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Identification and partial characterization of six members of the kinesin superfamily in *Drosophila*

(microtubules/polymerase chain reaction)

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ABSTRACT **Recent evidence has suggested that the principal polypeptide component of the microtubule motor protein kinesin may be a member of an extended superfamily of related motor proteins. To gain insight into how large the kinesin superfamily might be and to begin determining the potential functions in which various superfamily members might participate, we identified and partially characterized six additional members of the *Drosophila* kinesin superfamily. Genes encoding these proteins were identified by using the polymerase chain reaction with degenerate primers corresponding to highly conserved regions of the kinesin heavy-chain motor domain. Partial sequencing of the six genes revealed that they encode proteins that are 40–60% identical to the motor domain of the kinesin heavy-chain sequence. The cytogenetic locations as well as the developmental and tissue-specific expression patterns have been determined. The data suggest that each of these six kinesin-like proteins may have functions in a wide variety of cell types and tissues.**

The microtubule network is largely responsible for mediating the transport and positioning of the components of the eukaryotic cytoplasm. Microtubule-associated mechanochemical proteins generate the forces necessary for these movements, which may include vesicle transport, organelle transport, nuclear migration, chromosome segregation, and probably others. The microtubule-associated mechanochemical proteins identified thus far fall into three distinct classes of proteins: dynein (1, 2), dynamin (3), and kinesin (4).

The principal component of kinesin, the kinesin heavy chain (KHC), has recently been demonstrated to be a member of a superfamily of related proteins. This conclusion derives from the recent discovery of individual genes in several organisms (5–8) and two genes in *Drosophila* (9–11) that encode proteins closely related to KHC. The members of the kinesin superfamily all share a very similar protein domain of ≈350 amino acids. In the case of KHC, this shared domain is contained within the mechanochemical domain, which contains the elements required for microtubule motility *in vitro* (12). Therefore, the different members of the kinesin superfamily are likely to be microtubule-associated motors as well, although this has been experimentally demonstrated only for the kinesin-like† product of the *ncd* gene of *Drosophila* (13, 14).

Outside of the shared motor domain there are few apparent similarities among the kinesin superfamily members. Each known member of the kinesin superfamily has a tail domain of unique sequence attached to the motor domain. These tail domains vary in size, predicted structure, and position in the primary sequence of the proteins with respect to the motor domain. The tail domains and other unique features of the kinesin superfamily members are thought to be specialized adaptations that allow a common motor domain to carry out a wide array of specific transport processes (5, 7, 15). For example, the unique tail domains of each superfamily member may contain binding sites that allow each motor to recognize a specific cargo. If this hypothesis is correct, then the large number of cellular processes that depend upon translocation along microtubules, as well as the complexity of some movements that probably require multiple motors (e.g., meiosis and mitosis), suggest that the kinesin superfamily may have many members within all eukaryotes.

To gain insight into the dimensions of the kinesin superfamily in *Drosophila*, we searched for new members by taking advantage of the polymerase chain reaction (PCR) and the observation that there are strongly conserved regions of sequence within the kinesin-like motor domain. This approach was previously used successfully to identify and isolate the *ncd* gene of *Drosophila* (10). In this report we describe the identification and partial characterization of six additional members of the *Drosophila* kinesin superfamily.‡ This raises the number of proven KLPs in *Drosophila* to 11, including KHC, the products of the *ncd* (10) and *nod* (11) genes, and the products of two genes located near lethal(1)zeste–white 4 [*l(1)zw4*] (M. Goldberg, personal communication) and 52A (16).

MATERIALS AND METHODS

PCR. Four oligonucleotide primers were synthesized that corresponded to the regions of the kinesin-like motor domain indicated by the arrows in Fig. 1. Primers represented by arrows pointing to the right were complementary to the noncoding strand and primers represented by arrows pointing to the left were complementary to the coding strand. The nucleotide sequences (5′ to 3′) and degeneracies of the primers were as follows: primer 1, *GCGCGAATTC*-CT(T/C/G)-(A/T)A(T/C)-CT(T/C/G)-GT(T/C/G)-GA(T/C)-CT(T/C/G)-GCN-GG, 2592-fold degenerate; primer 2, *CGTCTAGA*(A/T)-(A/G)T(T/C)-NC(T/G)-(A/G)(A/T)A-NGG-(A/G)AT-(A/G)TG, 4096-fold degenerate; primer 3, AT(T/C)-TT(T/C)-GCN-TA(T/C)-GGN-CA(A/G)-ACC, 256-fold degenerate; primer 4, *GCGCGAATTC*-NTC-(A/C/G)(A/T)T-(A/G)TA-(A/T/G)AT-(T/C)TC, 288-fold degenerate. With the exception of primer 3, the primers had additional sequences (italics) encoding restriction sites (*Eco*RI or *Xba* I) to allow directional cloning of the amplification products. Either two or four G or C residues were also

