

Identification of Isoforms of a Mitotic Motor in Mammalian Spermatogenesis¹

Patrick M. Navolanic and Ann O. Sperry²

Department of Pharmacology, East Carolina University School of Medicine, Greenville, North Carolina 27858

ABSTRACT

We have isolated the full-length coding sequence for mouse *KIFC5A* (kinesin family c-terminal 5A) cDNA, encoding a motor protein found in the testes. The complete sequence of the *KIFC5A* cDNA is homologous to a group of carboxyl-terminal motors, including hamster *CHO2*, human *HSET*, and mouse *KIFC1* and *KIFC4*. The *KIFC5A* and *KIFC1* cDNAs are nearly identical except for the presence of two additional sequence blocks in the 5'-end of *KIFC5A* and a number of single base-pair differences in their motor domains. Polymerase chain reaction amplification and sequencing of the 5'-end of *KIFC5A* identified 3 distinct RNA species in testes and other tissues. Sequence comparison and genetic mapping confirmed the existence of a small multi-gene family in the mouse and suggest possible mechanisms of alternative splicing, genetic duplication, and separate genetic loci in the generation of these motors. In order to examine the possible role of these motors in germ cells of the testes, an antibody to a shared epitope was used to localize this group of proteins to different spermatogenic cell types. These experiments suggest that *KIFC5*-like motor proteins are associated with multiple microtubule complexes in male germ cells, including the meiotic spindle, the manchette, and the flagella.

INTRODUCTION

During spermatogenesis, a remarkable rearrangement of the microtubule cytoskeleton occurs in order to transform apolar spermatogonia into highly polarized spermatozoa. This cytoskeletal remodeling involves the formation and/or dissolution of several microtubular structures including the mitotic and meiotic spindles, the spermatid manchette, and the sperm axoneme. Although the importance of the microtubule cytoskeleton in this developmental pathway is clear, the identity of molecular motor proteins present in the testes and their role and regulation during spermatogenesis is not well understood. Motors of the kinesin and dynein superfamilies have been localized to microtubule structures in spermatogenic cells, and there is evidence that multiple kinesin motors are present during this process [1–4].

The kinesin superfamily of motor proteins is composed of more than 100 proteins that participate in a wide variety of movements within cells [5–7]. One reason for the large number of kinesin motors is to allow for modulation of function during development. Most differentiation programs, such as spermatogenesis, involve rearrangement of the cytoskeleton and probably require numerous molecular motors at specific times during the acquisition of the final cellular phenotype. In order to successfully achieve the final

differentiated state, the activity of numerous motors must be tightly regulated to participate in various complexes during development.

All kinesins have a conserved motor domain containing the sites for ATP hydrolysis and microtubule binding required for the generation of force against microtubules. The varying placement of the motor domain and its interaction with other parts of the protein are partially responsible for the direction in which a particular motor moves along microtubules [8]. In addition to variation in motor domain placement, the homology of superfamily members to one another decreases dramatically outside the motor domain. The divergent “tail” domains of these proteins are thought to specify the cargo to which they attach and move. Although alternative messenger RNA processing is a common method for generating diversity in structure and function in a variety of proteins, this mechanism does not appear to be commonly used by kinesin superfamily members. Only kinesin-associated proteins, such as KAP3, associated with KIF3A/3B [9], and the kinesin light chains [10, 11] have been shown to be the products of alternatively spliced genes.

We have previously identified six members of the kinesin superfamily that are expressed in the seminiferous epithelium of the rat [3]. Here we describe the cloning and initial characterization of one member of this group from the mouse, *KIFC5A*. We reasoned that the unique microtubule rearrangements of spermatogenesis might require specific motor activities. To investigate this idea, we have used molecular techniques to identify isoforms of a mitotic motor in the mouse testes. This study has identified another level of diversity that applies to motors related to *KIFC5A* and may be important for modulating their function during spermatogenesis. *KIFC5A* is part of a closely related group of motor proteins that comprise a small multigene family within the larger kinesin superfamily. The diversity of this group may be explained by both alternative splicing and the presence of separate genes. Strikingly, immunolocalization of related proteins in testicular cells reveals that some may function in several microtubule complexes in germ cells, including elements of the meiotic spindle, spermatid manchette, and sperm flagella.

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones

Recombinant lambda gt10 clones containing *KIFC5A* sequence were identified by hybridization screening of a mouse 15-day embryonic library (Clontech, Palo Alto, CA). Primary screening was done by Lofstrand Labs Inc. (Gaithersburg, MD) according to established techniques using the radiolabeled rat *KIFC1* homolog, *KRP1* head domain cDNA, as a probe for hybridization [3]. Twelve potential positives were identified in the initial screen. The insert size for each clone was determined by polymerase chain reaction (PCR) using oligonucleotide primers to either the vector sequences flanking the cloning site or the vector and head domain sequences. One clone was identi-

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²Correspondence: Ann O. Sperry, Dept. of Pharmacology, East Carolina University School of Medicine, 600 Moye Blvd., Greenville, NC 27858. FAX: 252 816 3203; e-mail: asperry@brody.med.ecu.edu

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fied with an insert size of about 2 kilobases (kb), sufficient to encode *KIFC1*, a related motor from the mouse, and was further purified by two rounds of infection, plating, and hybridization to the *KRP1* head probe.

The *KIFC5A* cDNA was sequenced, in both directions, with overlapping oligonucleotide primers (Gibco-BRL, Rockville, MD), either directly from the phage DNA or after subcloning into Bluescript (Stratagene, La Jolla, CA), using the *fmol* DNA Sequencing System from Promega (Madison, WI). Sequence analysis was performed using both Apple Macintosh (Cupertino, CA) and PC computers with Seqpup (public domain software) and Dnasis (Eastman Kodak, Rochester, NY) software packages, respectively. Alignments were also generated against sequences in the Genbank databases using the National Center for Biotechnology Information (NCBI, Bethesda, MD) Blast server. Multiple sequence alignment of *KIFC5A* and other kinesin motors, including *KIFC1*, *KIFC4*, *CHO2*, and *HSET*, was performed using both the Dnasis (Pharmacia LKB Biotechnology, Piscataway, NJ) and the Clustal W program [12].

DNA Manipulations

PCR primers were designed to amplify the divergent region in the *KIFC5A* sequence. The 5' primer (*KIFC1a*: ATCGTCCTGCCTGCTGCCTTTG) hybridizes to nucleotides 10–31 of both the *KIFC5A* and the *KIFC1* cDNAs, while the 3' primer (*KRP1*rev: GCAGCCGCTCTCCCTGAAGCTC) hybridizes to nucleotides 736–757 of *KIFC5A* and 547–558 of *KIFC1*. Recognition sites for *Hind*III (5' primer) or *Xba*I (3' primer) were included to permit subsequent subcloning of amplification products. Reverse transcription (RT)-PCR reactions were conducted essentially as previously described [3]. Total RNA, from various tissues, was first converted into cDNA using the RT-PCR kit from Stratagene. Briefly, 10 μ g RNA was mixed with random primers, annealed by slow cooling to room temperature from 65°C, and extended with Moloney murine leukemia virus (MMLV) reverse transcriptase at 37°C in the provided buffer. After heat denaturation, the cDNA was ready for amplification.

