

# Chromokinesin: a DNA-binding, Kinesin-like Nuclear Protein

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**Abstract.** Microtubule-associated mechanoenzymes have been proposed to play a fundamental role in chromosome movement. We have cloned and characterized the cDNA for a novel protein, named Chromokinesin, that fulfills several of the criteria expected of a mitotic motor. Chromokinesin contains both a kinesin motor-like domain and an unusual basic-leucine zipper DNA-binding domain. Its mRNA is readily detectable in proliferating cells,

but not in postmitotic cells. Immunocytochemical analysis with antibodies directed against the nonconserved COOH-terminal region of Chromokinesin indicates that the protein is localized in the nucleus, and primarily associated with chromosome arms in mitotic cells. These data suggest that Chromokinesin is likely to function as a microtubule-based mitotic motor with DNA as its cargo.

**M**ITOTIC division involves a sequence of complex and coordinated chromosome movements, such as congression at the metaphase plate and segregation towards the spindle poles. The mechanisms responsible for these movements have been the subject of extensive studies, but remain incompletely understood. It has been proposed that some of the necessary forces are generated by changes in the polymerization/depolymerization of spindle microtubules (Coue et al., 1991; Koshland, 1994), and/or by the action of motor molecules propelling chromosomes along microtubules (Gorbsky et al., 1987; Salmon, 1989; McIntosh and Pfarr, 1991; Bloom, 1993; Rieder and Salmon, 1994). Information about the identity and actual function of these putative mitotic motors is still fragmentary, but suggests that they constitute a heterogeneous group of molecules with complementary, redundant, or even antagonistic functions. Motor molecules directly tethering chromosomes to spindle microtubules, although presumed to exist (Hyman et al., 1992), have not been identified.

Candidate molecules for mitotic motors include microtubule-based mechanochemical enzymes known to move cellular elements towards either the fast- or the slow-growing (or "+" and "-") ends of microtubules. For example, cytoplasmic dynein, a minus end-directed motor, has been immunocytochemically detected at the kinetochore, centrosome, and spindle fibers (Pfarr et al., 1990; Steuer et al., 1990). Particularly relevant to this report, however, is the superfamily of kinesin-like proteins. The prototypical kinesin

is a plus end-directed motor consisting of two light chains and two heavy chains (Vale et al., 1985); the latter, in turn, contain an NH<sub>2</sub>-terminal globular head (motor domain), a coiled-coil stalk, and a globular tail (Yang et al., 1989; Kosik et al., 1990). Kinesin itself does not appear involved in mitotic movements (Saxton et al., 1991), but several kinesin-like proteins have been suggested to participate in centrosome separation and/or spindle formation (Enos and Morris, 1990; Endow, 1991; Goldstein, 1991; Theurkauf and Hawley, 1992; McIntosh, 1994). The family of kinesin-like proteins is defined by the presence in all its members of a conserved head region resembling the motor domain of *Drosophila* kinesin heavy chain (Endow, 1991; Skoufias and Scholey, 1993; Walker and Sheets, 1993). This region contains both ATP- and microtubule-binding sites, and is sufficient to generate force and motion relative to microtubules in an ATP-dependent manner (Scholey et al., 1989; McDonald et al., 1990). The various members of the kinesin-like family, on the other hand, differ significantly in regions outside the motor domain; it has been hypothesized that the variable tail regions are responsible for recognition and binding of appropriate cargos (Yang et al., 1989; Vale and Goldstein, 1990). Direct evidence for this motor: cargo-binding specificity, however, is still limited.

In this paper we report the characterization of Chromokinesin, a hitherto undescribed member of the kinesin-like family that contains both a kinesin motor-like domain and an unusual basic-leucine zipper DNA-binding domain. Its capacity to bind DNA was verified by South-Western analysis. In situ hybridization and immunocytochemical evidence showed its abundance in proliferating cells and its association with mitotic chromosomes. We postulate that Chromokinesin may link chromosomal DNA to spindle microtubules, and could function as a mitotic motor.

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## Materials and Methods

### Cloning of Chromokinesin

A previously cloned 1086-bp fragment (*sw3-3*) of *chromokinesin* (*chk*)<sup>1</sup> cDNA (Wang and Adler, 1994) was used as a probe for screening an unamplified E6 chick embryo retina cDNA library constructed with the SuperScript Lambda System (Bethesda Research Laboratories) primed with oligo dT and random hexamers. Six positive clones were obtained with inserts corresponding to a total of 3496 bp. The remaining 922 bp at the 5' end of the cDNA were obtained with the 5' RACE method (GIBCO BRL, Gaithersburg, MD) using first strand cDNA primed with *chk* specific oligonucleotides corresponding to the sequence between 1570 and 1590 bp. Both DNA strands were sequenced. A tentative assignment of a start ATG codon was made based on comparison with conserved motor domains at the NH<sub>2</sub>-terminal region of kinesin heavy chains from *Drosophila* and squid (Yang et al., 1989; Kosik et al., 1990).

### PCR Amplifications

PCR reactions aimed at determining whether the zip-b-zip and motor domain coexisted within the same molecule were carried out with first-strand cDNA synthesized from poly(A)<sup>+</sup> RNA isolated from E6 retinas, independently from that used for cDNA library and 5' RACE. An oligonucleotide primer corresponding to a region 3' to the PEST sequences was used. PCRs were carried out using as 5' primer either one of two oligonucleotides corresponding to different regions of the kinesin-like motor domain (GAGGCAG-GATTGGGAATTTG, starting at nucleotide 453, and CCTACAGAGACT-CAAAG, starting at nucleotide 935) of Chromokinesin, and as 3' primer an oligonucleotide corresponding to leucine zipper 2 in the zip-b-zip region (downstream primer CACTCCTGAGCTCCATCTCAG, starting at nucleotide 2575). After a "hot start," 35 cycles of amplification at 53°C for 30 s, 72°C for 1.5 min, and 95°C for 45 s were carried out, with a final annealing step at 53°C and extension at 72°C for 10 min.