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Abbreviations: KHC, kinesin heavy chain; KLP, kinesin-like protein.

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†We use the term kinesin heavy chain (KHC) to refer to proteins that have substantial amino acid sequence similarity throughout their length to *Drosophila*, squid, and sea urchin KHC; we use the term kinesin-like protein (KLP) to refer to proteins that have amino acid sequence similarity only to the motor domain of KHC.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M74427–M74432).

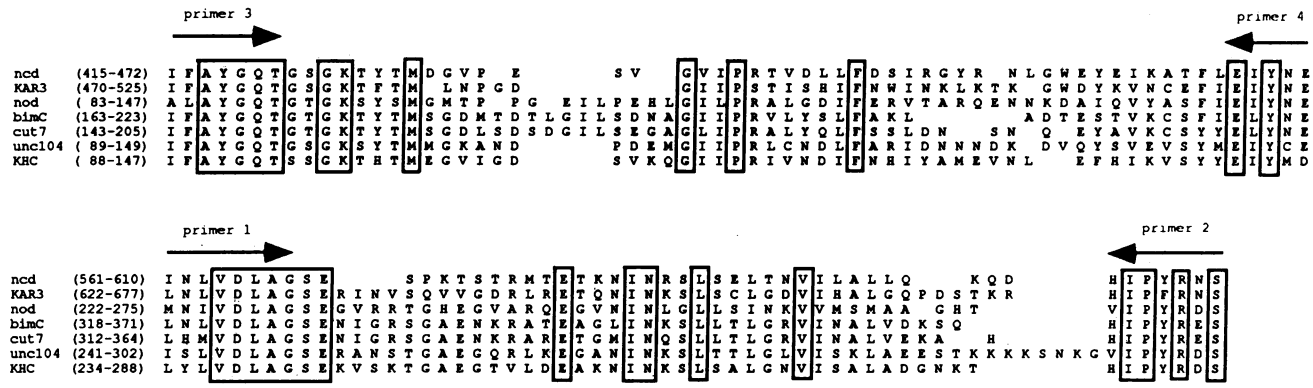

FIG. 1. Locations of primers used for PCR. Two sets of primers were designed that would amplify the two regions of the KHC motor domain indicated by the residue numbers. Amino acid residues that are completely conserved in all seven members of the kinesin superfamily are boxed. The nucleotide sequences of the primers are described in *Materials and Methods*.

included at the ends of the primers to increase the efficiency of the restriction enzyme digests of the amplification products.

Genomic DNA isolated from wild-type *Drosophila melanogaster* was amplified with primers 1 and 2, or with primers 3 and 4. Primers 1 and 2 were used at final concentrations of 15 and 30 μM, respectively, with the following temperature cycle: 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min, for 40 cycles. Primers 3 and 4 were used at final concentrations of 20 μM, with the following temperature cycle: 94°C for 1 min, 30°C for 1.5 min, and 72°C for 1 min, for 10 cycles, followed by 30 cycles of 94°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min. The first 10 cycles with primers 3 and 4 were at a lower annealing temperature because of the low initial melting temperature of primer 3. PCR was performed in a Perkin–Elmer/Cetus DNA thermal cycler with AmpliTaq DNA polymerase and the buffer suggested by the manufacturer.

Isolation and Sequencing of PCR Amplification Products. The major amplification products were purified from agarose gels, treated with the Klenow fragment of DNA polymerase I, and then digested either with *Eco*RI and *Xba* I (primers 1 and 2 reaction) or with *Xba* I (primers 3 and 4 reaction). The products of primers 1 and 2 were cloned into the *Eco*RI and *Xba* I sites of pBluescript SK (Stratagene). The products of primers 3 and 4 were cloned into the *Hin*cII and *Xba* I sites of pBluescript SK. To identify clones encoding KLPs, clones containing inserts were picked at random and sequenced using a Sequenase kit (United States Biochemical).