Typical PCR reactions contained 1–3 μ l of the cDNA reaction mixture, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 1 μ M each primer in a volume of 25 μ l. The reactions were preheated to 95°C for 5 min, and 2.5 U *Taq* polymerase (Gibco-BRL) added as the reaction cooled to 60°C for 5 min. Cycling conditions were 72°C, 3 min, with a 6-sec extension per cycle; 96°C, 45 sec; and 55°C, 4 min, for 30 cycles. Amplification products were separated by electrophoresis in low-melting point agarose (Gibco-BRL), and the DNA fragments were purified by extraction from the gel using the Qiaex kit from Qiagen (Valencia, CA). For subcloning, the purified DNA was digested with the appropriate restriction enzymes and ligated to Bluescript II SK. Recombinants were sequenced with universal primers using the *fmol* sequencing kit from Promega. For RT-PCR experiments using isoform specific oligonucleotides, the melting temperature (T_m ; temperature at which 50% of a population of oligonucleotide is removed from its complementary sequence) was calculated for hybridization to the desired isoform versus other isoforms. For example, the approximate T_m for the *KIFC5B* oligo (see Figure 4) is 67°C, while the T_m for the portion of this oligo comple-

mentary to *KIFC5A* was calculated to be 47°C. Annealing was done at 60°C in this case.

Northern Analysis

Messenger RNA was prepared from mouse tissues using the Poly(A)Pure Kit from Ambion (Austin, TX) and separated by electrophoresis through a denaturing formaldehyde gel. RNAs were transferred to Nytran nylon membrane (Schleicher and Schull, Keene, NH) and hybridized to one of three probes: 1) motor probe, an approximately 500-base pair (bp) fragment amplified by PCR from the head domain of *KIFC5A*; 2) *KIFC5B* (gaga) probe, a 23-residue oligonucleotide (CGCAGAGAAAGGGAAGGGAAGGG) specific for the GA-rich sequence found in the *KIFC5B* isoform; or 3) a 905-nucleotide (nt) fragment from the mouse glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*; Ambion). The specificity of the *KIFC5B* oligonucleotide was tested against RNA transcribed in vitro from *KIFC5A*, *KIFC5B*, and *KIFC5C* templates. This oligonucleotide recognized only *KIFC5B* on Northern blots of in vitro-transcribed *KIFC5A*, *KIFC5B*, and *KIFC5C* RNA (data not shown). The motor and *GAPDH* probes were labeled by the random hexamer protocol using the kit from Boehringer Mannheim (Indianapolis, IN) and [α ³²P]dCTP while the oligo probe was labeled with [α ³²P]dATP using terminal transferase (Boehringer Mannheim). Blots were hybridized to probe in Church and Gilbert solution overnight at 68°C for the motor probe and 50°C for the oligonucleotide [13]. After hybridization, blots were washed as described previously for the motor probe [3], and at 50°C with increasing stringency, with a final wash of 0.05-strength SSC (single-strength SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% SDS.

Cell Preparations and Indirect Immunofluorescence

Several different spermatogenic cell preparations were used for immunofluorescent localization of KIFC5-like proteins. Enriched populations of elongating spermatids and other cell types were prepared by extrusion of seminiferous tubules of defined stages according to the method of Parvinen and Hecht [14]. Testes were obtained from sexually mature rats (8–10 wk, Sprague-Dawley; Harlan, Indianapolis, IN), and the tunica albuginea was removed. The seminiferous tubules were then gently dissected apart using stainless steel probes. Tubules were viewed by transillumination, and sections approximately 2 mm in length representing stages VIII–XII, containing elongating spermatids, were identified as described by Parvinen and Hecht and removed, and the germ cells were extruded onto a glass slide for processing. For staining of meiotic cells, segments identified as between stages XIV and I were selected by transillumination. For staining of spermatozoa, the epididymis was removed from the testes, cut into 5–10 pieces, and placed in a small volume of Dulbecco's modified Eagle's medium (DMEM). After incubation for 15 min at room temperature, the fluid was removed, and sperm were isolated by centrifugation at 14 000 \times g for 5 min.

KIFC5-like proteins were localized within different spermatogenic cell types by indirect immunofluorescence using a monoclonal antibody against the *CHO2* kinesin-related protein (kind gift of Dr. Ryoko Kuriyama [15], Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, MN) according to established procedures. Spermatogenic cells isolated as above were attached to polylysine-coated coverslips or slides coated in Vecta-

bond (Vector Laboratories, Burlingame, CA) in PBS, pH 7.4, and fixed for 15 min at room temperature in 3% paraformaldehyde in 0.1 M cacodylic buffer (pH 6.2) with 1 mM CaCl₂ and 1 mM MgCl₂ followed by extensive washing in PBS [16]. The cells were then blocked in 2% BSA in TBST (20 mM Tris, pH 7.5, 154 mM NaCl, 2 mM EGTA, 2 mM MgCl₂, 0.1% Triton X-100) and incubated sequentially with primary and secondary antibodies. Fixed cells were incubated with the *CHO2* monoclonal antibody at a 1:1000 dilution in blocking buffer for 30 min and then rinsed 3 times in TBST. The primary antibody was detected with donkey anti-mouse IgG conjugated to Texas Red (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA), and microtubules were colocalized with anti- α tubulin conjugated to fluorescein isothiocyanate (FITC; 1:50 dilution; Sigma, St. Louis, MO). The intracellular localization of the *CHO2* antigen was observed with a Nikon (Melville, NY) E600 fluorescence microscope fit with appropriate filters. Controls for these experiments included a nonspecific antibody (anti-HIS6 monoclonal; Qiagen), omission of primary antibody, and preincubation of the *CHO2* antibody with *KIFC5A* protein. *KIFC5A* fused to an HIS6 tag was expressed in bacteria and purified by Nickel affinity (Qiagen). Ten micrograms of purified protein was incubated overnight at 4°C with 1 μ l *CHO2* antibody in 200 μ l TNT (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) according to the method of Tres and Kierszenbaum [17]. For use in immunofluorescence, the preincubated antibody was diluted to 1:1000.

RESULTS

Cloning of the Full-Length cDNA for Mouse KIFC5A

The *KRPI* head domain cDNA fragment was previously identified in an RT-PCR screen of rat testis RNA [3] and was found to be homologous to the C-terminal motors, *CHO2* [15], *KIFC1* [18], and *HSET* [19]. In fact, the rat *KRPI* head domain is 91% identical to that of mouse *KIFC1*, suggesting that *KRPI* might be the rat ortholog of *KIFC1* [18]. The rat *KRPI* cDNA encoding part of the motor domain was used to screen a lambda gt10 library constructed from Day 15 mouse embryos. Because of its strong homology to mitotic motors, *KRPI* was a good candidate for a motor involved in the cell divisions of spermatogenesis. Of the 14 positive clones detected, one of just over 2 kb was chosen for further analysis. This size was sufficient to encode a protein of similar size to the *KIFC1* and *CHO2* polypeptides. Sequence analysis showed that this clone, which we termed *KIFC5A*, encodes a polypeptide with a C-terminal motor domain, like its relatives *CHO2* and *KIFC1*, along with other sequences characteristic of kinesin-related motor proteins.

