

Subcellular Localization and Sequence of Sea Urchin Kinesin Heavy Chain: Evidence for its Association with Membranes in the Mitotic Apparatus and Interphase Cytoplasm

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Abstract. Kinesin was previously immunolocalized to mitotic apparatuses (MAs) of early sea urchin blastomeres (Scholey, J. M., M. E. Porter, P. M. Grissom, and J. R. McIntosh. 1985. *Nature [Lond.]* 318:483–486). Here we report evidence that this MA-associated motor protein is a conventional membrane-bound kinesin, rather than a kinesin-like protein. Our evidence includes the observation that the deduced amino acid sequence of this sea urchin kinesin heavy chain is characteristic of a conventional kinesin. In addition,

immunolocalizations using antibodies that distinguish kinesin from kinesin-like proteins confirm that conventional kinesin is concentrated in MAs. Finally, our immunocytochemical data further suggest that conventional kinesin is associated with membranes which accumulate in MAs and interphase asters of early sea urchin embryos, and with vesicles that are distributed in the perinuclear region of coelomocytes. Thus kinesin may function as a microtubule-based vesicle motor in some MAs, as well as in the interphase cytoplasm.

KINESIN is a heterotetrameric, two-headed motor protein that uses MgATP hydrolysis to move particles towards the plus ends of microtubule (MT)¹ tracks in vitro (Vale et al., 1985a, b; Cohn et al., 1989; Hackney, 1988; Bloom et al., 1988; Kuznetsov et al., 1989; Hirokawa et al., 1989; Scholey et al., 1989; Yang et al., 1989, 1990). It is likely that this mechanoenzyme participates in intracellular membrane transport (Vale et al., 1986; Vale 1987; Sheetz, 1987; Kelly, 1990) because immunocytochemical studies have suggested that kinesin is associated with cytoplasmic membrane vesicles or tubules (Pfister et al., 1989; Hollenbeck, 1989), and membrane transport can be inhibited in cells or cytoplasmic extracts treated with antibodies that directly block or immunoadsorb out kinesin activity (Vale et al., 1985b; Schroer et al., 1988; Brady et al., 1990; Hollenbeck and Swanson, 1990; Bomsel et al., 1990). Moreover, mutations in the *Drosophila* kinesin heavy chain gene can cause neuromuscular malfunctions that are consistent with defects in axonal vesicle transport (Saxton et al., 1991).

Kinesin has been isolated from sea urchin eggs and early embryos (Scholey et al., 1985) where MTs are primarily devoted to a role in mitosis (Bloom and Vallee, 1989), and affinity-purified kinesin heavy chain antibodies stained the mitotic apparatus (MA) in fixed mitotic sea urchin blasto-

meres, suggesting that kinesin may also function as a mitotic motor (Scholey et al., 1985; Leslie et al., 1987). However, the significance of this work has been called into question by studies showing that antikinesins do not stain MAs in some other cell types (e.g., Pfister et al., 1989; Hollenbeck et al., 1989), and to date, no direct evidence concerning the function of kinesin in the MA has been reported. Furthermore, kinesin may be associated with the large number of membranous vesicles that accumulate in the MAs of marine invertebrate embryos (Rebhun, 1960; Harris, 1962, 1975; Petzelt and Hafner, 1986; Henson et al., 1989).

While the function of kinesin in mitotic cells remains unclear, recent genetic studies have demonstrated that some mitotic movements are carried out by kinesin-like proteins (Enos and Morris, 1990; Meluh and Rose, 1990; Hagan and Yanagida, 1990; Endow et al., 1990; McDonald and Goldstein, 1990; Zhang et al., 1990). The deduced sequences of these kinesin-like proteins display similarity only to the motor domain of conventional kinesins (Yang et al., 1989; Kosik et al., 1990). Thus it is thought that eukaryotes contain a kinesin superfamily whose members share similar force-generating domains linked to different types of tails that confer unique cargo-binding properties (Vale and Goldstein, 1990). These results raise the possibility that a kinesin-like protein in the sea urchin mitotic spindle, rather than kinesin itself, may have bound our polyclonal kinesin antibody, giving rise to MA staining.

Here we describe molecular biological and cytological

1. *Abbreviations used in this paper:* MA, mitotic apparatus; MT, microtubule; PCR, polymerase chain reaction.

studies that improve our understanding of kinesin in the sea urchin MA. They support the hypothesis that conventional kinesin is a MT-based, membrane-bound motor in the MA of early sea urchin blastomeres as well as in the interphase cytoplasm.

Materials and Methods

Materials

Sea urchins *Lytechinus pictus*, *Strongylocentrotus Purpuratus*, or *Strongylocentrotus franciscanus* were obtained from Marinus Inc. (Long Beach, CA) or the University of California Davis Marine Biology Laboratory at Bodega Bay, CA whereas *Strongylocentrotus droebachiensis* were collected near the Mt. Desert Island Biological Laboratory (Salsbury Cove, ME). General biochemical and microscopic procedures and reagents were as described previously (Scholey et al., 1985; Porter et al., 1987; Ingold et al., 1988; Cohn et al., 1989; Johnson et al., 1990). Monoclonal and polyclonal antibodies to sea urchin kinesin heavy chain were described previously (Ingold et al., 1988). Rabbit anti-sea urchin calsequestrin antiserum was a generous gift from D. Begg (Harvard Medical School, Boston, MA) and B. Kaminer (Boston University School of Medicine, Boston, MA). Mouse monoclonal anti- β -tubulin was a generous gift from M. Klymkowski (University of Colorado, Boulder, CO).