Isolation of Genomic Clones. A *Drosophila* genomic library (strain *dp cn bw*), constructed by R. Padgett (Rutgers University) in the vector λ DASH II (Stratagene), was probed by standard methods (17). Probes were prepared by excision and gel purification of cloned PCR fragments from plasmids and then labeled by the random primer method (18).

***In Situ* Hybridization to Polytene Chromosomes.** With the exception of clones containing the KLP 1 gene, all the λ phage genomic clones were labeled with biotinylated dUTP by oligonucleotide priming (18). However, genomic clones containing the KLP 1 gene also contained repeated DNA, since *in situ* hybridization using the full-length KLP 1 genomic clones resulted in over 70 sites of hybridization on polytene chromosomes. A genomic KLP 1 clone digested with *Bam*HI and *Hin*dIII produced a 2.5-kilobase (kb) subfragment containing no repeated DNA, which was then excised and gel-purified for use as a probe. Polytene chromosomes squashes were made from either Canton S or *yw* strains of *D. melanogaster*. The sites of hybridization were visualized using a Vectastain Elite kit (Vector Laboratories).

Analysis of RNA Expression. Total RNA was isolated by standard techniques (17) from 0- to 12-hr embryos, mixed larval stages, mixed pupal stages, adult males, adult females, adult heads, Schneider S2 cultured cells, adult males without testes, testes, adult females without ovaries, and ovaries. Poly(A)$^+$ RNA was selected from total RNA with a Fasttrack kit (Invitrogen, San Diego). RNA samples were fractionated in 1.1% agarose gels in the presence of 6% formaldehyde and transferred to GeneScreen*Plus* (DuPont). Probes for the newly identified genes were prepared by excision and gel purification of the cloned PCR fragments from plasmids and labeled by random priming (18). Gel-purified full-length cDNAs were used as probes for the KHC, *ncd*, and *nod* genes. Hybridization conditions were as described by Church and Gilbert (19).

RESULTS

Identification of Genes Encoding KLPs in *Drosophila*. The KHC motor domain of the kinesin superfamily contains several regions where there is almost complete conservation of amino acid sequence among all known members of the kinesin superfamily. Two pairs of degenerate primers complementary to four of these regions (Fig. 1) were used in separate PCR experiments to amplify segments of genes encoding KLPs from *Drosophila* genomic DNA. Since primer 3 encodes a portion of an ATP-binding consensus sequence (20), the DNA segments amplified with primers 3 and 4 are likely to encode a region of the KLPs that contains at least part of the ATP binding site. Primers 1 and 2 correspond to sequences within a region of the KHC motor domain that may interact with microtubules (21).

Amplification of *Drosophila* genomic DNA with primers 1 and 2 resulted in a major product of ≈170 base pairs (bp), the size expected from the known cDNA sequences encoding KLPs. In addition to the major amplification product, there were several minor products both larger and smaller than the major product (data not shown). Control amplification reactions containing only primer 1 or primer 2 demonstrated that some of the minor products were amplified with the same primer at both ends and therefore were not likely to be sequences related to KHC. PCR amplification of genomic sequences by a single primer of a pair of degenerate primers has been reported previously (10). Some of the minor products, however, may encode KLPs of an unusual size or that are interrupted by short introns.

To identify genes encoding new KLPs, the ≈170-bp products generated by amplification with primers 1 and 2 were purified and cloned. From a total of 54 PCR fragments that were cloned and sequenced, 34 were found to encode KHC or KLPs. Fifteen of the remaining 20 clones encoded se-

quences that were not recognizably related to KHC, although they contained one or both of the primers. The remaining five clones contained neither primer. The PCR fragments encoding KLPs fell into five classes. Each class encoded a predicted protein that was 40–60% identical to KHC in the region between the primers (Fig. 2*a*). Although we do not know whether the five classes have sequence similarity to KHC outside of the motor domain, we have designated them as KLPs 1–5 for simplicity. In addition to sequences encoding KLPs 1–5, segments of the KHC and *ncd* genes were recovered. However, no sequences from the *nod* gene were found, indicating that the screen was not complete, although reactions with the degenerate primers and a *nod* cDNA plasmid demonstrated that the primers were capable of amplifying *nod* sequences.