### Expression and Purification of Fusion Polypeptides

Three *chk* cDNA fragments (see Fig. 4 A), derived from the *sw3-3* fragment (see Fig. 3), were ligated (through SstI-HindIII sites) to DNA corresponding to the NH<sub>2</sub>-terminal 260 amino acids of T7 gene 10 in pGEMEX-1 (Promega). The recombinant plasmids were transformed into *Escherichia coli* JM109. *E. coli* BL21(DE3) pLysS, (Novagen, Inc., Madison, WI) was used for expression. Harvested cells were lysed by two passes through a French Press (16,000 psi) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1% Na-deoxycholate. Since the overexpressed polypep-

1. *Abbreviations used in this paper:* E6, embryonic day 6; *chk*, chromokinesin; RT-PCR, reverse transcription-polymerase chain reaction.

ptides were always insoluble in this buffer, the pellets were washed twice, solubilized with 8 M urea, and dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl at 4°C. Remaining insoluble materials were removed by centrifugation. Purified polypeptides were analyzed by SDS-PAGE.

### DNA Binding Assays

Purified fusion polypeptides were subjected to SDS-PAGE with ~0.4 µg protein loaded in each lane. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, renatured in the presence of 1% Triton X-100 in reaction buffer (33 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1 mM EDTA), prehybridized with 25 µg poly(dI-dC)/poly(dI-dC) and 400 µg tRNA at room temperature in reaction buffer for 1 h, and hybridized with 50 ng MboII-digested chicken genomic DNA labeled with <sup>32</sup>P by random priming using a DNA-labeling kit (Boehringer Mannheim). Five washes were done with the same buffer for a total of 1 h at room temperature. The membrane was then air dried and exposed to x-ray film. After autoradiography, the membrane was incubated with affinity-purified antibody against T7 gene 10 polypeptide (as shown in Fig. 4 B, lane 8), followed by protein A-peroxidase and enhanced chemiluminescence substrate (Amersham Corp., Arlington Heights, IL).

### In Situ mRNA Hybridization

Sense and antisense dioxigenin-labeled RNA probes were prepared from plasmids pSPT 18 and pSPT 19 with an insert of 1086 bp corresponding to the *sw3-3* fragment (see Fig. 3) of *chk*, using the Genius RNA Labeling Kit according to the manufacturer's directions (Boehringer Mannheim Corp., Indianapolis, IN). In situ mRNA hybridization protocols have been previously described in detail (Wang and Adler, 1994). For double labeling experiments with BrdU E5 embryos were injected with 50 µg of BrdU; 3 h later they were fixed in paraformaldehyde, and cross sections were processed first for in situ mRNA hybridization (see above), and then reacted with anti-BrdU antibodies and rhodamine-labeled secondary antibodies essentially as described by Biffo et al. (1992).

### Immunocytochemistry

Polyclonal antibodies against Chromokinesin were produced in rabbits against a SDS-PAGE gel purified fusion polypeptide containing nonconserved sequence at the tail region (derived from *sw3-3* fragment, see construct P in Fig. 4 A). Anti-Chromokinesin antibodies were purified by preabsorbing sequentially the crude serum with total *E. coli* lysate, purified T7 gene 10 polypeptide (see Fig. 4 B), and purified fusion polypeptide from construct BZP (see Fig. 4, A and B). Antibodies bound to purified gene 10 polypeptide and to fusion polypeptide BZP were eluted and found to react specifically to gene 10 and Chromokinesin, respectively (data not shown). Affinity purified anti-Chromokinesin antibody and anti-gene 10 antibody were used as primary antibodies, followed by the ABC-peroxidase

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MVREEEKGLPVRVVRCPRLVPKETSSEGCQMCLSFVPGEPQVIVGSDKAFETYDYVDFRPSVE 60
QEEVENTAVAPLIRGIFKGYNATVLAAYGOTGSGKTYSMGGTYTASQEHDRSMGVIPRVIK 120
LLFKEKEQRQDWEFVFLKVSYLEIYNEDILDLICSSRRERSQISIREDPKEGIKIVGLTER 180
NVASARDTVSCLREQNCRVTASTMNSQSSRSJHALFTICIDOKKKNPKNSSFHSLHLV 240
DLAGSERQKKTAKBGDRLEKGININRGLLCLGNVIALGEEENKKGGFVVPYRDSKILTRLLQ 300
DSLGGNSHTLMTACVSPADSNLEETLNTLRVADRARKIKNKPINVNDPQAAELNHLKQOV 360
QQLQVLLQAHGGTLPVINSMAPSENQLSLMEKNQSLMEENEKLSRGLSEAAGQTAQML 420
ERITVTEQENEKMAKLEQLQQAHAVCKLDLQKLLTVEDEELKENVEVIRNLQOVLAQFQ 480
SESAAAAEAATEMANAEQDAAGEAETGQVTKRSSDDFTQHALRQAQMSKELVELNKAALA 540
LKEALAKKMIQNDSQLEPIQSQYQTNIKDLEVSNLQKEKEELILALSMAKKDVNQAKL 600
SERRRRLQLEGGQINELKKKLNEQAKLLKKESTERTVSKLNQETIREMKNQVRQLMRQM 660
KEDAERFRQWKQKQKDEVIQLKERDRKRQYELLKLERDFQKQASVLRRTTEAAAAANKRL 720
KDALQKQREAADKRKESQNRGMEGVAARVKSWSLANEVEVLVSTEEARRHLADLLEDRKIL 780
AOELLQKKEKESGENPPSKLRRRTYSITDLOASEMDLSLSKOIESLETETMELRSAOIAD 840
LOOKLLDADNGDRVQQRWDNIATILEAKCALKYLLGELVSSKVQESKLESGLQQSKTNC 900
DIQKMLIEERNHATEMEAEFQNLQLLQEQHQQEVLYLLSQFQKQKQKQKQKQKQKQKQKQK 960
KQMQRERLKFQEKELKEMREICEKNQELLQENDVLRKQKMLLVQVQASGQKLRDQVSPESP 1020
DSPFDYIPPKPKTRRQTVAKPRAPTPEMNVVEELFSDSEESGEEEDAEWVVPVKAAGTKKS 1080
ATGCFCKGRCGNRQCGCRKQKVGCTAGCSCDSTKCRNRDPGFQDATVCEQDQTKDSEGSFK 1140
LEDLRDVTAGETFFQPVYSPPTMKVLKIDITDQGVFMKPKSTAASLLVRDEESQENQLPFV 1200
KKKKRMLSSNTSFFSGCTPIKEEID
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Figure 1. Deduced protein sequence of Chromokinesin. Main features in the primary structure of Chromokinesin include a kinesin-like motor domain (dotted underlining), a leucine zipper-basic domain-leucine zipper structure (solid underlining), and two PEST sequences (double underlining); the latter are usually found in short-lived proteins (Rogers et al., 1986). These sequence data are available from EMBL/GenBank/DBJ under accession number U18309.

method (Vector Laboratories) as described by the manufacturer with 3-amino-9-ethylcarbazole as chromogen. Glial cultures were derived from E8 chick embryo retinas as described (Threlkeld et al., 1989). Preparation of cultured cells for immunocytochemistry was as described by Earnshaw et al. (1989), except that no colcemide was used and the hypotonic treatment was done on ice.

