with these varied microtubule arrays, cytoplasmic dynein may power the transport of a variety of cellular components during embryonic development.

Does the early accumulation of dynein in the oocyte support a role for the dynein motor in the differentiation of the oocyte and the specification of embryonic axes? The distinct localization of the dynein heavy chain polypeptide in the oocyte, compared to the presence of the dynein transcript in the nurse cell complex, suggests that dynein may transport itself into the oocyte. An intriguing possibility is that the dynein motor also powers the transport of morphogenic molecules and signals from the nurse cell complex into the oocyte. In this regard, we have shown that the Drosophila cytoplasmic dynein motor can be purified from ovaries (Fig. 8 C) and embryos (Hays et al., 1994) by its increased affinity for microtubules in the absence of MgATP. This property reflects the enzymatic activity required for the minus-end directed microtubule translocation that we have previously shown for the Drosophila cytoplasmic dynein (Hays et al., 1994).

The movement of the dynein motor from the nurse cell cytoplasm where it is synthesized into the oocyte is likely to occur along a polarized array of microtubules. Recent evidence supports the existence of a microtubule array with the necessary polarity to account for the translocation of dynein into the oocyte (Theurkauf et al., 1993; see also Pokrywka and Stephenson, 1991; Koch and Spitzer, 1983). The Drosophila oocyte develops within a cyst of 16 cells that are interconnected by cytoplasmic bridges or ring canals (King, 1970). Shortly after the formation of the syncytial 16 cell cyst in the gerarium, the MTOC of the 15 cells destined to become nurse cells degenerate, or lose their association with the germline nuclei, and are transported through the ring canals to the single cell that will develop as the oocyte (Mahowald and Strassheim, 1970). The resulting single MTOC complex is first detected in region 2a-2b of the gerarium and organizes a polar array of microtubules (Theurkauf et al., 1993). The minus ends of microtubules are apparently focused to the single MTOC located in the oocyte, while the distal plus ends of the microtubules extend through the ring canals and into the nurse cells. In the presence of this polar microtubule scaffold, the dynein motor is predicted to "walk" into the oocyte. Our results show that the differential accumulation of cytoplasmic dynein in the oocyte begins in region 2b of the gerarium and so is temporally coincident with the establishment of microtubule polarity in the early egg chamber. The differential localization of the dynein heavy chain in the oocyte is maintained throughout oogenesis. Our analyses of ovaries from Bic-D and egl homozygous mutant flies indicate that the corresponding gene products are required for proper accumulation of dynein to the oocyte. This might readily be explained by the apparent participation of the Bic-D and egl gene products in the establishment and maintenance of the polarized microtubule array in the 16-cell syncytial cyst (Theurkauf et al., 1994). In the absence of this polar array of microtubules, the directed migration of the dynein motor into the oocyte would not occur. Previous studies have shown that the microtubule inhibitor colchicine, similar to mutations in Bic-D and egl, can disrupt the polarized microtubule array and block oocyte differentiation (Theurkauf et al., 1993; Koch and Spitzer, 1983). Preliminary data show that the accumulation of cytoplasmic dynein in the oocyte is also disrupted by colchicine (unpublished observations). Given that recessive mutations in Bic-D and egl block dynein transport to the oocyte and oocyte differentiation, our results are consistent with the view that dynein mediates the microtubule-based transport of cargoes that are required for oocyte differentiation.

A second pattern of dynein distribution is observed later in stage 8-10 egg chambers. In the somatic follicle cell epithelia that surrounds the oocyte, dynein is concentrated at the apical surface of the columnar cells. This polarized epithelium participates in the secretion of the multilayered egg shell and is known to provide signals that are involved in the specification of both the dorso-ventral and the anterior-posterior axes in the oocyte (for reviews see Schüpbach et al., 1991; Ruohola-Baker et al., 1994). It will be interesting to determine if dynein-mediated polarized secretion by the follicle cells is a component of the signaling mechanism between the soma and germline during oogenesis. Within the oocyte, the dynein antigen becomes enriched at the posterior pole during stage 9 of oogenesis. These changes in dynein distribution may reflect a further reorganization of the oocyte microtubule cytoskeleton. In stage 7-8
egg chambers, the now posteriorly located MTOC degenerates and the apparent center and highest density of microtubule nucleation appears to shift to the anterior cortex of the oocyte (Theurkauf, 1993). Theurkauf and coworkers (1993) have shown that after brief treatments with microtubule-destabilizing agents, short microtubules remain at the anterior of the oocyte. Based on these results, the authors suggest that the more stable minus ends of the microtubules lie at the anterior cortex with the distal plus ends extending into the ooplasm. If the inference regarding microtubule polarity is correct, then it is not clear how, or if, the reorganization of microtubule polarity contributes to the apparent posterior enrichment of dynein. Given that dynein is a minus-end directed motor one might have predicted an anterior enrichment of dynein. One potential explanation is that the dynein motor may selectively bind and translocate along a subset of microtubules of opposite polarity to that inferred from the colchicine studies. Alternatively, the posterior enrichment of dynein may occur earlier in stages 2-7 as dynein actively translocates to and accumulates at the posterior MTOC. This earlier enrichment at the posterior pole might be masked by the elevated level of soluble dynein present throughout the ooplasm at these stages. Later during stages 9 and 10 the increased growth of the oocyte may effectively lower the concentration of soluble dynein and reveal the localized enrichment of dynein at the posterior pole. On this scenario, the gradual reorganization of microtubule polarity during stages 7-10 and the initiation of cytoplasmic streaming in stage 10b might contribute to the reduction in dynein localization at the posterior pole that we observe in oocytes after stage 10.