Since the first screen with primers 1 and 2 was incomplete, a second screen was conducted using degenerate primers 3 and 4. Amplification of *Drosophila* genomic DNA with primers 3 and 4 resulted in a major amplification product of ≈180 bp, as expected from the cDNA sequences encoding known KLPs. As with primers 1 and 2, primers 3 and 4 produced minor amplification products in addition to the major product. As before, the major amplification product was purified and cloned. Of 70 cloned PCR fragments that were sequenced, 51 were found to encode KHC or KLPs. However, in addition to segments of the KHC and *ncd* genes, only two classes of fragments encoding unique KLPs were identified. Again, no *nod* segments were found. The remaining 19 sequenced fragments had no recognizable similarity to KHC.

Isolation and Cytogenetic Localization of the KLP Genes. Characterization of KLPs 1–5 was begun by using the cloned PCR segments isolated in the screen with primers 1 and 2 to isolate genomic clones corresponding to each of the genes encoding KLPs 1–5. Since the regions of the motor domain that were amplified in the two PCR screens were nonoverlapping, the genomic clones for KLPs 1–5 were probed with the two unique PCR fragments amplified from genomic DNA with primers 3 and 4. One of the unique PCR fragments hybridized with a genomic clone for KLP 5, while the other unique PCR fragment did not hybridize with any of the genomic clones for KLPs 1–5. It was therefore designated KLP 6 (Fig. 2*b*). The cloned KLP 6 PCR fragment was then used to isolate a genomic clone.

The identities of the isolated KLP genomic clones were confirmed by sequencing of the appropriate regions. Primers complementary to sequences inside of the amplified regions were constructed for each of these KLPs and used to sequence the region of the genomic clones between the PCR primers, as well as the regions under the primers, and short regions beyond the primers (Fig. 2). Additional regions of strong amino acid sequence conservation (e.g., SSRSH) were found outside of the PCR-amplified regions, further confirming that these genes are bona fide kinesin superfamily members. (Nucleotide sequences and genomic clones are available upon request.)

To determine whether these KLP genes might correspond to genes identified by previously characterized mutations, and to facilitate future genetic analysis, the KLP genomic clones were used to locate the KLP genes in the *Drosophila* genome by *in situ* hybridization to polytene chromosomes. Six unique locations were determined (Table 1). None of these KLP genes are located in regions that contain well-characterized mutations with phenotypes that suggest an obvious defect in a motor protein function.

Expression of the KLPs. Since the expression patterns of the KLP genes could provide a preliminary suggestion of potential function, the six KLP genes were further characterized by determining the pattern of their expression in several developmental stages and in several adult tissues. The developmental stages examined were 0- to 12-hr embryos, mixed larval stages, mixed pupal stages, adult males, and adult females. The adult tissues examined were heads, testes, males without testes, ovaries, and females without ovaries. Schneider S2 cultured cells were also studied. RNA from each of these sources was used to prepare Northern blots, which were probed with a cloned PCR fragment corresponding to each of the six KLP genes. For standardization of the amount of RNA loaded, the Northern blots were probed with a cDNA encoding RP49, a ribosomal protein (22).

For comparative purposes the Northern blots were also probed with cDNA clones encoding the KHC, *ncd*, and *nod* proteins. We found that KHC is expressed in all of the tissues examined (Fig. 3), as might be expected since Western blot analysis suggests that KHC is ubiquitously distributed in *Drosophila* (23). Interestingly, KHC is expressed much more abundantly in testes and ovaries than in the other tissues (Table 1). In contrast to KHC, expression of the *ncd* and *nod* genes is more limited—neither is detectably expressed in nonproliferating tissue, i.e., heads and adult bodies without gonads (data not shown; see also refs. 11 and 24). Under our conditions the distribution of the *nod* transcript is more limited than that of the *ncd* transcript, since *nod* mRNA was not detected in all developmental stages or in cultured cells and testes.

