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Cloning by insertional mutagenesis of a cDNA encoding *Caenorhabditis elegans* kinesin heavy chain

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ABSTRACT **An additional genetic locus in *Caenorhabditis elegans*, *unc-116*, was identified in a screen for mutations resulting in defective locomotion. *unc-116* was cloned by use of a transposon insertion mutant and the physical and genetic map of the genome. The cDNA sequence predicts an 815-amino acid protein. Based upon sequence comparison and secondary structure predictions, *unc-116* encodes all three domains of the kinesin heavy chain: the motor, stalk, and tail. While the motor and tail domains have a high degree of identity to the equivalent domains of cloned kinesin heavy chains, the rodII domain of the stalk is significantly shorter than those previously reported and is not predicted to form a coiled-coil α-helix. Analysis of mutational defects in two *C. elegans* genes encoding anterograde motor molecules, *unc-116* and *unc-104*, should provide insight into the *in vivo* functions of these members of the kinesin heavy chain superfamily.**

Intracellular transport of membrane-bound organelles is essential for processes common to all eukaryotic cells and for specialized functions such as secretory vesicle movement in endocrine cells. The molecular motor, kinesin, is implicated in the microtubule-based anterograde transport of membrane-bound organelles. Although kinesin has been shown to transport a number of specific organelles, the identity of additional cargo in other cell types and the particular biological processes that require kinesin-mediated transport *in vivo* remain to be defined conclusively.

Vale *et al.* (1, 2) initially identified and purified kinesin from squid axoplasm based on an *in vitro* assay for (+)-end-directed transport along microtubules. Kinesin is localized to a variety of tissue and cell types, and to subcellular organelles in cultured cells and *in vivo*. *In vitro* assays and the distribution of kinesin are consistent with the hypothesis that kinesin functions as an anterograde microtubule-based motor in neuronal and nonneuronal cells.

The subunit composition of the kinesin molecule and functional domains within the kinesin heavy chain (khc) have been characterized. Native kinesin isolated from bovine brain (3) or sea urchin (4) consists of two heavy chains and two light chains. The heavy chain contains three domains: (*i*) an amino terminal globular motor domain that is necessary and sufficient for motility (5, 6) and contains a consensus ATP binding site and a microtubule-binding region (7); (*ii*) an α-helical region predicted to form a coiled coil (8), which allows for dimerization of the khc; and (*iii*) a globular tail domain (7, 9). The tail domain serves as a binding site for the light chain, appears to interact with membrane-bound organelles (9, 10), and also can bind microtubules in cell culture (11).

The complexity of the systems in which kinesin has been studied and the nature of *in vitro* preparations have precluded a full understanding of kinesin function. We report the identification of the *unc-116* locus by mutation, the cloning of the *unc-116* gene, and the determination of the cDNA sequence,§ and we show that *unc-116* encodes the khc in *Caenorhabditis elegans*, which has a simple anatomy and well-described development. *C. elegans* khc differs from other khcs in the rodII domain in length and predicted structure, but the molecule is otherwise quite similar to *Drosophila* (7), sea urchin (12), squid (13), and human (11) khcs. In addition, khc mutation alters embryogenesis, larval development, and neuromuscular function in *C. elegans*.

MATERIALS AND METHODS

Isolation of *e2281*. Seventy-two thousand individuals of the TR679 strain were screened by touching the animal on the tail and on the head in turn and observing the elicited response. Individuals exhibiting abnormal backward locomotion and normal forward locomotion relative to wild type were isolated and subsequently outcrossed 14 times into the standard N2 Bristol strain to eliminate background transposon insertions and to stabilize the insertion. One of the mutations, *e2281*, mapped to a previously unrecognized locus within the genome. For historical reasons, two additional *e* numbers, *e2282* and *e2310*, were allocated to the *e2281* allele; for the sake of consistency we have agreed to use *e2281* from now on.

Genetic Mapping of the *e2281* Locus. *e2281* was mapped to the third chromosome by linkage to *dpy-17* (*e164*) and absence of linkage to other markers on chromosomes I, II, IV, V, and X. *e2281* failed to complement previously identified loci within the region (*unc-47, unc-69, unc-25, sma-2, sma-3, dpy-19*, and *lin-21*). The gene maps to the cluster of chromosome III between *unc-86* and *sma-2*, 0.5 ± 0.2 map unit to the right of *unc-86*. From estimates of distance in the cluster, the gene was expected to lie within an interval of 60 kilobases (kb), ≈150 kb to the right of *unc-86*. Recombinant strains generated during mapping were used for Southern analysis.