While we favor a model in which the dynein motor actively translocates to the posterior MTOC, we cannot eliminate alternative models. For example, the dynein motor could be complexed with a plus-end directed kinesin motor and so potentially be transported to the posterior pole as a cargo of the kinesin motor. However, to date, no native kinesin motor has been shown to accumulate at the posterior end of the oocyte. Yet, an ectopically expressed fusion protein in which β-galactosidase is attached to the motor portion of the Drosophila kinesin heavy chain has been reported to be transiently localized to the posterior end of the oocyte (Clark et al., 1994). The posterior localization of dynein could also result from the differential binding and stability of dynein at the posterior pole and the degradation of unbound dynein. Regardless of the mechanism of localization, the dynein motor must be added to the list of endogenous cytoplasmic components that are asymmetrically positioned to the posterior pole of the oocyte. Significantly, dynein is the only member of the list for which an intrinsic motor activity has been demonstrated.

The accumulation of dynein to the posterior pole plasm of stage 9 oocytes is distinct from the earlier accumulation of dynein to the pro-oocyte beginning in region 2b of the germarium. This is dramatically demonstrated in the ovaries of flies homozygous for the capu<sup>kn22</sup> and spire<sup>kn9</sup> mutations. In these ovaries the posterior accumulation of dynein fails to occur, while the earlier transport of dynein to the pro-oocyte occurs normally. Similarly, only the posterior localization of dynein is affected in N<sup>o</sup>/N<sup>o</sup> ovariess. The mislocalization of dynein to the center of the oocyte is similar to the mispositioning of staufen protein and osk RNA in N<sup>o</sup>/N<sup>o</sup> ovariess. Given the requirement for capu, spire, and Notch in the proper assembly of the posterior pole plasm it is tempting to speculate that the dynein motor is also required for pole plasm assembly. In contrast dynein distribution is undisturbed in ovaries from homozygous staufen<sup>kn5</sup> Ries, suggesting that dynein is not a component of the polar plasm itself, but a component of the localization machinery. Together with the disruption of dynein accumulation in the pro-oocyte by recessive mutations in Bic-D and egl, our observations suggest that dynein might participate in multiple steps during oogenesis. In early oogenesis, dynein might play an active role in some aspect of oocyte determination, while in later stages it might function in the correct spatial distribution of morphogens involved in the specification of the embryonic axis and pole cell formation. However, whether dynein motor activity is directly responsible for transport of any components of the posterior pole plasm remains to be determined.

In summary, while the role of cytoplasmic dynein in the transport of vesicles and organelles is well established, there is some evidence to support the speculation that dynein may also function to transport informational macromolecules in the form of mRNAs, RNP complexes, and proteins (for review see Wilhelm and Vale, 1993). Genetic studies have identified a number of genes (e.g., Bic-D, egl, oskar, bicoid, staufen, cyclin B, nanos, swallow, orb, and fs(1)K10) that participate in oocyte differentiation and/or the establishment of the embryonic axes in Drosophila (for reviews see Macdonald, 1992; Ruohola-Baker et al., 1994; Spradling, 1993; Ding and Lipshitz, 1993; St. Johnston and Nusslein-Volhard, 1992; Lasko, 1992). Several of these genes encode products (mRNAs and/or proteins) that show patterns of spatial distribution during oogenesis that are similar to those that we observe for cytoplasmic dynein. For example, oskar, cyclin B, Bic-D, fs(1)K10, swallow, and orb transcripts also appear to be synthesized in the nurse cells and accumulate preferentially in the presumptive oocyte (for reviews see Macdonald, 1992; Lasko, 1992; Ruohola-Baker et al., 1994; Spradling, 1993; Ding and Lipshitz, 1993; St. Johnston and Nusslein-Volhard, 1992). In addition, the posterior enrichment of staufen protein, as well as oskar and cyclin B mRNAs, is quite similar to the pattern seen for the dynein heavy chain polypeptide. In addition to describing the first evidence for the accumulation of an endogenous molecular motor protein in the Drosophila oocyte, we demonstrate several genetic requirements for proper dynein localization. The correlation between the requirement for the gene products egl, Bic-D, capu, spire, and Notch in oocyte determination and differentiation, and their requirement for the asymmetric localizations of cytoplasmic dynein in oogenesis, supports a model in which the motor protein is actively involved in the transport of morphogenetic determinants during oogenesis. The cloning and molecular characterization of the dynein heavy chain gene provides the necessary foothold to begin to understand the relationship between the structure of the Drosophila dynein motor and its function. Mutations in the dynein heavy chain gene are currently being characterized (Gepner, J., M.-G. Li, C. Kortas, S. Ludmann, M. McGrail, and T. S. Hays, manuscript in preparation) to establish the range of developmental processes that require dynein function and to directly address whether dynein-based microtubule transport is intimately coupled to oocyte differentiation and axis specification.
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