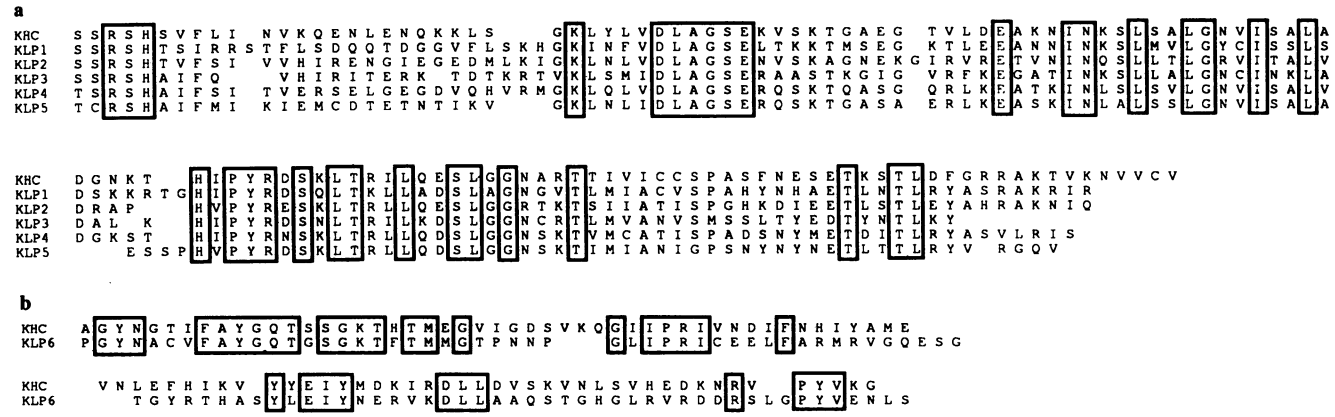

FIG. 2. Partial amino acid sequences of KLPs 1–6. For comparison the comparable KHC sequence is shown on the top line. Genomic clones corresponding to KLP 1–6 genes, or appropriate regions of the genomic clones subcloned into pBluescript, were sequenced to confirm the sequence between the PCR primers and to obtain additional sequences outside of the PCR-amplified regions. (*a*) Sequences of KLPs 1–5, which were identified with primers 1 and 2. (*b*) Sequence of KLP 6, which was identified with primers 3 and 4. Residues that are completely conserved in all of the genes are boxed.

Table 1. Expression of KHC and KLP transcripts

Gene	Cytogenetic location	Transcript size, kb	mRNA expression										
			Embryos	Larvae	Pupae	Males	Females	Cultured cells	Heads	Male bodies	Testes	Female bodies	Ovaries
KHC	52F	4.0	++	++	++	++	++	+++	++	+	++	+	++
ncd	99B	2.3	++	+	+	+	+	+++	ND	ND	+	ND	++
nod	10A	2.4	+++	ND	ND	ND	+	ND	ND	ND	ND	ND	+++
KLP 1	54D	?	+*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
KLP 2	61F	3.9, 3.7	++	+	+	+	+	+++	ND	ND	++	ND	++
KLP 3	67A	3.0, 2.8	+	+	+	++	+	++	ND	ND	+++	ND	++
KLP 4	64D	2.6	+	+	+	++	+	+	+++	ND	ND	ND	++
KLP 5	68D	3.1	+	+	+	++	+	++	+++	+	+	+	+++
KLP 6	98A	?	+*	ND	ND	ND	ND	ND	+*	ND	ND	ND	ND

ND, not detected.
*Expression data were obtained by PCR-amplifying 0- to 4-hr embryo and adult head cDNA libraries with primers specific for these KLP genes.

The expression of the six KLP genes, like that of the KHC, *ncd*, and *nod* genes, is apparently not limited to a specific developmental stage or tissue (Table 1). For example, the KLP 5 message is expressed throughout development and in all adult tissues we examined (Fig. 3). In adults, the KLP 5 gene appears to be expressed most abundantly in heads and ovaries. The KLP 4 gene has an expression pattern similar to that of the KLP 5 gene; it is expressed throughout development, and in adults it is also most abundant in heads and ovaries (Fig. 3). However, expression of the KLP 4 gene is not apparent in testes and adult bodies, although longer exposure or a higher-specific-activity probe might have revealed expression in these tissues. The KLP 2 and 3 genes are expressed throughout development, but in adults are expressed only in the gonads (Fig. 3). The messages for KLP 2 and 3 were relatively abundant in Schneider S2 cultured cells. The KLP 2 probe recognizes two transcripts (3.9 and 3.7 kb) in embryos and possibly in cultured cells. The KLP 3 probe recognizes a different-size transcript in males and testes (3.0 kb) than in females and ovaries (2.8 kb). In both cases we do not know whether the multiple signals represent alternative transcripts or cross-hybridization with as yet unidentified kinesin superfamily members.