Preparation of DNA. Nematodes were cultured, and DNA was extracted by previously described procedures (14). Cosmid and plasmid DNA were extracted by standard methods (15). Phage DNA was obtained by using LambdaSorb (Promega) and following the supplier's protocol.

Southern Blot Hybridization. Southern blotting techniques were essentially as described (15); probes were synthesized by the random-primer method with ^{32}P. Cosmid clones were provided by A. Coulson and J. Sulston (Medical Research Council, Cambridge, U.K.) from their genomic library, and restriction fragments were generated by standard subcloning methods (15). Tc5 probes were synthesized from the TR31A and TR33 clones.

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Abbreviations: khc, kinesin heavy chain; RFLP, restriction fragment-length polymorphism.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L19120).

cDNA Cloning. Duplicate filters of a cDNA mixed-stage hermaphrodite library were screened with cosmids RO5D3 and ZC262 (15). The longest of four double-positive clones (≈1.4 kb) contained an endogenous *Eco*RI site at one end and so presumably was not full-length. A well-methylated cDNA mixed-stage hermaphrodite library in λgt10 was screened with a nonradioactively labeled probe (Genius kit; Boehringer Mannheim) of the 1.4-kb clone. Of 20 purified clones characterized for size, the 3 longest were excised from the vector with *Eco*RI, and the resulting *Eco*RI fragments were subcloned into pBS KSII(+) (Stratagene).

Sequencing. The three *Eco*RI fragments of a single cDNA clone (0.4, 0.9, and 2.2 kb) were sequenced by the dideoxy method (16) with the phage T7 polymerase kit (Pharmacia) on double-stranded templates. Both strands were completely sequenced with a combination of nested *Exo* III deletions (17) and oligonucleotide primers. The ends of the three *Eco*RI fragments from a second, shorter clone were also sequenced, and no discrepancies were found.

The 1.7-kb *Hin*dIII genomic fragment from cosmid RO5D3 and a 2.7-kb *Eco*RI fragment of ZC291 (a smaller cosmid spanning the region) were each subcloned into pBS KSII(+), and unidirectional deletions were made. The complete sequence of the 1.7-kb fragment and partial sequence of the 2.7-kb fragment were obtained by automated sequencing and were aligned with the *unc-116* cDNA sequence. The 1.7-kb sequence contained the two internal *Eco*RI sites and so permitted ordered assembly of the cDNA *Eco*RI fragment sequences.

Localization of Tc5 in *e2281*. We constructed a size-selected (3.2–3.8 kb) *Eco*RI library from *e2281* genomic DNA in Lambda ZAP (Stratagene) and screened ≈110,000 plaque-forming units by using the 1.67-kb *Hin*dIII genomic fragment, which is polymorphic in *e2281*. Three of 10 positives were subcloned into pBS SKII(+) by using the *in vivo* excision protocol of the manufacturer. The Tc5–*unc-116* junction was sequenced as described above by using T3 and T7 primers and an *unc-116*-specific primer.

Computer Analysis. Sequence assembly and alignments of nucleotide and protein sequences were executed on a VAX by using the University of Wisconsin Genetics Computer Group programs (18) BESTFIT and ASSEMBLE. Searches of GenBank, National Biomedical Research Foundation/Protein Identification Resource, and SwissProt data banks were performed by using the FASTA algorithm (19). The MAP program was used for conceptual translations of nucleotide sequence. For secondary structure analysis, the PLOTSTRUCTURE program was used (20). Multiple alignments were generated with the CLUSTAL program (21).

RESULTS

Identification and Genetic Mapping of the *unc-116* Locus. We identified a previously unrecognized locus, *unc-116*, in a screen for mutants with specific locomotor defects. The allele, *e2281*, was isolated from the TR679 strain of *C. elegans* in which native transposons within the genome are mobile (22, 23). *e2281* specimens responded to stimulation by a head tap with little, abnormal, or no backward locomotion, while wild-type animals move 1–3 body lengths backward.