## Results

### *chk* Encodes a Kinesin-like Protein with a DNA-binding Domain

A 1086-bp fragment of *chk* cDNA was initially isolated from embryonic chick retina using the reverse transcription-polymerase chain reaction (RT-PCR), and temporarily named *sw3-3* (Wang and Adler, 1994). The deduced amino acid sequence of this fragment contains a region of 59 residues that shows ~20% sequence identity with members of the basic-leucine zipper (b-zip) family of transcriptional regulators (Landschulz et al., 1988). However, while all known members of this family have a single leucine zipper motif following a basic domain, *Sw3-3* was found to contain two leucine zippers, one on each side of the basic domain (i.e., zip-b-zip) (Wang and Adler, 1994). Towards the COOH-terminal end, the *Sw3-3* fragment contains two PEST sequences, usually found in proteins with short half-lives (Rogers et al., 1986). In order to further characterize this potentially interesting molecule, we used the *sw3-3* fragment as a probe for screening an embryonic day 6 (E6) chicken retina cDNA library.

The RACE (rapid amplification of cDNA ends) method was used to obtain additional information on the 5' region of the *chk* cDNA. The resulting cDNA sequence is 4418 bp long, with an open reading frame corresponding to a protein of 1225 amino acids (Fig. 1).

Analysis of the deduced protein sequence indicates that the NH<sub>2</sub>-terminal 350 amino acids of Chromokinesin (which is located 400 residues NH<sub>2</sub>-terminal to the zip-b-zip structure) is highly homologous to kinesin motor domains (Fig. 2), with 41 and 42% sequence identity to the kinesin heavy chains of *Drosophila* (Yang et al., 1989) and squid (Kosik et al., 1990), respectively, and 81% identity to a *Xenopus* kinesin-like protein, XKlpl (Vernos et al., 1993). Homology among all these proteins includes several perfectly matching segments, of five or more consecutive residues, within the putative ATP- and microtubule-binding regions (Fig. 2); they further identify Chromokinesin as a novel member of the kinesin-like protein family. As is the rule among members of this family, Chromokinesin does not show any obvious similarities to other published kinesin-like proteins outside the motor domain. There is, however a 69% overall sequence identity between Chromokinesin and the full-length *Xklpl* deposited by Vernos, Hirano, Raats, Wylie, and Karsent in the EMBL database (accession number x82012). This suggests that *Xklpl* may be the *Xenopus* homologue of Chromokinesin.

To verify that the apparent coexistence of a kinesin-like motor domain and a zip-b-zip structure in Chromokinesin

	1		
Chk	MVREEEKGIPVRVRCRPLVPKETS	EGCQMCLSFV	PGE PQVIVGSDKAFTYDYVFDPSVE
XKlpl	VRVA*****NN**K***T**		***Q*****TE*S*****A**
DROME	MSAEREIPAEDSIKVC*F***NDS*	EKA*SKFVVK*PNNV*ENC*SIAG*	VYLF*K**K*NAS
Squid	MDVSECNIKVI*RV***NEA*ERA*	SKFI*K*P	TDDS*SIAG*V*VF*K*LK*N*S
		ATP-binding	
	61		
Chk	QEEVFNTAVAPLIRGIFKGYNATV	LAYGQTGSGKTYSMGGTYTASQE	HDPSMGVIPRVIK
XKlpl	***Y*S*****K*L*****A**	HN**NE*TV*****TVI	
DROME	**K*Y*E*AKSIVTDVLA***G*IF*	*****S***HT*E*VIGDSVKQ	*I***IVN
Squid	**Y*Y*VGAK*IVADV*S*C*G*IF*	*****HT*E*VLDKPSM*	*I***IVQ
	91		
Chk	LLFKEK EQRQDWEFVLKVSYLEIYN	EDILDLLCSSRERSQISIREDPKEGI	KIVGLTER
XKlpl	A**R*I H**PE***N*****E**	***YAA*DKTNT**L*****C*****	
DROME	DI*NHIYAMEVNL**HI****Y***	MDK*R***DV*KV NL*VH**KNRV	PYVK*A***
Squid	DI*NYIYGMDENL**HI*I**Y***	LDK*R***DVTKT NLAVH**KNRV	PYVK*A***
			? ———
	181		
Chk	NVASARDTVSCLEQGNCRVASTAMNS	QSSRSHAI FTICIDQKKNDKNSS	FHSKLHLV
XKlpl	D*KT*L*L*****SS*****D*S*	*****R*EG**N**R*****	
DROME	F*S*PE*VFVEVI*E*KSN*HI*V*N*	EH*****SV*L*NVK*ENL	ENQKKLSG**Y**
Squid	F*S*PEEVMVIDE*K*N*H**V*N*	EH*****SV*L*NVK*ENV	ETQKKLSG**Y**
	241		
Chk	DLAGSERQKKTKAEGDRLKEGININR	GLLCLGNVISALGEENKGGFV	PYRDSKLTRLLQ
XKlpl	*****S*****D*S*****I**		
DROME	*****KVS**G***TV*D*AK***	KS*SA*****ADG**TH	I*****I**
Squid	*****KVS**G***AV*D*AK***	KS*SA*****ADG**SH	*****I**
	301		
Chk	DSLGGNSHTLMIACVSPADSNLEETL	NLTLRYADRARKIKNKPIVNVDP	QAAE 352
XKlpl	*****M*****I**		
DROME	E*****AR*TVI*C***SF*ES**	KS**DFGR**KTV**VVC**EELT*E*	347
Squid	E*****AR*T*VI*C***SY*ES**	KS**LFGQ**KT**VVS**EELT*D*	340