Relationship of KIFC5A to CHO2 and KIFC1

We found that *KIFC5A* is closely related to both *CHO2* and *KIFC1*; however, sequence alignment revealed additional features concerning the relationship between these cDNAs (schematized in Fig. 1A and shown in detail in Fig. 1B; [15, 18]). The *KIFC5A* nucleotide sequence was almost identical to that of *KIFC1* throughout most of its length. We were able to detect a handful of single base-pair differences between the head domain of *KIFC5A* and the published sequence of *KIFC1*. However, the most striking difference appeared when the tail domains of these two cDNAs were compared: *KIFC5A* contains two approxi-

mately 100-bp inserts missing in the *KIFC1* sequence. Both sequence inserts are highly homologous to sequences found in the *CHO2* cDNA (Fig. 1B; [15]). It is also interesting to note, when comparing these sequences, that there is a deletion of 27 bp between insert 1 and 2 in the *CHO2* cDNA compared to the other two cDNAs (Fig. 1B). These observations suggest that the *KIFC5A* and *KIFC1* messages, and perhaps also *CHO2*, might be derived from alternative splicing of a common gene.

The *KIFC5A* sequence retains the Kozak consensus translation start site present in the *KIFC1* message (filled arrowheads Fig. 1, A–C; [20]). However, 126 bp with high homology to the 5'-end of the *CHO2* gene are inserted immediately after the *KIFC1* start site (insert 1, Fig. 1C). Insert 1 is 80% identical to *CHO2* at the amino acid level and 73% identical at the nucleotide level. The peptide encoded by this insert has a very basic pI predicted to be 11.5; 9 of the 41 amino acids are lysine or arginine (underlined in Fig. 1C). The concentration of these amino acids in this short peptide is suggestive of a basic nuclear localization signal (NLS; [21]). No sequence within the first insert matches the consensus NLS exactly; however, one 4-amino acid peptide is the precise reverse of the consensus (Fig. 1C, asterisk). The second insert of 63 bp at position 532 of the *KIFC1* cDNA is also homologous to *CHO2*, but less so than insert 1. The second insert has no striking structural features, although both inserts contain putative protein kinase C phosphorylation sites.

KIFC5A Is a Member of a Closely Related Group of Motor Proteins

The cloning and characterization of the *KIFC5A* cDNA revealed sequence heterogeneity in the tail domain relative to other family members. In order to determine whether this region is the site of other sequence variants, we conducted an RT-PCR experiment using primers flanking this region to amplify RNA from different tissues (Fig. 2A). PCR products amplified from this region differed in size between different tissues, such as brain and testes, and multiple species were detected within an individual tissue (Fig. 2, B and C). These two panels show results representative of numerous amplification reactions. Some variability in the number of bands visualized was seen (compare Fig. 2B, primer 1+2, with Fig. 2C, primer 1+2). To identify the sequence composition of these amplification products and to compare them to related species, PCR products were gel-purified, cloned, and sequenced. A total of 3 distinct sequence variants were detected by sequence analysis of the amplification products, including the tail domain of the original *KIFC5A* sequence, and are shown schematized in Figure 3A and aligned in Figure 3, B and C. We have chosen to call these species *KIFC5A*, *KIFC5B*, and *KIFC5C*. This nomenclature is designed to avoid confusion with the various kinesin-related head domains and related genetic loci that have been detected recently until the correct "head" can be connected to the correct "tail" (see *Discussion* and [22]).

The *KIFC5A* tail sequence was identified in clones from brain cDNA, and the original clone was isolated from an embryonic library. Other sequences detected were very similar to that of *KIFC5A*; all contained the inserts not present in *KIFC1*. *KIFC5B* was detected in brain and spleen and is notable for the replacement of 12 bp in the first insert with a 9 nucleotide GA-rich sequence. *KIFC5B* is 89% identical to *KIFC5A* at the nucleotide level and 87% at the