An additional allele of *unc-116*, *rh24*, was independently isolated during a screen for mutants with nervous system defects (J. Plenefisch and E. Hedgecock, personal communication). Of the *rh24* embryos, 77% died before hatching; the lethality is a strictly maternal effect (our data; also J. Plenefisch, personal communication). *rh24* specimens exhibited a progressive loss of mobility throughout the larval stages. Adults retained the ability to make minimal head movements and exhibited pharyngeal pumping, but displayed neither spontaneous nor elicited locomotion. *rh24* specimens failed to reach their full length and became increasingly dumpy in appearance relative to age-matched wild-type specimens. *rh24/rh24* progeny of heterozygous hermaphrodites displayed the same larval and adult phenotype as progeny of homozygous hermaphrodites but displayed no embryonic lethality.

The *unc-116* locus was mapped genetically to chromosome III by use of markers on each of the six chromosomes and then was mapped within chromosome III to the *LG* cluster between the *unc-86* and *sma-2* loci. Cosmids that spanned the region were identified on a physical and genetic map of the *C. elegans* genome (24, 25).

The *e2281* Allele Contains a Tc5 Transposon Insertion. The transposon-tagged allele, *e2281*, was used to clone the *unc-116* locus. Southern blots of genomic DNA from a series of *e2281* recombinant strains were probed with cosmids ZC262, ZK251, RO5D3, ZK345, and ZK353 to identify a restriction fragment-length polymorphism (RFLP) in the transposon insertion mutant. Cosmids ZC262, ZK251, and RO5D3 recognized a RFLP on blots of *Hin*dIII- and *Eco*RI-digested DNA, while ZK345 and ZK353 did not, placing the insertion to the left of these latter cosmids. When the *Hin*dIII blot was probed with RO5D3, a 1.7-kb band was visible in recombinant strains wild type for the *unc-116* phenotype (Fig. 1*A*, lanes 1–7) but was absent in recombinant strains that retained the *unc-116* mutant phenotype. However, the mutant strains contained an extra band at 4.9 kb (lanes 8–14). The polymorphism consistently segregated with the phenotype after a series of recombinations, leading us to conclude that the molecular defect was a 3.2-kb insertion into a 1.7-kb *Hin*dIII fragment of the *unc-116* locus.

We identified the insertion as a Tc5 transposon by Southern analysis of *unc-116* recombinant strains. With a Tc5 probe, an extra 3.6-kb band was present in the mutant strains (Fig. 1*B*, lanes 8–14) and was absent in the wild-type strains (lanes 1–7). The extra Tc5 band cosegregated with the phenotype in all cases. In addition, on Northern blots, both a Tc5 and *unc-116* probe recognized an additional mRNA in *e2281* that was ≈3.2 kb longer than the wild-type message (data not shown).

Isolation and Characterization of Unc-116 cDNAs. Two minimally overlapping cosmids, RO5D3 and ZC262, which identified the RFLP on Southern blots, were used to probe a mixed-stage hermaphrodite cDNA library, and double-positive clones were selected. The longest of these contained an endogenous *Eco*RI site and was therefore used to probe a well-methylated cDNA library. Both strands of a putative full-length cDNA (≈3.5 kb) were sequenced. By cosmid blot analysis, the cDNA was mapped to the genomic region that is polymorphic in the *e2281* allele (data not shown).

Conceptual translation of the 3566-nucleotide sequence revealed a 2445-base-pair open reading frame, predicted to encode an 815-amino acid protein (Fig. 2*A*). The AUG start codon of this open reading frame has numerous stop codons 5′ to it in all three forward frames, and the proximal upstream bases to the AUG closely match the translation start consensus sequence derived for *Drosophila* genes (26). The sequenced cDNA has ≈500 and ≈620 base pairs of 5′ and 3′ untranslated regions, respectively. A search of GenBank and National Biomedical Research Foundation/Protein Identification Resource (NBRF), SwissProt, and newNBRF data bases (April 1991) revealed a strong homology between *unc-116* nucleotide sequence and the *Drosophila* khc (7).