Figure 2. Comparative alignment of the NH<sub>2</sub>-terminal region of Chromokinesin (*Chk*) with sequences from kinesin heavy chain motor domains of *Drosophila* (*DROME*, Yang et al., 1989) and squid (Kosik et al., 1990), and with a *Xenopus* kinesin-like protein *XKlpl* (Vernos et al., 1993). Identical residues are indicated by asterisks. Gaps, represented by spaces, were introduced to maximize homology. The putative ATP-binding site, and the region in kinesin heavy chain of *Drosophila* known to bind microtubules (underlined), are indicated. A question mark indicates uncertainty regarding the NH<sub>2</sub>-terminal boundary of the microtubule-binding region (Yang et al., 1989; Endow et al., 1990).

was not due to a cloning artifact, PCR was employed using a downstream primer corresponding to the zip-b-zip region, and either one of two upstream primers corresponding to regions in the motor domain. The template was first-strand cDNA prepared from samples of E6 retina mRNA, different from those used for cloning *chk*. PCR products of the predicted size were observed (Fig. 3) confirming that both domains are present in the same mRNA molecule.

### Chromokinesin Binds Genomic DNA

The DNA-binding properties of the zip-b-zip region of Chromokinesin were investigated by South-Western analysis using radioactively labeled chicken genomic DNA and bacterially expressed fusion (Chromokinesin polypeptides (derived from the *sw3-3* fragment, Figs. 3 and 4 A), that were purified to near homogeneity (Fig. 4 B). The ZBZP polypeptide, that contains both leucine zipper motifs flanking the basic domain, showed strong binding (Fig. 4 C, I), while none of the other polypeptides did so. Weak bands, probably representing background, were obtained with construct P, which lacks the zip-b-zip structure, and with construct BZP, which lacks leucine zipper no. 1, the extra leucine zipper that is absent in regular b-zip proteins (Fig. 4, B and C). This suggests that, under our assay conditions, the basic domain and leucine zipper no. 1 are required for efficient binding to DNA. The role of leucine zipper no. 2, which is present in all known members of the family of b-zip proteins, was not investigated in our study.

### *chk* Is Expressed Preferentially, if Not Exclusively, in Proliferating Cells

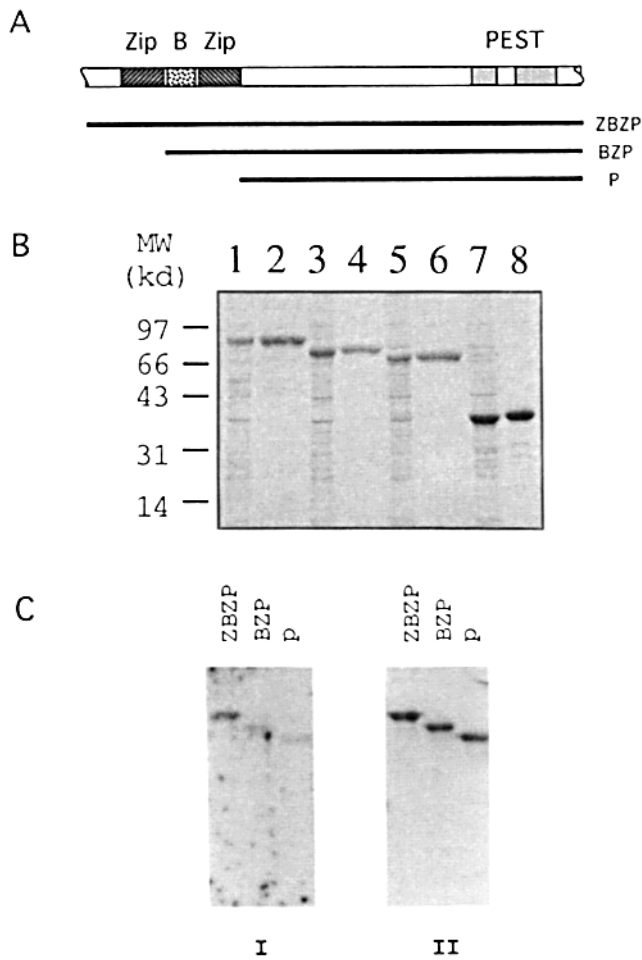
In situ mRNA hybridization using RNA probes against the 1086-bp *sw3-3* fragment (Fig. 3) of the *chk* cDNA showed that the gene is expressed predominantly in proliferating cells. In the developing CNS of chick embryos, *chk* mRNA was detected exclusively in the region of the neuroepithelium known to contain mitotically competent cells (Fig. 5 A). No signal was seen in the mantle zone, where cells migrate after terminal mitosis. A similar correlation has been observed in the retina and lens (Wang and Adler, 1994), as well as in non-neural tissues such as limb bud and mesenchyme (data not shown). Further analysis showed that *chk* mRNA is detectable not only in cells that are generally competent to divide, but also in those that are actually undergoing proliferation. After embryos were pulse-labeled with BrdU for three hours and sequentially processed for in situ hybridization and BrdU immunocytochemistry, strong *chk* mRNA signals were observed in many BrdU-labeled cells (i.e., those undergoing S-phase during the BrdU-labeling period; Fig. 5 C). Moreover, *chk* mRNA could also be seen in cells going through metaphase, telophase, or cytokinesis (Fig. 5, D-F).

### Chromokinesin Is a Nuclear Protein Associated with Chromosomes in Mitotic Cells

Immunohistochemical analysis of the chick embryo neural tube using affinity purified antibodies against a region of



**Figure 3.** Verification by PCR analysis of the coexistence of a kinesin-like motor domain and a zip-b-zip domain in Chromokinesin. As described in detail in Materials and Methods, the reactions were carried out with first-strand cDNA synthesized from E6 retina mRNA, isolated independently from that used for cDNA library and 5' RACE. The two bottom lines indicate the predicted size of PCR products, based on the position of the corresponding primers (see Materials and Methods). As shown in the negative image of an ethidium bromide-stained agarose gel, amplified products of the correct size (2.12 and 1.64 kb) were obtained. The relative position of the *sw3-3* fragment (Wang and Adler, 1994) is indicated.