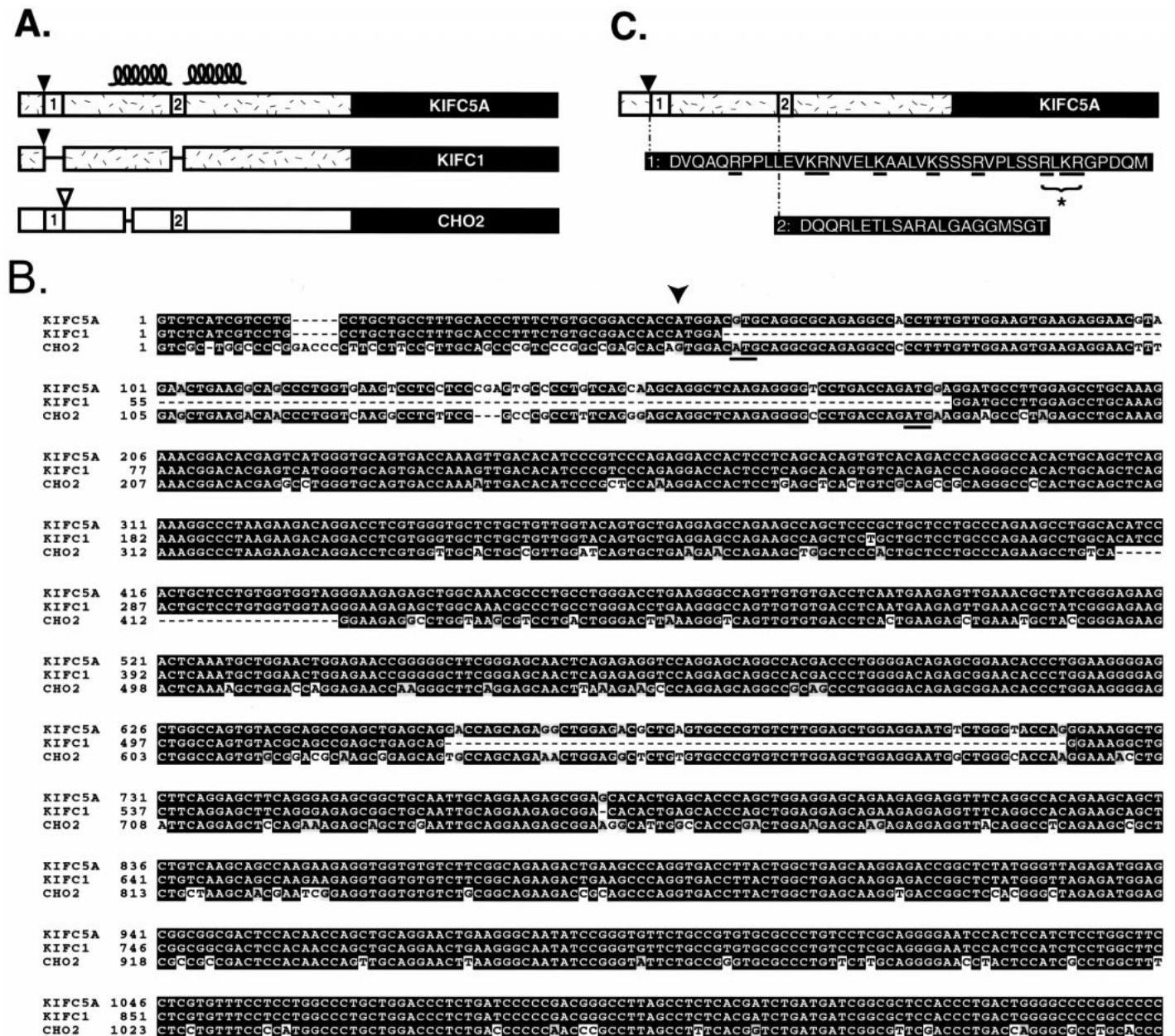


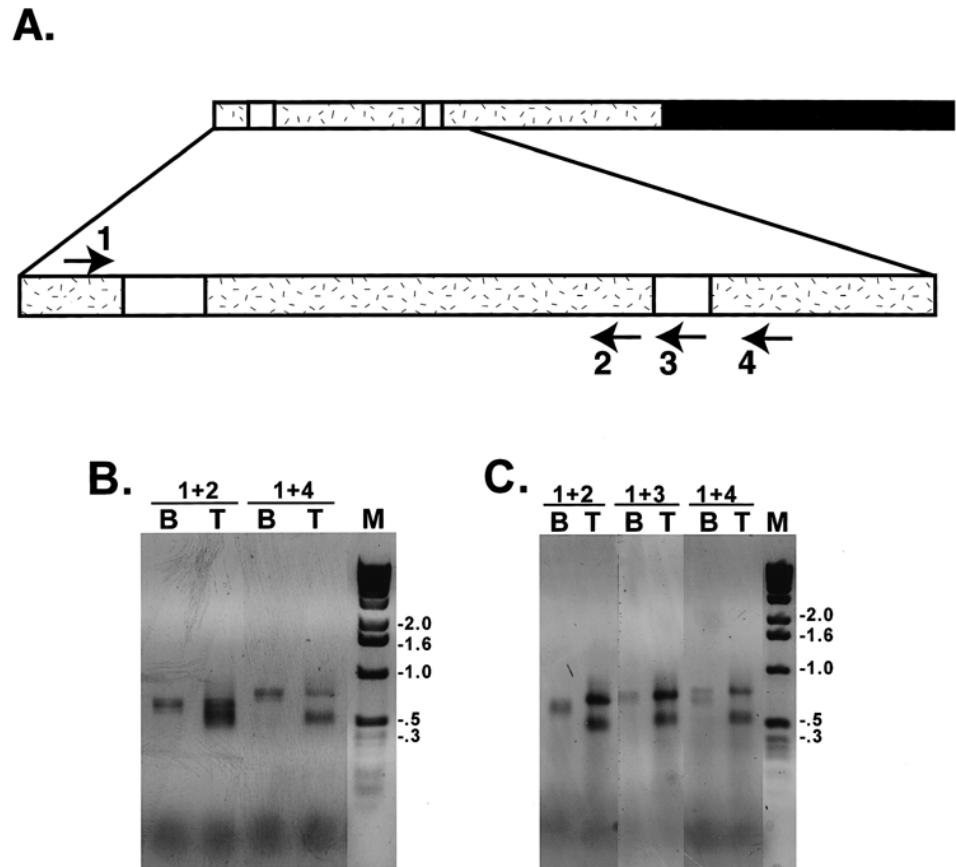
FIG. 1. Comparison of *KIFC5A* with *KIFC1* and *CHO2*. A) A schematic representation of the relationship of these three cDNAs. The inserted sequences in *KIFC5A* are indicated by numbered white boxes. Regions with a high probability to adopt a coiled-coil structure are indicated by coils above the schematic [30]. The stippled boxes in *KIFC5A* and *KIFC1* indicate the high level of homology between these two cDNAs compared to that with *CHO2*. The nucleotide sequence of the 5' half of the *KIFC5A* cDNA is aligned with the corresponding regions from the *KIFC1* and *CHO2* cDNAs in B. The putative start codons for *CHO2* translation are underlined. C) A schematic of the predicted *KIFC5A* protein with the amino acid sequences of the inserts shown. The head domain is indicated by a black box, and the relative positions of the inserts are shown by numbered boxes. The black arrowheads indicate the Kozak consensus ATG start codons while the open one (in A) denotes the start site for the *CHO2* protein, which lacks a Kozak consensus. Lysine and arginine residues in the first insert are underlined, with a possible nuclear localization signal indicated with a bracket and an asterisk. The complete DNA sequence of *KIFC5A* is available from GenBank/EMBL/DDJB under accession number AF221102.

amino acid level. This species has a deletion of 54 bp at nucleotide 255 of the *KIFC5A* cDNA in addition to base substitutions throughout the amplified region. Another species called *KIFC5C*, amplified from liver, spleen, and testes RNA, is 92% and 83% identical to *KIFC5A* at the nucleotide and amino acid levels, respectively. In none of the *KIFC5* variants do the base substitutions appear to be clustered, except for the deletion and the GA-rich region in *KIFC5B*.

KIFC5 Variant Tail Domains Are Connected to a Putative Head Domain

In order to determine whether the sequence variants detected in our RT-PCR experiments might reflect full-length kinesin-related transcripts, we designed oligonucleotide primers specific to the tail region of selected isoforms and to the *KIFC5A* head domain to amplify transcripts from spleen, brain, and testes (Fig. 4A). Because of the consid-

FIG. 2. Detection of KIFC5-like isoforms by RT-PCR. **A**) PCR primers designed to amplify the region containing the inserts found in *KIFC5A*. Primer 1 hybridizes to both *KIFC1* and *KIFC5A* cDNAs and was used in all amplification reactions. The remaining primers were designed to amplify increasingly longer products and to detect the presence of insert 2. **B, C**) Two examples of PCR reactions using these primers to amplify messages from brain (B) and testes (T). DNA molecular weight markers, sized in kb, are shown in the lane marked M. The primers used in each reaction are indicated above the respective lanes. Control reactions without added template resulted in no amplification product (not shown).



erable homology between KIFC5 isoforms, we were able to make oligonucleotides specific only for the GA-rich region of *KIFC5B* (Fig. 4B). We also designed a *KIFC1* "junction" oligonucleotide to amplify both the *KIFC1* transcript, by hybridization across the insert junction, and the KIFC5 variants, through hybridization of the 3'-end of the oligonucleotide (see Fig. 4C). The annealing conditions used in this experiment were designed to greatly favor amplification of specific messages over that of other KIFC5 variants. Indeed, both oligos, in conjunction with the *KIFC5A* head primer, were able to support amplification of fragments of approximately 1.8 and 1.6 kb. These size fragments are consistent with those expected for amplification from KIFC5 variants and *KIFC1* cDNA, respectively.

To further investigate whether the isoforms we detected by RT-PCR represent transcribed species and to determine whether any isoform is testes-specific, we used a sequence found only in *KIFC5B* to design an oligonucleotide probe for Northern analysis. The KIFC5 isoforms are extremely similar in sequence; however, *KIFC5B* contains an inserted GA-rich sequence that is unique to this message (see Fig. 3B). The *KIFC5B*-specific probe recognized an approximately 1.9-kb transcript enriched in the testes compared to brain (Fig. 5). This size is sufficient to encode a KIFC5 isoform. The *KIFC5A* motor domain probe recognized a species in testis RNA approximately the same size as that of the *KIFC5B* signal.

CHO2 Antigen Is Part of the Meiotic Spindle

We found that *KIFC5A* bears extensive homology to the mitotic motors CHO2 [15] and XCTK2 [23]. These motors are concentrated at spindle poles and have demonstrated microtubule bundling activities. On the basis of these ob-

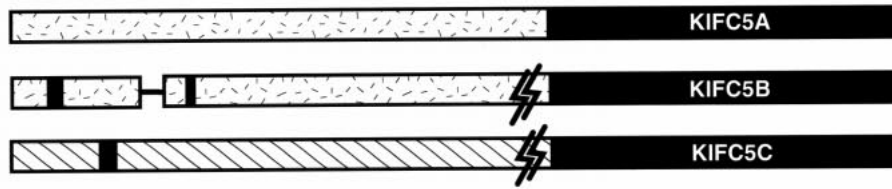
servations, we were particularly interested in the localization of the CHO2 antigen in meiotically dividing cells of the testes. A monoclonal antibody raised against the CHO2 protein (a generous gift of Dr. Ryoko Kuriyama) was used to localize this protein, and related proteins, in mixed populations enriched in specific spermatogenic cell types. This antibody recognizes bacterially expressed *KIFC5A* (unpublished results). We were able to identify several cells in our preparation with spindle structures (Fig. 6A). These cells were isolated from stage XIV tubules and likely represented meiotic cells. In these cells, the CHO2 monoclonal clearly stains centrosomes and spindle fibers in meiotic cells (Fig. 6A, arrowheads), with an apparent concentration toward spindle poles.