Unc-116 Sequence Analysis. Based upon sequence comparison as well as secondary structure predictions *unc-116* encodes the three domains of the khc: motor, stalk, and tail. Amino acids 1–358 of unc-116 are 75% identical to the mechanochemical domain of *Drosophila* khc (amino acids 1–362); the consensus ATP binding site ["sequence A" (27); "segment I" (28)] indicated in Fig. 2*A* is 100% identical

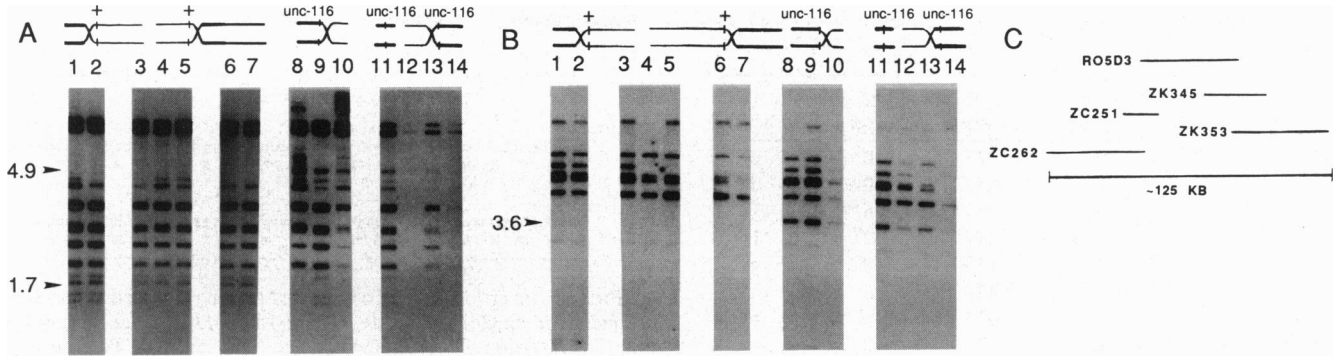

FIG. 1. Detection of RFLPs on Southern blots of recombinant *e2281* DNA by genomic cosmid RO5D3 and by transposon Tc5. (*A*) Recombinant *e2281* genomic DNA digested with *Hin*dIII was electrophoresed, transferred to a nylon membrane, and hybridized to random-primer-labeled cosmid RO5D3. The polymorphic fragment (1.7 kb in wild-type strains, 4.9 kb in *unc-116* mutant strains) that cosegregated with the phenotype is indicated. Recombinant strain genotypes determined by phenotypic markers are summarized at the top: tick marks indicate the *unc-116* locus; boldface lines correspond to the *e2281*-containing chromosome; normal lines correspond to the wild-type (N2) chromosome. Lanes: 1–7, wild type for *unc-116* (1–3, mutant for nearby markers to the left; 4–7, mutant for nearby markers to the right); 8–10, mutant for *unc-116*, wild type to the right; 11, *unc-116* mutant (nonrecombinant); 12–14, mutant at *unc-116*, wild type to the left. Lanes from a single blot were rearranged for clarity of presentation. (*B*) DNA samples from the same strains as in *A* were digested with *Eco*RI, separated and transferred as in *A*, and were hybridized to random-primer-labeled Tc5 DNA. Arrowhead indicates the Tc5 copy that cosegregated with the phenotype; DNA length is given in kb. Other fragments that hybridized to the Tc5 probe but did not cosegregate with the phenotype represent additional irrelevant copies of Tc5. Lanes from a single blot were reordered as in *A* for clarity; genotype schema is the same as in *A*. (*C*) Cosmids in the region to which *unc-116* maps are schematically illustrated.

between the two. The amino-terminal domain of unc-116 is 71–73% identical to the motor domain of khc from squid, human, and sea urchin and has significantly less identity with other khc-like molecules [e.g., 41% identical to the motor domain of *C. elegans* unc-104 (29)]. The putative motor domain of unc-116 also resembles the globular motor domain of the khc (7, 9) on the basis of predicted secondary structure consisting of alternating α-helix, β-sheet, and turns (Fig. 2*B*).

Among khcs, the domain following the motor domain shows little sequence conservation but a similar structure of two coiled coils connected by a hinge region (13). In unc-116, the 260 amino acids following the motor domain of unc-116 (amino acids ≈420–680) are predicted to form an extended α-helix, with an interruption between amino acids 554 and 598 (Fig. 2*B*). Within the small non-α-helical (hinge) region, the two proline and three glycine residues would minimally cause kinks and flexibility in the α-helix. The hinge also lacks extended α-helical structure and instead comprises alternating turns, β-sheet, and short stretches of α-helix, consistent with the predicted and observed structures of other khcs.