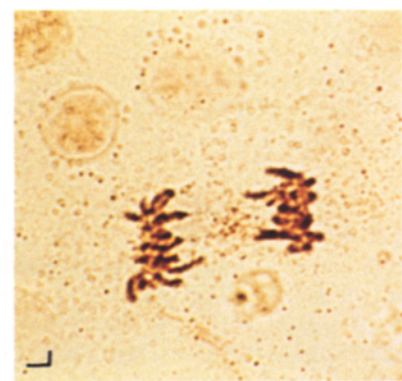
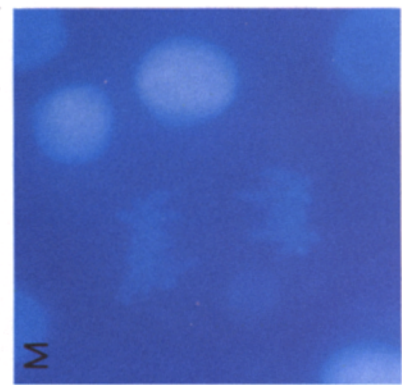
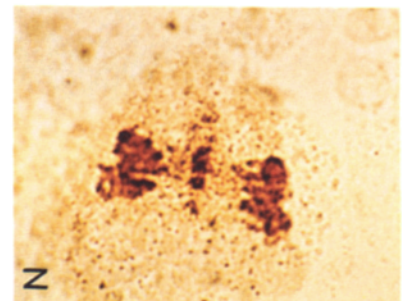
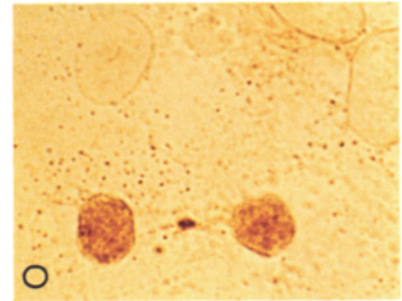
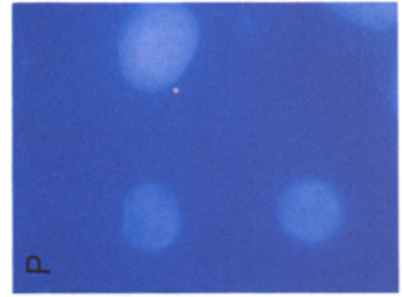
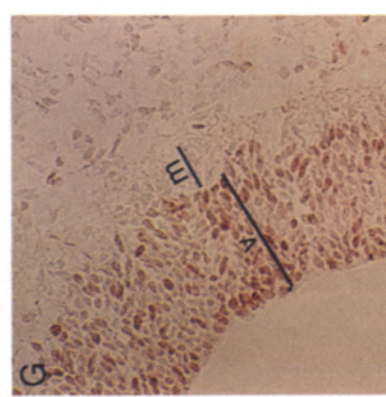
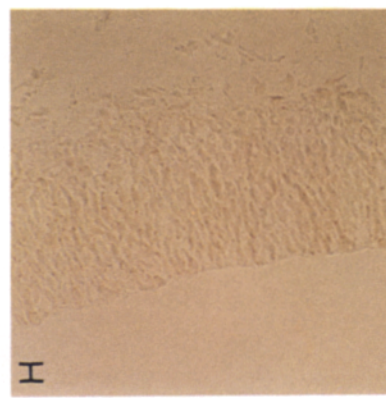
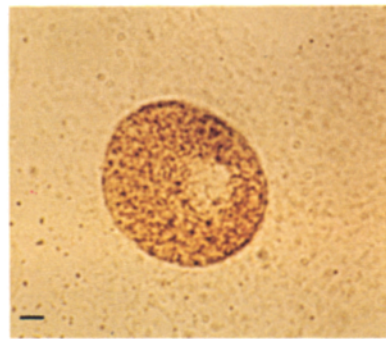
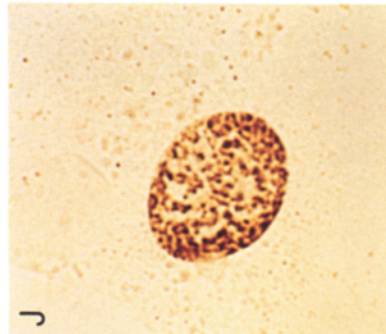
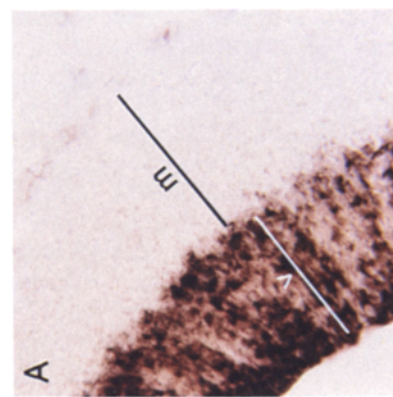
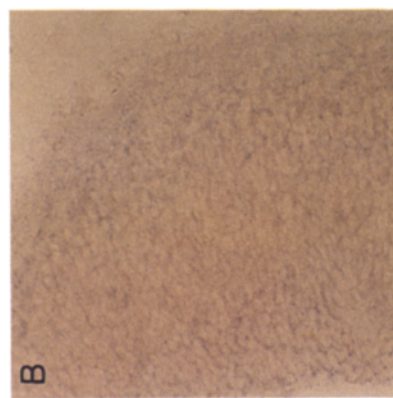
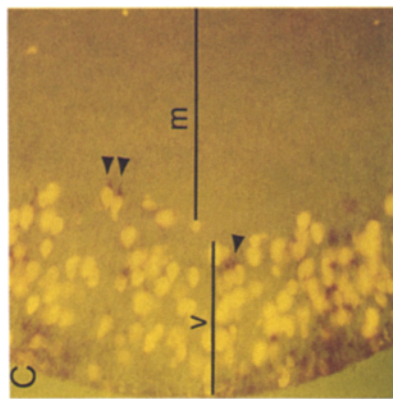
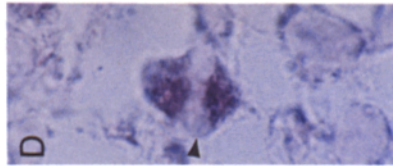
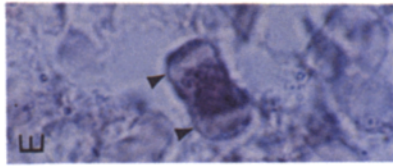
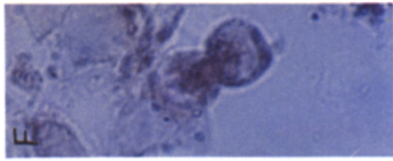
**Figure 4.** South-Western analysis of the DNA-binding properties of bacterially expressed Chromokinesin polypeptides. (A) Schematic illustration of the relative length and composition of three polypeptides generated by expression of cDNA fragments in an expression system (see Materials and Methods). ZBZP was coded for by the 1086 bp *sw3-3* cDNA fragment (Fig. 3), and contains both leucine zippers and the basic domain; BZB corresponds to a 916-bp fragment, and lacks leucine zipper no. 1; polypeptide P (corresponding to a 766-bp cDNA fragment) lacks the entire zip-b-zip region. (B) Coomassie blue-stained SDS-PAGE gel of proteins from *E. coli* harboring expression plasmids with various *chk* inserts (see A). Lanes 1, 3, 5, and 7, total protein; lanes 2, 4, 6 and 8, purified fusion polypeptides. Lanes 1 and 2, polypeptide ZBZP; lanes 3 and 4, polypeptide BZP. Lanes 5 and 6, polypeptide P. Lanes 7 and 8, vector polypeptide (T7 gene 10). (C) DNA binding assayed by South-Western hybridization. (I) DNA binding by fusion Chromokinesin polypeptides. Only ZBZP yielded positive signals above background (i.e., that of polypeptide P). (II) The same membrane reacted with affinity purified antibody against vector protein (T7 gene 10, which is present in all constructs), showing the relative amount of fusion polypeptides present in each lane.