The expression pattern and transcript size are not known for the KLP 1 and 6 genes, since these did not give signals on the same Northern blots when probes comparable to the other KLP gene probes were used. However, these KLP genes are likely to be expressed, since we have been able to use PCR to amplify fragments of the correct size from a 0- to 4-hr embryo cDNA library with specific primers for KLP 1, and from both a 0- to 4-hr embryo cDNA library and an adult head cDNA library with specific primers for KLP 6 (data not shown).

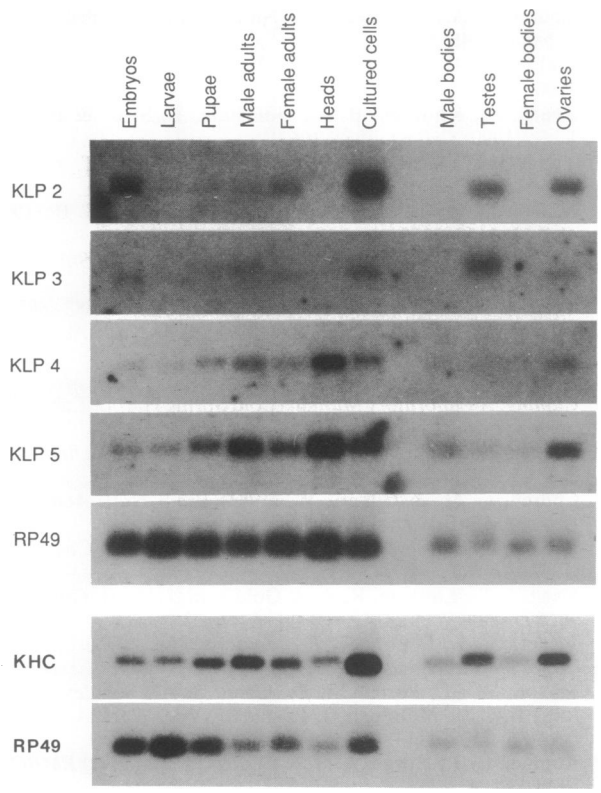

FIG. 3. Developmental and tissue expression patterns of KLPs 2–5 and KHC. Samples (1 μg) of poly(A)$^+$ RNA from the developmental stages, adult heads, and cultured cells or samples (40 μg) of total RNA from gonads and adult bodies without gonads were analyzed by Northern blotting using the cloned PCR fragments as probes. The developmental stages were 0- to 12-hr embryos, mixed larval stages, mixed pupal stages, and adult males and females. The same blot was probed, stripped, and reprobed with clones for KLPs 2–5. A separate blot with the same samples was probed with KHC cDNA. Each blot was probed with a cDNA encoding ribosomal protein RP49 to quantitate the amount of RNA loaded.

DISCUSSION

Kinesin Superfamily Size in *Drosophila*. Our findings imply that the kinesin superfamily in *Drosophila* is much larger than was previously thought. In this work, we have identified six more members of the kinesin superfamily in *Drosophila* on the basis of their sequence similarity to highly conserved regions of the KHC motor domain. These six, in addition to two recently reported and confirmed KLP genes near *l(1)zw4* (M. Goldberg, personal communication) and at 52A (16), raise the total number of bona fide KLP genes in *Drosophila* to 11 (including KHC, *ncd*, and *nod*), which establishes that the kinesin superfamily in any single organism can be quite large. However, four arguments suggest that our PCR screen is incomplete and that the number of KLPs in *Drosophila* is larger still. (*i*) No segments of the *nod* gene were recovered even though *nod* sequences should have been amplified with our degenerate primers. (*ii*) Unique KLP sequences were identified in two independent PCR screens and only one of the new KLP sequences was isolated in both screens. (*iii*) By isolating only PCR fragments of a size expected from cDNA sequences encoding known KLPs, we may have missed segments of KLP genes that have an unusual size or those that are interrupted by introns. (*iv*) It was recently reported (16) that a mixture of PCR fragments obtained by amplifying cDNA libraries with degenerate KHC primers similar to ours hybridized to 35 sites in the *Drosophila* genome. Four of these sites were directly confirmed by sequence analysis to encode bona fide KLPs [KHC, *ncd*, a gene near *l(1)zw4*, and