The first 130 amino acids of the α-helical region (rodI) contain a heptapeptide repeat characteristic of coiled-coil α-helices (30, 31) (Table 1). Over 18 repeats, positions a and d in the heptad tend to be hydrophobic in 61% and 63% of the cases, respectively. The remaining heptad positions frequently contain either a positively or negatively charged residue (50–63%). Therefore, rodI is predicted to form a coiled coil, which would allow *C. elegans* khc to dimerize and also would serve as a relatively rigid spacer between the motor and rodII/tail domains of the molecule.

When the remaining helix beyond the hinge (rodII, residues 597–680) is tabulated as a heptapeptide structure, all posi-

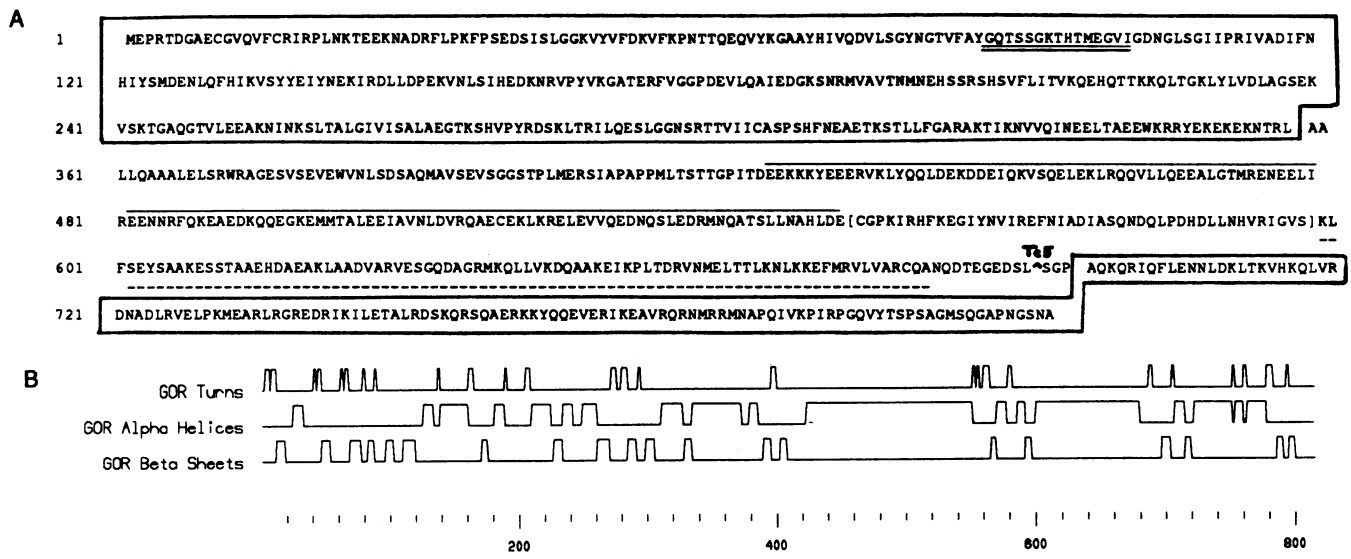

FIG. 2. Deduced amino acid sequence encoded by *unc-116* cDNA and predicted secondary structure. (*A*) Approximate borders of khc domains are indicated as follows: the motor domain by a large box (positions 1–358); the consensus ATP binding site by double underlines (88–102); rodI by an overline (423–553); hinge by brackets (554–596); rodII by a broken underline (599–682); and the tail domain by a box (696–815). The site of the Tc5 insertion is also indicated. (*B*) Secondary structure predictions of turn, α-helix, and β-sheet are plotted against the amino acid number; GOR refers to Garnier, Osguthorpe, and Robson algorithms (20).