Chromokinesin devoid of obvious similarities with any published proteins in the database (Fig. 4 A, construct P) showed a distribution of immunoreactive cells similar to that seen with *in situ* mRNA hybridization, i.e., restriction of positive cells to the proliferating zone (Fig. 5 G). The immunocytochemical signal, moreover, appeared almost exclusively localized to the nucleus of the cells. Sections stained with

anti-gene 10 antibody, that was affinity purified from the same antisera as the anti-Chromokinesin antibody (See Materials and Methods), were consistently negative (Fig. 5 H). Further analysis of proliferating cells in retinal glial cultures (Fig. 5, I–P) showed that Chromokinesin immunoreactivity is strongly associated with chromosomes at all stages of mitosis. While centromeres appear negative, immunostaining was seen throughout the arms of the chromosomes, which frequently had a beaded appearance due to the presence of some darker areas (Fig. 5, J–N). Some immunoreactivity was also detected at the interzone in anaphase B/telophase (Fig. 5 N) and at the midbody during cytokinesis (Fig. 5 O); these regions are known to contain remains of the two sets of polar microtubules. No immunoreactivity was obvious in interphase cells (Fig. 5, L and M, and O and P; and data not shown). As in the case of the neural tube, immunostaining of cultured glial cells with control antibody against gene 10 protein gave negative results (data not shown).

## Discussion

Several lines of evidence suggest that Chromokinesin should be considered a bona fide member of the kinesin-like family of proteins. Previously described members of this family have been reported to share a region of ~340 amino acids showing 35–45% identity to the kinesin heavy chain of *Drosophila*, with highly conserved segments separated by regions of low sequence similarity (Goldstein, 1991). This so-called “motor domain” is capable of ATP-dependent microtubule translocation, and is usually located near the NH<sub>2</sub>-terminus of the molecule (for exceptions see Vale and Goldstein, 1990). These features are also present in Chromokinesin; its first 350 amino acids show 41% sequence identity with the canonical *Drosophila* kinesin motor domain, and six segments within this Chromokinesin region, including the ATP-binding site, have at least five consecutive residues that are perfectly conserved in the kinesin heavy chains of *Drosophila* and squid, and in a kinesin-like protein from *Xenopus* (Xklp1) (Yang et al., 1989; Kosik et al., 1990; Vernos et al., 1993). As already indicated in Results, Xklp1 may in fact be the *Xenopus* homologue of Chromokinesin, since there is 69% identity between the latter and the Xklp1 full-length sequence deposited by Vernos et al. in the EMBL database. Another feature shared by Chromokinesin and other members of the kinesin-like protein family is the lack of conservation in their tail regions. It has been hypothesized that each kinesin-like protein participates in the transport of a specific subset of intracellular elements, such as organelles, vesicles, or chromosomes; the diverging tail regions would provide the physical substratum for these specific motor/cargo interactions (Yang et al., 1989; Vale and Goldstein, 1991). This model is conceptually appealing, and has received support from experiments showing the binding of kinesin-like proteins, such as KAR3 and CENP-E, to their presumed cargoes (Meluh and Rose, 1990; Liao et al., 1994). The evidence reported here suggests that genomic DNA may be a cargo for Chromokinesin. This possibility was suggested by the immunocytochemical localization of Chromokinesin to mitotic chromosomes, and corroborated by the binding of the tail region of Chromokinesin to genomic DNA observed in South-Western studies. The DNA-



binding capacity of Chromokinesin could be associated with a region located ~400 amino acids COOH-terminal to the conserved motor domain, consisting of two leucine zipper motifs separated by a basic domain (zip-b-zip). This region shows some similarities with the b-zip family of transcription factors, in which the basic domain (which mediates DNA binding) is adjacent to a single leucine zipper motif (a region of 30–40 residues containing a heptad repeat of leucine residues, and believed to mediate protein–protein interaction) (Kouzarides and Ziff, 1988; Turner and Tjian, 1988). Distinguishing features of Chromokinesin, however, include the presence of an additional leucine zipper motif, and the presence of helix disturbing residues (glycine and proline) at positions respectively occupied by conserved asparagine and alanine residues in the basic domain of conventional b-zip proteins (Wang and Adler, 1994). The observed binding of the Chromokinesin zip-b-zip region to DNA occurred only with Chromokinesin tail fragments containing the basic domain and both leucine zippers (construct ZBZP), but not with fragments lacking the extra leucine zipper (construct BZP), or lacking the basic domain and both leucine zippers (construct P). These results are suggestive of a specific interaction between Chromokinesin and DNA but, given that our *in vitro* South-Western assays do not necessarily reflect physiological conditions, further studies are necessary to verify this possibility.