CHO2 Antigen Is a Constituent of Two Other Microtubule-Based Structures

Several additional microtubule complexes are essential for the formation of viable sperm, including the spermatid manchette and the flagella. In order to investigate the localization of KIFC5-like proteins in these structures, germ cells were isolated from seminiferous tubule fragments of stages VIII to XII containing elongating spermatids and were triple-stained for the CHO2 antigen, α -tubulin, and DNA. The CHO2 antigen is present in several cell types and colocalizes with microtubules in spermatocytes and spermatids. The most striking finding, however, was the localization of this group of proteins to the spermatid manchette and sperm flagella.

A conspicuous localization of the CHO2 antigen with microtubules of the spermatid manchette was observed in intermediate-stage spermatids, about step 12, isolated by transillumination aided microdissection (Fig. 6B). In these

A.



B.

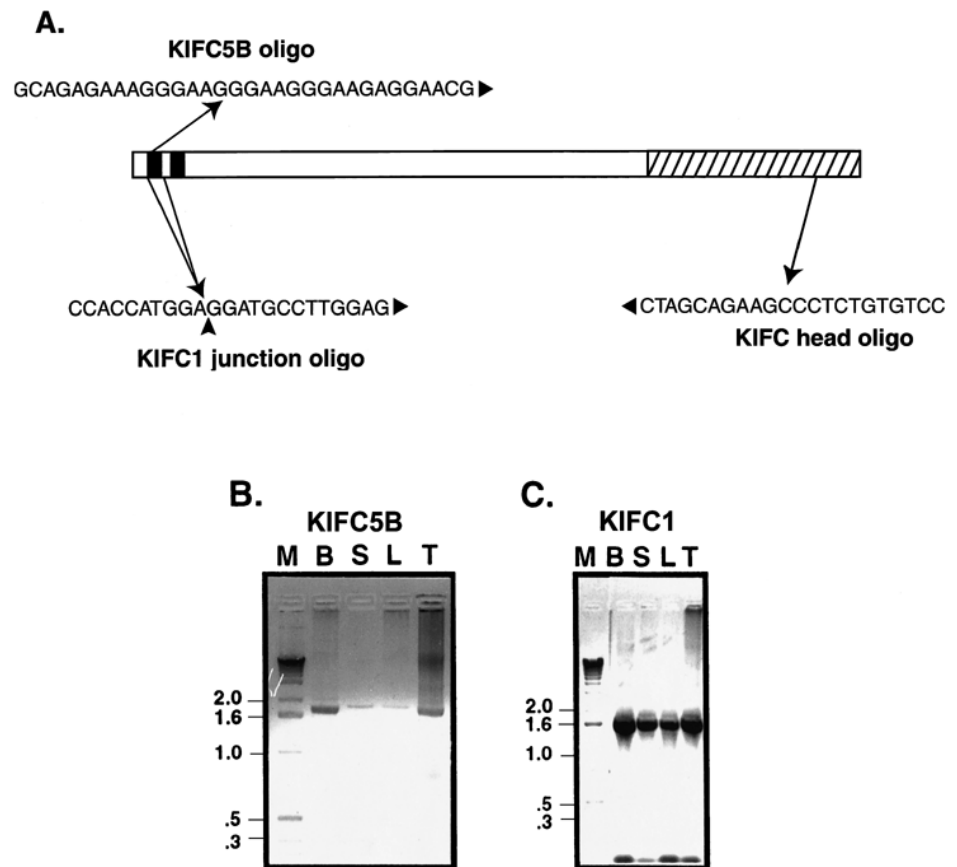
KIFC5A	1	ATCGTCTCGCTGCTGCCTTTGCA	CCCTT	CTGTGCGGACCACCATGGACGTGCAGGCG
KIFC5B	1	ATCGTCTCGCTGCTGCCTTTGCATCCCT	T	CTGTGCGGACCACCATGGACGTGCAGGCG
KIF5C	1	ATCGTCTCGCTGCTGCCTTTGCATCCCT	T	CTGTGCGGACCACCATGGACGTGCAGGCG
KIFC5A	60	CAGAGGCCACCTTTGTTGGAAAGTGAAGAGGAACCTAGA	ACTGAAGGCAGCCCTGGTCAAG	
KIFC5B	61	CAGAG---	AAAGGGAACGGAAGCGAAGAGGAACCTAGA	ACTGAAGGCAGCCCTGGTCAAG
KIF5C	61	CAGAGGCCACCTTTGTTGGAAAGTGAAGAGGAATGTAGA	CTGAAGACATG	CCTGGTCAAG
KIFC5A	120	TCCTCCTCCCGAGTGCCCTGTGTCAGCAAGCAGGCTCAAGAGG		GGTCTGACCAGATGGA
KIFC5B	118	TCCTCCTCCCGAGTGCCCTGTGTCAGCAAGCAGC	CTCAAGAGG	GGTCTGACCAGATGGA
KIF5C	121	TCCTCCTCCCGAGTGCCCTGTGTCAGCAAGCAGGCTCAAGAGT		GGTCTGACCAGATGGA
KIFC5A	179	GGATGCCTTGGAGCCTGCAAAAGAACGGACACGAGTCATGGGTG	CAGTGACCAAAGTTGA	
KIFC5B	177	GGATGCCTTGGAGCCTGCAAAAGAACGGACACGAGTCATGGGTG	CAGTGACCAAAGTTGA	
KIF5C	180	GGATGCCTTGGAGCCCTAAAGAACGGACACGAGC	CATGGGTG	CAGTGACCAAAGTTGA
KIFC5A	239	CACATCCCGTCCCAGAGGACCACTCCTCAGCACAGTGTACAGACCC	AGGGCCACACTGC	
KIFC5B	237	CACATCCCGTCCCAGAGGACCACTCCTCAGCACAGTGTACAGACCC	AGGGCCACAAATGC	
KIF5C	240	CAATCCCGTCCCAGAGGACCACTCTCAGCACAGTGTACAGACCC	AGGGCCACACTGC	
KIFC5A	299	AGCTCAGAAAGGCCCTAAGAAGACAGGACCTCTGGGT		GCTCTCTGTTGGTACAGTGC
KIFC5B	297	AG-----		TGC
KIF5C	300	AGCTCAGAAAGGCCCTAAGAAG-CAGGACCTCTGGGT		GCTCTCTGTTGGTACAGTGC
KIFC5A	358	TGAGGAGCCAGAAGCCAGCTCCCGTGTCTGCCAGAAAGCCTGGCACATCCACTGCTC		
KIFC5B	302	TGAGGAGCCAGAAGCCAGCTCCCGTGTCTCCCTGC		GAAGCCTGGCACATCCACTGCTC
KIF5C	359	TGAGGANCCAAA--CCA	CTCCCCTGTCTGCCAGAAAGCCTGGCACATCCACTGCTC	
KIFC5A	418	CTGTGGTGGTAGGGAAGAGAGCTGGCAAACGCCCTGCCTGGGACCTGAAGGGCCAGTTGT		
KIFC5B	359	CTGTGGTGGTAGGGAAGAGAGCTGGCAAACGCCCTGCCTGGGACCTGAAGGGCCAGTTGT		
KIF5C	416	CTGTGTGGTAGGGAAGAGAGCTGGCAAACGCCCTGCCTGGGANCTGAAGGGCCAGTTGT		
KIFC5A	478	GTGACCTCAATGAAGAGTTGAAACGCTATCGGGAGAAGACTCAAATGCTGGAAC	TGGAGA	
KIFC5B	419	GTGACCTCAATGAAGAGTTGAAACGCTATCGGGAGAAGACTGAAACGCTGGAAC	TGGAGA	
KIF5C	476	TGAACTCACTGAAGAGTTGAAACGCTATCGGGAGAAGATCAAACGCTGGAAC	TGGAGA	
KIFC5A	538	ACCGGGGGCTTCGGGAGCAACTCAGAGAGGTCAGGAGCAGGCCACGACCCTGGGGACAG		
KIFC5B	479	ACCGGGGGCTTCGGGAGCAACTCAGAGAGGTCAGGAGCAGGCCACGACCCTGGGGACAG		
KIF5C	536	ACCGGGGGCTTCGGGANCACTCAGAGAGGTCAGGAGCAGGCCACGACCCTGGGGACAG		
KIFC5A	598	AGCGGAACACCCTGGAAGGGGAGCTGGCCAGTGTACGCAGCCGAGCTGAGCAGGACCAGC		
KIFC5B	539	AGCGGAACACCCTGGAAGGGGAGCTGGCCAGTGTACGCAGCCGAGCTGAGCAGGACCAGC		
KIF5C	596	AGCAGACACCCTGGAAGGGGAGCTGGCCAGTGTACGCACCCGAGCTGAGCAGGACCAGC		
KIFC5A	658	AGAGGCTGGAGACGCTGAGTGCCCTGTCTTGGAGCTGGAGGAATGCTGGGTACCAGGG		
KIFC5B	599	AGAGGCTGGAGACGCTGAGTGCCCTGTCTTGGAGCTGGAGGAATGCTGGGTACCAGGG		
KIF5C	656	AGAGCTGGAGATGCTGAGTGCCCTGTCTTGGAGCTGGAGGAATCTGGGTACCAGGG		
KIFC5A	718	AAAGGCTGCTTCAGGAGCTTCAGGGAGAGCGGGCTG		
KIFC5B	659	AAAGGCTGCTTCAGGAGCTTCAGGGAGAGCGGGCTG		
KIF5C	716	AAAGGTTGGTTCAGGAGCTTCAGGGAGAGCGGGCTG		