Table 1. Residue composition of stalk α-helices

α-Helices	Residues, %			
	Hydrophobic	(+)-Charged	(−)-Charged	Position in heptad
RodI (aa 423–553)	47	32	16	c
	63	21	0	d
	16	21	26	e
	11	11	47	f
	16	5	58	g
	61	11	6	a
	11	11	39	b
RodII (aa 599–682)	67	17	8	—
	50	33	8	—
	42	17	25	—
	42	33	17	—
	25	17	25	—
	25	17	8	—
	50	0	17	—

RodI and rodII of the stalk domain were each analyzed for the presence of a seven-residue (a, b, c, d, e, f, g) repeating motif characteristic of α-helical coiled-coils (30, 31). For each, residues were aligned continuously in units of seven with no gaps permitted. The percentage of each type of residue appearing in a given position (a–g) was calculated; for rodI, assignment of position in the heptad was based upon the periodicity of the hydrophobic residues [amino acid (aa) 423 is in position c]. For rodII no assignment of position could be made.

tions are frequently or predominantly hydrophobic (25–67%). Since rodII does not display a periodic distribution of hydrophobic and charged residues, it is not predicted to participate in a coiled-coil structure. In addition, rodII contains only 93 amino acids, while *Drosophila* and squid rodII each have ≈230 residues (7, 13).

The carboxyl-terminal domain of unc-116 (amino acids 683–815), like the amino-terminal domain, is predicted to be globular (Fig. 2*B*). In earlier studies of *Drosophila* khc, a tail domain of 80 amino acids was proposed (7); however, sequence comparisons among khcs from *Drosophila*, squid, sea urchin, and human with the unc-116 protein suggest a more extensive tail domain of nearly 120 residues (Fig. 3). Amino acids 696–815 of unc-116 are 59–63% identical to the carboxyl-terminal residues of the khc from these species. The unc-116 tail domain contains 23% basic residues and has a net charge of +13, like the tail domain of khc (13). In summary, the predicted unc-116 molecule contains each of the three khc domains and as such represents the *C. elegans* equivalent of the khc.

Localization of Tc5 in *e2281*. To confirm that the sequenced gene represents the mutated locus, we localized the Tc5 insertion in the transposon tagged allele, *e2281*: when the genomic insertion site is aligned with the unc-116 cDNA, the transposon appears between base pairs 2576–2577 (Fig. 4). The target site sequence, TCA, is duplicated on the other side of the insertion. The target site matches the consensus for Tc5 insertions, TNA, and the duplication event has been observed in other known Tc5 insertion events (J. Collins, personal communication). In addition, a spontaneous revertant of *e2281* was obtained in which the phenotype is reverted and the insertion is absent, as determined by Northern analysis and sequencing of PCR-amplified genomic DNA (data not shown).

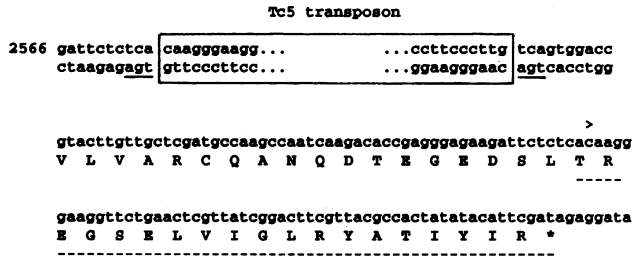

FIG. 4. Insertion site of Tc5 in *e2281* and the deduced amino acid sequence in single-letter code. (*Upper*) *Unc-116* sequence flanking the Tc5 insertion. The boxed sequence represents the Tc5 transposon, of which only the ends are shown. The three bases of *unc-116* DNA duplicated by the insertion event are underlined. (*Lower*) Sequence of *e2281* at the Tc5–*unc-116* junction and the deduced amino acid sequence. The sequence begins at base pair 2521 of the unc-116 cDNA. Additional amino acids encoded from the inserted Tc5 sequence, including a stop codon (asterisk) in the open reading frame of unc-116, are underlined; ">" indicates the start of the Tc5 sequence in the DNA.

According to the insertion site in the DNA and Tc5 sequence, the hybrid mRNA transcribed in e2281 would encode a truncated khc missing the tail domain but including an extension of 19 amino acids derived from Tc5 prior to truncation (Fig. 4). The variability in penetrance of the *e2281* phenotype and relative weakness suggest either that rodII functions in conjunction with the tail in cargo recognition and binding, so that a tail-less khc retains some function, or that some wild-type khc is produced by somatic excision of the transposon.