The existence of multiple members of the kinesin-like family in each animal species has raised questions about the need for cell type- and developmental stage-specific regulation of their expression (Goldstein, 1991). Information pertinent to these questions is scarce, although there are examples in the literature for both broadly distributed and cell type-restricted kinesin-like molecules (Niclas et al., 1994). Chromokinesin appears to have fairly broad tissue distribution, but its expression is developmentally regulated with a pattern that seems to correlate with the mitotic activity of the cells. For example, *in situ* mRNA hybridization analysis of embryonic retina and CNS organs using probes against the 3' end of *chk*, show positive cells only in regions occupied by proliferating cells; they are consistently absent from the mantle layer, where cells migrate after terminal mitosis. A similar pattern has been observed in the lens, where there is a clear equatorial demarcation between the anterior epithelium occupied by proliferating, *chk* mRNA (+) cells, and

the posterior region, where postmitotic, *chk* mRNA (–) cells are found (Wang and Adler, 1994). The spatio-temporal pattern of distribution of immunoreactive materials detected with antibodies against the non-conserved tail region of Chromokinesin is very similar to the distribution of *chk* mRNA observed by *in situ* hybridization. The remarkable sharpness of the demarcation between territories occupied by + and – cells observed with both immunocytochemistry and *in situ* hybridization suggests that the Chromokinesin protein and its corresponding mRNA have fairly short half-lives, and/or that the expression of the *chk* gene may be tightly regulated at the transcriptional level. While further studies will be necessary to investigate each one of these possibilities, it is noteworthy that the COOH-terminal region of Chromokinesin sequence contains two PEST motifs, which are usually associated with proteins with a very short half-life. They could make Chromokinesin susceptible to ubiquitin proteolysis, as reported for cyclins and other proteins (Hershko and Ciechanover, 1992; Holloway et al., 1993).

The possibility that Chromokinesin could be involved in chromosome movement during mitosis is suggested by its predominant (if not exclusive) expression in mitotically competent cells, by the presence in its sequence of both a kinesin-like motor domain and a DNA-binding domain, by its capacity to bind DNA, and by its association with the arms of mitotic chromosomes. Given the importance of kinetochore/microtubule interactions in chromosome movements, the apparent absence of Chromokinesin immunoreactivity from kinetochores could be relevant. Our studies, however, cannot exclude the possibility that Chromokinesin could be present in the kinetochores at concentrations below the limits of detection of our techniques, or that the epitopes recognized by our antibodies could be masked by other kinetochore proteins. On the other hand, the abundance of Chromokinesin in chromosome arms is by itself compatible with a role of this protein in mitosis, since there is considerable evidence suggesting that non-kinetochore chromosomal regions interact with non-kinetochore microtubules during mitosis (Ault et al., 1991; Leslie, 1992; Rieder and Salmon, 1994). Examples of such interactions are microtubule-dependent “ejection” forces (Carlson, 1938; McNeill and Berns, 1981; Rieder et al., 1986), that have been proposed to play an important role in chromosome congression at the metaphase plate (Salmon, 1989; McIntosh and Pfarr, 1991;

**Figure 5.** Distribution of *chk* mRNA studied by *in situ* hybridization (A–F), and of Chromokinesin immunoreactive materials in embryonic tissues (G–H) and cultured glial cells (I–P). (A) A section of E6 CNS neuroepithelium hybridized with a digoxigenin-labeled antisense RNA probe prepared from the 1086-bp *sw3-3* fragment of *chk* (Fig. 3). Positive signals (purple color) are concentrated in the ventricular zone (*v*; the proliferating zone), whereas the mantle zone (*m*), in which postmitotic cells accumulate, is negative. (B) Similar section hybridized with a sense RNA probe; no signals are observed. (C) CNS neuroepithelium from an E5 embryo, processed for BrdU immunocytochemistry and *chk* *in situ* hybridization. *chk* mRNA is only detectable in the region occupied by BrdU-positive cells. Arrowheads point to double-labeled cells. (D–F) Mesenchymal cells in mitosis, in which chromosomes are indicated by arrowheads. In metaphase (D) *chk* mRNA can be seen on both sides of the equatorial chromosomes; in anaphase (E), *chk* mRNA accumulates in the region delineated by the chromosomes; a similar distribution is observed during cytokinesis (F). (G) Cryosection of E4 CNS neuroepithelium processed for immunocytochemistry with an affinity purified anti-Chromokinesin antibody; immunoreactivity appears concentrated to cell nuclei in the ventricular zone (*v*), and is essentially undetectable in the emerging mantle zone (*m*). Some positive cells can also be seen in the mesenchyme. (H) A control section stained with affinity-purified antibody against vector T7 gene 10 polypeptide; no signals are observed. (I–P) Cultured glial cells immunoreacted with an affinity purified antibody against Chromokinesin. Immunoreactivity appears associated with chromosomes throughout mitosis. I, prophase; J, late prophase; K, prometaphase/metaphase; L–M, anaphase visualized by bright field (L) and Hoechst dye no. 33258 fluorescence (M); N, telophase showing immunoreactivity in the interzone; O–P, cytokinesis showing immunoreactivity in the midbody (O), and Hoechst dye no. 33258 staining of the same cells (P). No positive signals were observed if cultured glial cells were immunostained with anti-gene 10 antibody (data not shown).

Alberts et al., 1994). Further studies are now needed to elucidate the actual role of Chromokinesin in the mitotic process.