C.

KIFC5A	1	MDVQAQRPPLEVKRNVELKAALVKSSSRVPLSASRLKRGPDQMEDALEPAKRRTRVMGA
KIFC5B	1	MDVQAQRK-GRPCKRNVELKAALVKSSSRVPLSASSLKRGPDPQMEDALEPAKRRTRVMGA
KIFC5C	1	MDVQAQRPPLEVKRNVELKTSLVKFSRRVPRQQAGSRVGPDPQMEDALEPVKRRTRGMGA
KIFC5A	61	VTKVDTSRPRGPLLSTVVSQTQGHATAAQKGPKKTGPRGCSAVGTVLSRQKPAAPAAQKPG
KIFC5B	60	VTKVDTSRPRGPLLSTVVSQTQGHNAV-----LSQKPAAPAAAKPG
KIFC5C	61	VTKVDTSRPRGPLFSTVVSQTQGHATAAQKGPKKDLLGCSAVGTVLSRQKPAAPAAQKPG
KIFC5A	121	TSTAPVVVVKRAGKRPADLKGQLCDLNEELKRYREKTMLEENRGLRQLREVQEQAT
KIFC5B	101	TSTAPVVVVKRAGKRPADLKGQLCDLNEELKRYREKTELEENRGLRQLREVQEQAT
KIFC5C	120	TSTAPVVVVKRAGKRPADLKGQLFELSEELKRYREKNTLEENRGLRKLREVQEQAT
KIFC5A	181	TLGTERNTLEGELASVRSRAEQDQRLETLASRVLEEECLGTRELLQELQGERL
KIFC5B	161	TLGTERNTLEGELASVRSRAEQDQRLETLASRVLEEECLGTRELLQELQGERL
KIFC5C	180	TLGTEQSTLEGELANVRRAEQDQRLEMLSAQVLEEEVLTGTERLVQELQGERL

FIG. 3. Comparison of KIFC5-like isoforms. A) A schematic of the KIFC5-like cDNAs detected by PCR. The head domain, shown in black at right, is located at the carboxyl terminus of these motors, with the double zigzag indicating that *KIFC5B* and *KIFC5C* are partial clones containing only the tail domain. The sequence differences are indicated by small black boxes. *KIFC5B* contains a deletion when compared to the other isoforms. The hatched region in *KIFC5C* indicates the presence of multiple nucleotide changes throughout this region. The aligned nucleotide sequence of the isoform tail domains is shown in detail in B and the aligned amino acid sequence shown in C. The *KIFC5B* and *KIFC5C* sequences are available from GenBank/EMBL/DDJB under accession numbers AF221103 and AF221104.

FIG. 4. Amplification of KIFC5 isoforms from various tissues. *CHO2* subfamily members *KIFC5B* and *KIFC1* were amplified with specific oligonucleotides, at the 5'-end, and with a conserved KIFC head oligonucleotide as the 3'-primer (outlined in A). The *KIFC1* junction primer was designed to span the site of insertion of the first sequence (arrowhead). B) Results of amplification using the *KIFC5B* primer and the KIFC head primer using RNA derived from brain (B), spleen (S), liver (L), and testes (T). C) A similar amplification using the *KIFC1* junction primer and the *KIFC5* head primer. DNA molecular weight markers, sized in kb, are shown in the lane marked M in both panels.



cells, the nucleus is quite elongated and the cell has little residual cytoplasm. The CHO2 antigen is seen to colocalize with manchette microtubules (Fig. 6B, center panel). This staining persists through all stages in which the manchette is present, transforming from a cap-like structure in early spermatids to a band at the distal end of the spermatid nucleus in more mature spermatids (data not shown). In order to determine whether KIFC5 variants are also found in spermatozoa, the CHO2 antibody was used to stain spermatozoa isolated from rat epididymis (Fig. 6, E and F). The CHO2 antigen was found throughout the flagellar tail of the sperm.

DISCUSSION

Identification of new members of the kinesin superfamily of motor proteins has increased at an almost exponential rate in recent years. Two possible explanations for the large number of motors are the necessity for functional redundancy and the requirement for specialized motors during cellular development. In this report, we describe the cloning and initial characterization of the *KIFC5A* motor cDNA. These studies revealed multiple mRNA species closely related to this motor in the testes and the presence of related proteins in different microtubule complexes during spermatogenesis.