a gene at 52A], one mapped near the *nod* gene, and two mapped near genes that are reported here [KLP 2 and KLP 5; however, we found expression of the KLP 2 and KLP 5 genes in testes whereas previous workers (16) did not, causing us to doubt whether the genes described in the two reports are in fact the same]. Although our recovery of sequences not related to KHC with degenerate primers suggests that some of the remaining unconfirmed locations reported (16) may not encode KLPs, many of the reported locations could correspond to true KLPs. Hence, taken together, the data suggest that the kinesin superfamily in *Drosophila*, and by extension in other eukaryotes, is large and contains a minimum of 11 members.

KLP Function. One potential indication of function of KLPs 1–6 would be strong sequence homology to a previously identified KLP that has a genetically defined function. For example, genes encoding KHC have been cloned and sequenced from sea urchin (25) and squid (26) in addition to *Drosophila* (21). Comparison of the region of the KHC sequences between primers 1 and 2 reveals that sea urchin and squid KHC are 95% and 96% identical, respectively, to *Drosophila* KHC. Therefore, it might be expected that KLPs that were identified genetically in other organisms—e.g., the *bimC* (5), *cut7* (6), *KAR3* (7), and *unc-104* (8) gene products—might have homologs in *Drosophila* with a similar degree of sequence conservation. However, the sequence identities between the six KLPs reported here and *bimC*, *cut7*, *KAR3*, and *unc-104* are only 40–60% in the region between primers 1 and 2. Therefore, none of the six KLPs we have identified are conspicuous *Drosophila* homologs of the KLPs identified in other organisms, and conclusions about function cannot be drawn from the sequence information.

Specific functions for KLPs 1–6 are not suggested by their patterns of expression either, since none of them are restricted in expression to a specific tissue or developmental stage that we have examined. However, *Drosophila* KLPs 1–6 can be broadly categorized as having an expression pattern similar to KHC or similar to the *ncd* and *nod* genes. An important distinction can be drawn between the expression of the KHC gene and that of the *ncd* and *nod* genes—the KHC gene is expressed ubiquitously, whereas the *ncd* (ref. 24 and our data) and *nod* (ref. 11 and our data) genes are not detectably expressed in nonproliferating tissues (i.e., heads, and adults without gonads). The KLP 4 and 5 genes are similar to the KHC gene in that they are expressed in nonproliferating tissue, which suggests that they may have a primary cellular function not related to mitosis or cell division. Both are also relatively more abundantly expressed in heads, which may be significant; perhaps they have neurological functions like the *unc-104* protein and KHC. In contrast, the KLP 2 and 3 genes are apparently not expressed in nonproliferating tissues. Therefore, the KLP 2 and 3 genes, like the *ncd* and *nod* genes, may be involved primarily in cell division, although other interpretations are possible.

A final, general conclusion about KLP functions emerges from our and others' expression data. In short, the patterns of expression of the KHC, *ncd*, and *nod* genes are not well correlated with their genetically defined functions. Thus, KHC null mutants in *Drosophila* appear to have primarily neurological defects (27), even though the KHC gene is not expressed any more abundantly in heads than in other tissues and, in fact, is expressed most abundantly in testes and ovaries. Similarly, although genetic analysis suggests that the *ncd* gene product has an essential role only in the proper segregation of chromosomes during female meiosis and possibly during early embryonic mitoses (28), the *ncd* gene is expressed throughout development and even in testes and cultured cells. Similar arguments have been discussed for the *nod* gene (11). The expression of the KHC, *ncd*, and *nod* genes outside of the tissues expected from the phenotypes of their mutants suggests that all of these proteins may have additional, nonessential functions. It is possible that these functions can be performed by redundant motors in the absence of the KHC, *ncd*, or *nod* gene products. Similarly, the relatively general expression of the KLP 1–6 genes raises the possibility that they may also have functions that overlap with each other or overlap with the functions of the KHC, *ncd*, or *nod* genes.

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