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## References

- Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1994. *In Molecular Biology of the Cell*. Third Edition. Garland Publishing, Inc., New York. 925-927.
- Ault, J. G., A. J. Demarco, E. D. Salmon, and C. L. Rieder. 1991. Studies on the ejection properties of asters: astral microtubule turnover influences the oscillatory behavior and positioning of mono-oriented chromosomes. *J. Cell Sci.* 99:701-710.
- Biffo, S., L. Verdun di Cantogno, and A. J. Fasolo. 1992. Double labeling with non-isotopic *in situ* hybridization and BrdU immunohistochemistry: calmodulin (CaM) mRNA expression in post-mitotic neurons of the olfactory system. *Histochem. Cytochem.* 40:535-540.
- Bloom, K. 1993. The centromere frontier: kinetochore components, microtubule-based motility, and the CEN-value paradox. *Cell.* 73:621-624.
- Carlson, J. G. 1938. Mitotic behavior of induced chromosomal fragments lacking spindle attachments in the neuroblasts of the grasshopper. *Proc. Natl. Acad. Sci. USA.* 24:500-507.
- Coue, M., V. A. Lombillo, and J. R. McIntosh. 1991. Microtubule depolymerization promotes particle and chromosome movement *in vitro*. *J. Cell Biol.* 112:1165-1175.
- Earnshaw, W. C., H. Ratrie, III, and G. Stetten. 1989. Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads. *Chromosoma.* 98:1-12.
- Endow, S. A. 1991. The emerging kinesin family of microtubule motor proteins. *Trends Biochem. Sci.* 16:221-225.
- Endow, S. A., S. Henikoff, and L. Soler-Niedziela. 1990. Mediation of meiotic and early mitotic chromosome segregation in *Drosophila* by a protein related to kinesin. *Nature (Lond.)* 345:81-83.
- Enos, A. P., and N. R. Morris. 1990. Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in *A. nidulans*. *Cell.* 60:1019-1027.
- Goldstein, L. S. B. 1991. The kinesin superfamily: tails of functional redundancy. *Trends Cell Biol.* 1:93-98.
- Gorbsky, G. J., P. J. Sammak, and G. G. Borisy. 1987. Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. *J. Cell Biol.* 104:9-18.
- Hershko, A., and A. Ciechanover. 1992. The ubiquitin system for protein degradation. *Annu. Rev. Biochem.* 61:761-807.
- Holloway, S. L., M. Glotzer, R. W. King, and A. W. Murray. 1993. Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell.* 73:1393-1402.
- Hyman, A. A., K. Middleton, M. Centola, T. J. Mitchison, and J. Carbon. 1992. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature (Lond.)* 359:533-535.
- Koshland, D. 1994. Mitosis: back to the basics. *Cell.* 77:951-954.
- Kosik, K. S., L. D. Orecchio, B. Schnapp, H. Inouye, and R. L. Neve. 1990. The primary structure and analysis of the squid kinesin heavy chain. *J. Biol. Chem.* 265:3278-3283.
- Kouzarides, T., and E. Ziff. 1988. The role of the leucine zipper in the fos-jun interaction. *Nature (Lond.)* 336:646-651.
- Landschultz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science (Wash. DC)* 240:1759-1764.
- Leslie R. J. 1992. Chromosomes attain a metaphase position on half-spindles in the absence of and opposing spindle pole. *J. Cell Sci.* 103:125-130.
- Liao, H., G. Li, and T. J. Yen. 1994. Mitotic regulation of microtubule cross-linking activity of CENP-E kinetochore protein. *Science (Wash. DC)* 265:394-398.
- McDonald, H. B., R. J. Stewart, and L. S. B. Goldstein. 1990. The kinesin-like *ncd* protein of *Drosophila* is a minus end-directed microtubule motor. *Cell.* 63:1159-1165.
- McIntosh, J. R. 1994. The roles of microtubules in chromosome movement. *In Microtubules*. Wiley-Liss, New York. 413-434.
- McIntosh, J. R., and C. F. Pfarr. 1991. Mitotic motors. *J. Cell Biol.* 115:577-585.
- McNeill, P. A., and M. W. Berns. 1981. Chromosome behavior after laser microirradiation of a single kinetochore in mitotic PtK<sub>2</sub> cells. *J. Cell Biol.* 88:543-553.
- Meluh, P. B., and M. D. Rose. 1990. *KAR3*, a kinesin-related gene required for yeast nuclear fusion. *Cell.* 60:1029-1041.
- Niclas, J., F. Navone, N. Hom-Booher, and R. D. Vale. 1994. Cloning and localization of a conventional kinesin motor expressed exclusively in neurons. *Neuron.* 12:1059-1072.
- Pfarr, C. M., M. Coue, P. M. Grissom, T. S. Hays, M. E. Porter and J. R. McIntosh. 1990. Cytoplasmic dynein is localized to kinetochore during mitosis. *Nature (Lond.)* 345:263-265.
- Rieder, C. L., and E. D. Salmon. 1994. Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *J. Cell Biol.* 124:223-233.
- Rieder, C. L., E. A. Davison, L. C. W. Jensen, L. Cassimeris and E. D. Salmon. 1986. Oscillatory movements of mono-oriented chromosomes and their position relative to the spindle pole result from the ejection properties of the aster and half-spindle. *J. Cell Biol.* 103:581-591.
- Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science (Wash. DC)* 234:364-368.
- Salmon, E. D. 1989. Metaphase chromosome congression and anaphase poleward movement. *In Cell Movement*. Vol. 2, F. D. Warner and J. R. McIntosh editors. Alan R. Liss Inc., New York. 431-440.
- Saxton, W. M., J. Hicks, L. S. B. Goldstein, and E. Raff. 1991. Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila*, but mutants show no defects in mitosis. *Cell.* 64:1093-1102.
- Scholey, J. M., J. Heuser, J. T. Yang, and L. S. B. Goldstein. 1989. Identification of globular mechanochemical heads of kinesin. *Nature (Lond.)* 338:355-357.
- Skoufias, D. A., and J. M. Scholey. 1993. Cytoplasmic microtubule-based motor proteins. *Curr. Opin. Cell Biol.* 5:95-104.
- Steuer, E. R., L. Wordeman, T. A. Schroer, and M. P. Sheetz. 1990. Location of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature (Lond.)* 345:266-268.
- Theurkauf, W. E., and R. S. Hawley. 1992. Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the *nod* kinesin-like protein. *J. Cell Biol.* 116:1167-1180.
- Threlkeld, A., R. Adler and A. T. Hewitt. 1989. Proteoglycan biosynthesis by chick embryo retina glial-like cells. *Devel. Biol.* 132:559-568.
- Turner, R., and R. Tjian. 1988. Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science (Wash. DC)* 243:1689-1694.
- Vale, R. D., and L. S. B. Goldstein. 1990. One motor, many tails: an expanding repertoire of force-generating enzymes. *Cell.* 60:883-885.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39-50.
- Vernos, I., J. Heasman, and C. Wylie. 1993. Multiple kinesin-like transcripts in *Xenopus* Oocytes. *Devel. Biol.* 157:232-239.
- Walker, R. A., and M. P. Sheetz. 1993. Cytoplasmic microtubule-associated motors. *Annu. Rev. Biochem.* 62:429-451.
- Wang, S.-Z., and R. Adler. 1994. A developmentally regulated basic-leucine zipper-like gene and its expression in embryonic retina and lens. *Proc. Natl. Acad. Sci. USA.* 91:1351-1355.
- Yang, J. T., R. A. Laymon, and L. S. B. Goldstein. 1989. A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analysis. *Cell.* 56:879-889.