Comparison of the full-length *KIFC5A* sequence identified in our screen with two other members of this subfamily, *KIFC1* and *CHO2*, revealed an interesting relationship between these motors. The *KIFC5A* sequence is identical to *KIFC1* except for the presence of two additional sequence blocks in the 5'-end of the *KIFC5A* cDNA and a number of nucleotide differences in their head domains. Because the origin of these different species is still unclear,

we will refer to the two sequence blocks found in *KIFC5A* as "inserts" relative to the *KIFC1* sequence. The genetic relationship between these cDNAs is complicated by the presence of a small multigene family of KIFC5-like motors (see below).

KIFC5A is also homologous to two additional cDNA fragments found in the database, *HSET* [19] and *KIFC4* [22]. *KIFC5A* is closely related to *KIFC4*, showing 98% sequence identity to the head domain of this protein. The relationship between these proteins will be clarified when the entire coding sequence of *KIFC4* is reported. The *KIFC4* head domain sequence may, in fact, be genetically linked to one of the KIFC5-related sequences reported here. However, on the basis of the number of new tail sequences, we expect additional KIFC5-related genes to be cloned. The entire human *HSET* gene was recently cloned as part of a genome-sequencing effort, and the amino terminus is available for comparison [24]. *KIFC5A* is much less identical to *HSET* than it is to *KIFC4*, being 79% identical in the head domain, with this homology dropping off to 59% in the tail.

The relationship between the *KIFC5A* cDNA and the *KIFC1* cDNA suggested initially that they might be alternatively spliced products of a common precursor RNA. Such an arrangement would predict that probes specific for *KIFC5A* (inserts 1 and 2 in Fig. 1) would map to the same genomic location as sequences common to both *KIFC1* and *KIFC5A*. Both probe types do, in fact, localize to identical locations on chromosomes 13 and 17 (N.G. Copeland and N.A. Jenkins, personal communication). Although cosegregation of these markers supports alternative splicing, this interpretation is complicated by the finding of at least 4 loci encoding related cDNAs [22, 25]. Our mapping studies,

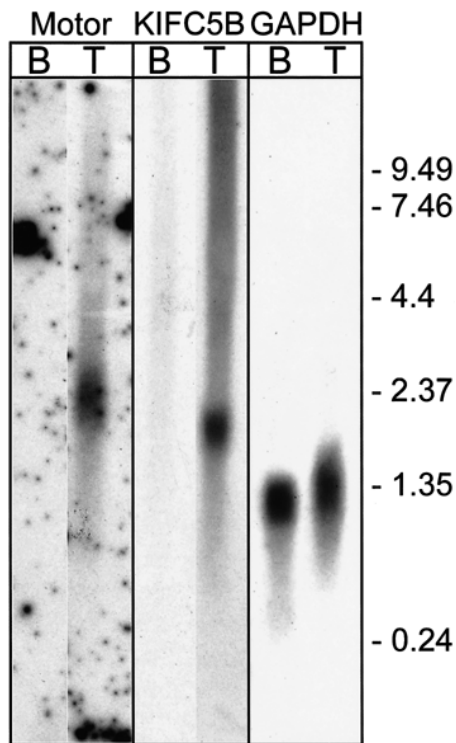


FIG. 5. Northern analysis of KIFC5 isoforms. Messenger RNA (10 μ g per lane, 3 sets) from brain (B) and testes (T) was separated by denaturing gel electrophoresis and transferred to nylon membrane. Motor lanes: Immobilized RNA was hybridized to an approximately 500-nt labeled fragment from the head domain of *KIFC5A*. KIFC5B lanes: RNA was probed with the KIFC5B oligonucleotide (see *Materials and Methods* for details). GAPDH lanes: RNA was hybridized with a probe to glyceraldehyde dehydrogenase (GAPDH) as a loading control. The sizes, in kb, of RNA markers are indicated to the right.

along with those of others, demonstrate the presence of a small multigene family of highly related motor genes: two on chromosome 17, one on each of chromosomes 10 and 13. Indeed, sequencing of the *KIFC5A* cDNA clone revealed several base-pair changes when compared to the published *KIFC1* and *KIFC4* sequences, suggestive of separate genetic loci. Confirmation of these differences in the *KIFC1* and *KIFC4* sequences, and the development of specific probes, will be necessary to clarify the relationship between the *KIFC1*, *KIFC4*, and *KIFC5* mRNAs and to determine their chromosomal location.

That multiple genetic loci are detected with *KIFC5A* probes is consistent with our finding of several divergent tail domain sequences by RT-PCR. Each isoform differs from the *KIFC1* cDNA, previously reported, by the presence of inserts at the same position as those of *KIFC5A*. In addition, these species have numerous nucleotide substitutions in the approximately 750-nt region we examined, consistent with the encoding of these species by separate genes. However, one identified sequence variant, *KIFC5B*, differs from *KIFC5A* in both the insertion and deletion of sequence blocks. Both alterations occur before the beginning of the predicted coiled-coil region at approximately nucleotide 432 or amino acid 144 in the predicted *KIFC5A* ORF. *KIFC5B* may represent a distinct KIFC5-like gene that is itself alternatively spliced.

The KIFC5-related coding sequences described here are very similar except for insertions, deletions, and nucleotide substitutions with respect to each other and to the *KIFC1* sequence. That these changes occur in the tail domain of

these motors has important implications for their function, as this region is important for cargo binding. The highly basic first insert present in the KIFC5 isoforms and absent in *KIFC1* might mediate interaction with acidic proteins, thereby targeting these motors to specific microtubule complexes. Another possible role of the first insert is as a nuclear localization signal. The presence of a such a signal in members of this subfamily of motors is particularly intriguing given our finding that the CHO2 antigen is a constituent of both cytoplasmic and nuclear microtubule structures during spermatogenesis.

Several members of this subfamily have deletions in their tail domains compared to other members. This suggests that the spacing of sequence elements in the tail domains of these motors might be important for their function. For example, *KIFC5B* contains a 19-amino acid deletion, while CHO2 lacks 8 amino acids compared to other motor sequences. Both deletions occur in the tail domain prior to sequences with a high probability of forming a coiled-coil structure. These spacing differences might modulate the ability of different motors to interact with their intended target complex. For instance, proper interaction of microtubules in the manchette might require motor proteins with a different arrangement of microtubule binding sites than is necessary for formation of the spindle.

This idea that the *KIF5B* transcript is alternatively spliced is supported by examination of a genomic locus containing a *KIFC1*-related gene on chromosome 17 that was entered recently into the database [26]. We have aligned the *KIFC5A* cDNA against this genomic sequence (unpublished observations). It is notable that the deletion in *KIFC5B* compared to the other cDNAs is encoded entirely by one exon in the genetic locus, consistent with formation of *KIFC5B* by alternative splicing. Comparison of *KIFC1* and *KIFC5A* with the genomic locus revealed extensive sequence identity with about a 5% difference in their coding sequence. Furthermore, 16 expressed sequence tags (ESTs) were detected in a search of the mouse EST database (unpublished observations). These sequences, although extremely related, fell into at least 4 classes. This is further evidence of a small multigene family in the mouse.

The sequences unique to the *KIFC5A* message show significant identity to sequences in the 5'-end of the *CHO2* cDNA. Besides raising the possibility of a common genetic precursor for these messages, the identification of the *KIFC5A* cDNA makes the prediction of the translation start site for *CHO2* and *KIFC5A* ambiguous. The *CHO2* cDNA contains two, in-frame, ATG codons, 117 bp apart, with the second identified previously as the start site of translation for the *CHO2* protein [15]. Neither of these start codons is part of a Kozak consensus [20]. In *KIFC5A*, however, a sequence similar to the *CHO2* sequence between the two methionine residues is placed just downstream of the ATG for the *KIFC1*, which is a Kozak consensus start site. This insert is a distinguishing feature of the KIFC5 cDNAs and may be functionally significant.