DISCUSSION

Although kinesin has been found in nearly every organism examined, previous attempts to purify microtubule-dependent motors in *C. elegans* revealed only the presence of a molecule tentatively identified as cytoplasmic dynein (32). The discovery of unc-104 in *C. elegans* (29) initially led to the speculation that unc-104 might be a modified equivalent of khc in *C. elegans* since the *unc-104* product is similar to the khc only in the motor domain. However, EM characterization of unc-104 mutants demonstrated a limited role for unc-104 in intracellular transport as a putative neuron-specific motor dedicated to the translocation of synaptic vesicles (33). We have identified the locus encoding khc in *C. elegans* by mutation and subsequent cloning and sequencing of the gene.

Our data indicate that *C. elegans* khc is similar to khcs from other species with the exception of the rodII domain. The sequences of the motor and tail domains have a high degree of sequence identity with those of cloned khcs and predicted

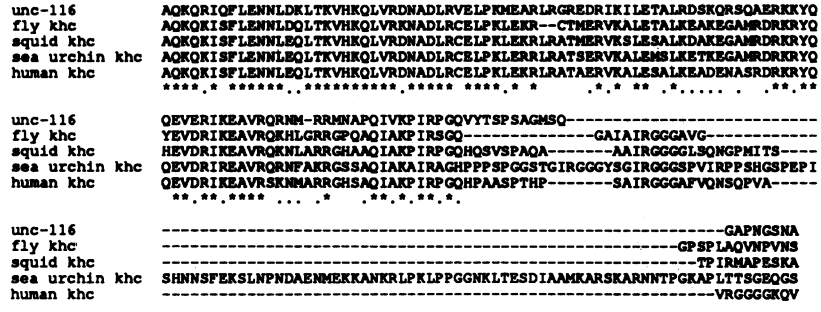

FIG. 3. Alignment of the carboxyl termini of unc-116 (amino acids 695–815) and khcs of fly (amino acids 848–975), squid (amino acids 828–967), sea urchin (amino acids 833–1031), and human (amino acids 827–963). Identical (asterisk) or conserved (dot) residues among the proteins are shown.

secondary structures consistent both with those of *Drosophila* khc (7) and with EM observations of bovine kinesin (9). The rodI and hinge regions of the stalk domain are also like those of characterized khcs in predicted structure and in size.

In contrast, the rodII region of the stalk domain differs from characterized khcs both in predicted structure and in size. Unlike khc from other species, rodII is unlikely to form a coiled coil and may form a relatively weak α-helix. The difference in the rodII domain of *C. elegans* khc suggests that either the rodII domain does not participate directly in the binding of the light chains or cargo or that the khc in *C. elegans* interacts with these components by a different mechanism than do other khcs.

The common features of khc mutants in *C. elegans* and *Drosophila* larvae (34), such as reduced size and progressive paralysis, suggest that the requirements for the khc in development and neuronal functioning may be similar across species. In *Drosophila* the paralysis does not seem to be the result of impaired synaptic vesicle transport (35). Similarly, synaptic vesicles are appropriately distributed in *rh24* specimens, as determined by EM analysis (David H. Hall, personal communication). In *C. elegans*, khc is required at two discrete times in development: once during very early embryogenesis, when it is supplied maternally, and the other during larval development, when the khc is transcribed from the zygotic genome. The requirement for khc during embryogenesis in *C. elegans* differs from observations made of *Drosophila* khc mutants, which exhibit apparently normal embryogenesis.

The molecular and phenotypic data on *unc-116* (our data and David H. Hall, personal communication) and on *unc-104* (29, 33) suggest that anterograde transport is mediated by at least two molecular motors (the *unc-116* and *unc-104* gene products), each of which may translocate a separate class of organelles. Based on *in vitro* and cell culture studies of kinesin, kinesin is a logical candidate motor for the transport of at least some components not transported by the unc-104 molecule, such as secretory granules and mitochondria. Additional specific subsets of membrane-bound organelles may be transported by other kinesin-like family members whose functions and putative cargo have not yet been characterized (36, 37).

Kinesin may not serve identical functions in all organisms, a supposition supported by the apparently unique role of kinesin in sea urchin embryos (12) and by differences between the phenotypes of *Drosophila* (34) and *C. elegans* khc mutants. Differences at the amino acid level such as the extended tail domain of sea urchin khc and the short uncoiled rodII of *C. elegans* khc may account for some differences in function. The study of khc mutants in multiple species is essential for understanding the full range of kinesin functions, and comparisons of the mutants should allow us to discern the common roles played by khc in different organisms and species-specific functions.

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