Alternative splicing offers one possible mechanism for the regulation of motor protein attachment to intended targets. It would provide a means of combining head domains with similar force-generating properties with different tail domains. Furthermore, there is a precedent for the use of alternative splicing as a mode of regulation of protein function in the testes. The CREB (cAMP response element-binding protein) precursor mRNA is differentially spliced in mid to late pachytene spermatocytes, resulting

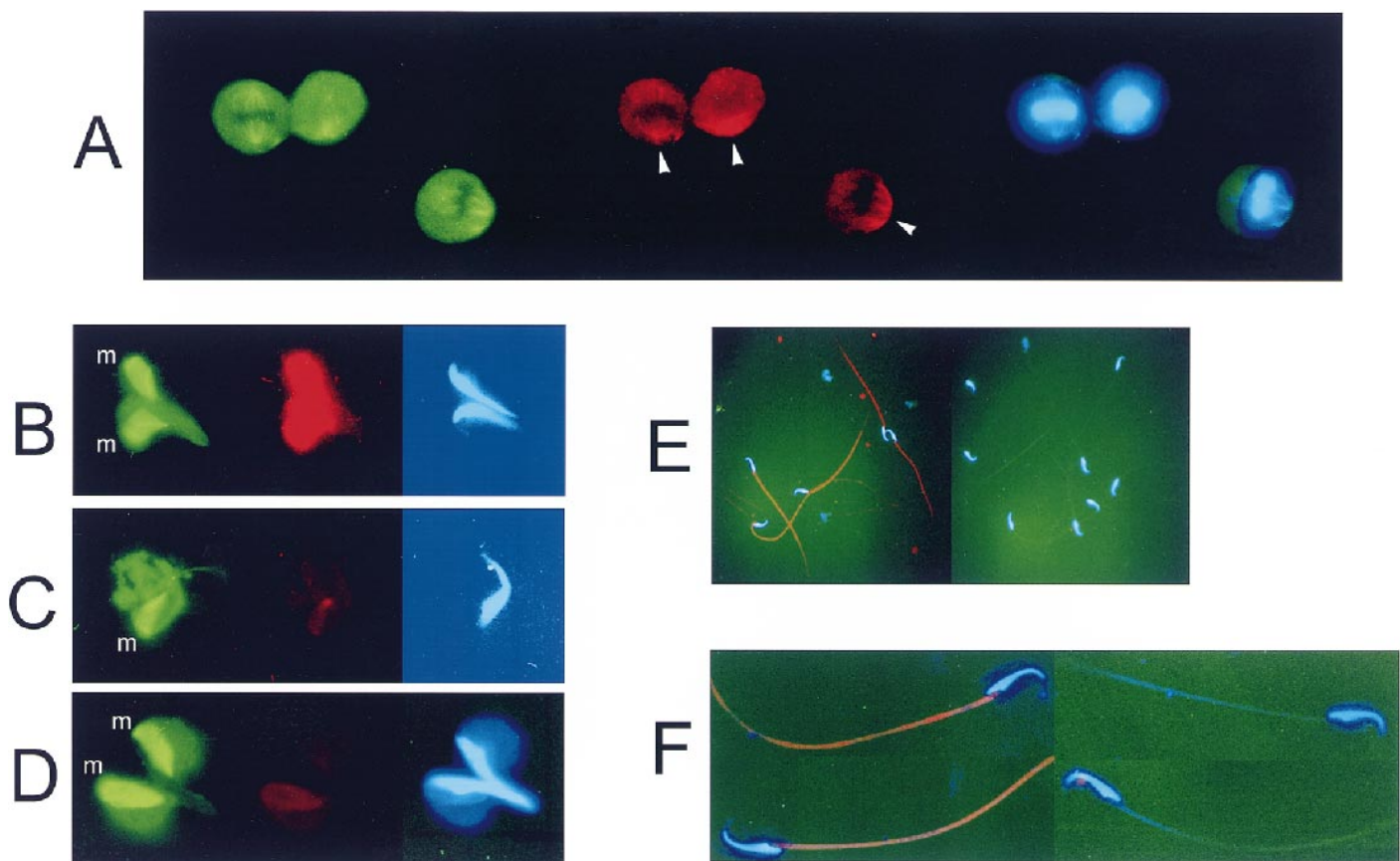


FIG. 6. Immunolocalization of the *CHO2* antigen in microtubule structures during spermatogenesis. **A**) Meiotic cells. **B-D**) Elongating spermatids. Cells were triple-stained with an FITC-conjugated antibody against α -tubulin (green), the *CHO2* monoclonal antibody was visualized with a Texas Red-conjugated secondary antibody (red), and the DNA was stained with DAPI (blue). Arrowheads in **A** indicate areas of intense staining with the *CHO2* monoclonal antibody at the position of the spindle poles. The positions of manchettes in **B-D** are indicated with an "m." **C**) An elongating spermatid stained as in **B** except that an irrelevant primary antibody was used in place of the *CHO2* antibody. An additional control is shown in **D**, in which the *CHO2* antibody was preincubated with bacterially expressed *KIFC5A* before use in immunofluorescence (see *Materials and Methods*). **E**) Localization of the *CHO2* antigen to the sperm tail (left panel) and a negative control in which the *CHO2* monoclonal antibody was replaced with an irrelevant antibody used as primary (right panel). The preincubated primary antibody was also used for immunofluorescence with isolated sperm in **F** (right panel) compared to unincubated antibody (left panel). The cells in **E** were photographed at $\times 100$, all others at $\times 250$ magnification (published at 90%).

in the conversion of this protein from a transcriptional activator to an inhibitor [27]. This cell-type specific splicing event results in the insertion of a 5' exon, causing a translation switch to a downstream ATG. This is especially pertinent to the results reported here, in which the insertion of a 5' exon might introduce an upstream ATG to begin the *KIFC5A* open reading frame. In the case of *KIFC5A*, such differential splicing might result in insertion of a highly basic peptide that may have functional importance.

We have localized a group of putative minus-end directed motors to multiple microtubule complexes in spermatogenesis. Several microtubule networks in the testes might require unique motors, including the meiotic spindle, the spermatid manchette, the flagella, and elements of the Sertoli cytoskeleton. These motors we have identified are almost identical except for their cargo-binding tail domains. The finding that the manchette contains motors related to those shown to be important for spindle stability is particularly intriguing. The manchette, like the spindle, displays complex motility that most likely relies on numerous motor activities [4, 28]. During its brief lifetime, the manchette assembles around the nucleus of step 7 spermatids, moves along the nuclear membrane towards its caudal end, con-

stricts during nuclear elongation, and finally disassembles in step 14 spermatids. The fact that other members of the COOH terminal subfamily—such as *XCTK2* [23], *CHO2* [15], and *ncd* [29]—have been shown to bundle microtubules and to promote the stability of microtubule networks leads us to propose that the motors described here may play a similar role in various microtubule complexes, including the manchette, during spermatogenesis. The divergent tail domains might serve to modulate their association with, and motor function in, different complexes. Further examination of the role of members of the *CHO2* subfamily in the biogenesis and function of the spindle, manchette, and flagella should reveal commonalities of motor function as well as explaining how different motor environments can result in such different forms of motility.

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