Library Construction and Screening

RNA prepared from unfertilized eggs of *S. purpuratus* (Chirgwin et al., 1979) was poly(A)⁺ selected by two passages over oligo dT cellulose, copied into cDNA, and size selected for cDNA molecules >1.0 kb. These were inserted into the EcoRI site of λ ZAP (Stratagene, La Jolla, CA). The resulting unamplified library contained $\sim 6 \times 10^6$ unique clones. Approximately 1.0×10^6 of these clones were screened with antikinesins (Ingold et al., 1988) preabsorbed with bacterial lysates, using the procedure of Mierendorf et al. (1987).

Clones that reacted with a polyclonal antikinesin, but not the corresponding preimmune, through duplicate screens, were plaque purified. These "positive" clones were further rescreened using a mixture of the monoclonal antikinesins, SUK2 and SUK4, which react with different epitopes on the heavy chain (Ingold et al., 1988), yielding 54 positives, 19 of which were plaque purified and further screened with SUK2 and SUK4 separately. 16 clones reacted with SUK2 alone, 2 reacted with SUK4 alone, but only 1, designated pKHC5, reacted with both SUK2 and SUK4.

The positive clones were subcloned into pBluescript and grown in *Escherichia coli* host, XLI-Blue. Antikinesin-immunoreactive proteins were expressed with or without IPTG induction (see also Yang et al., 1988). cDNA inserts from the positive clones were characterized by restriction mapping (Sambrook et al., 1989) and sequencing. The data obtained showed that pKHC5, which is 4.8 kb long and flanked by EcoRI sites, contains an internal EcoRI site lying between the SUK2 and SUK4 epitopes, and is missing ~ 450 nts of coding sequence, based on comparison to the *Drosophila* heavy chain sequence. It seems likely that inefficient methylation during library construction resulted in cleavage of a full length cDNA by EcoRI into pKHC5, and the further subdigestion of pKHC5 into the positive clones that contain only the SUK2 or SUK4 epitopes.

Polymerase Chain Reaction (PCR) Procedures

The 5' end of the sea urchin kinesin heavy chain-encoding cDNA (Fig. 1) was amplified from cDNA which was reverse transcribed from total sea urchin egg RNA. Briefly, 3 μ g total sea urchin egg RNA was first reverse transcribed using AMV reverse transcriptase (United States Biochemical Corp., Cleveland, OH) primed with 10 pmol pKHC5-specific primer and 0.5 mM each dNTP in 1 \times PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) containing 1 U RNase Block II (Stratagene), at 42°C for 1.5 h, 52°C for 0.5 h, and 95°C for 5 min. Removal of excess primer and the subsequent tailing reaction were done according to the procedure of Frohman et al. (1988), yielding 250 μ l of poly(A)-tailed (–) strand cDNA pool. Two rounds of amplification of this template were accomplished using additional pKHC5-specific primers under conditions recommended by Perkin-Elmer/Cetus and Frohman et al. (1988). The first and second round products were found to be of the size expected for a successful amplification of the 5' end of the kinesin heavy chain. Asymmetric PCR (Gyllenstein and Ehrlich, 1988; Frohman and Martin, 1989) using the first round product as a template yielded cDNAs, which were then sequenced, after removal of excess primers.

Similar PCR procedures were used to resolve an artifactual stop codon in pKHC5 (Fig. 1). Details of the primers and methods used for PCR can be obtained from K. P. Wedaman in J. M. Scholey's laboratory.

Sequencing Methods and Computer-assisted Sequence Analysis Procedures

DNA sequencing was performed by the dideoxy procedure of Sanger et al. (1977) using the sequencing strategy described in Fig. 1. Sequence analysis was done in consultation with Dr. Paul Schneeman of University of California Davis Computing Services, using standard procedures (Doolittle, 1987) and the GCG sequence analysis software package (Devereux et al., 1984).

Expression of Kinesin Fragments and Antibody Mapping

A 1.4-kb sea urchin kinesin cDNA fragment encoding the tail domain and a portion of the stalk domain (Fig. 1) was prepared by a ClaI digestion of pKHC5, blunt ending with mung bean nuclease, and digestion with NcoI. The fragment was isolated from a low-melt agarose gel and ligated to the plasmid vector T7 that had been cut with NcoI and StuI. A 0.8-kb fragment encoding a portion of the sea urchin kinesin stalk domain (Fig. 1) was prepared by digestion of pKHC5 with NcoI and HindIII. The fragment was isolated from a low-melt agarose gel and ligated to the plasmid vector T7 that had been cut with NcoI and HindIII. The ligation products were transformed into bacterial strain DH5 α . Positive subclones were identified by restriction enzyme analysis. Plasmid DNAs were prepared by the alkaline-lysis method (Sambrook et al., 1989). The plasmids were transformed into the *E. coli* strain BL-21 (DE3)pLysS and protein was expressed as described (Studier et al., 1990).

Protein extracts of induced transformants containing the expressed stalk and stalk/tail fragments were electrophoresed on SDS gels, blotted to nitrocellulose filters, and probed with monoclonal antibodies, SUK1-7, to map the domain specificity of the antibody binding sites (Figs. 3, 4). In addition, protein blots were used to affinity purify domain-specific polyclonal antibodies (data not shown). Appropriate bands were excised and used as ligands for affinity purification as previously described (Olmsted, 1981; Scholey et al., 1985). These antibodies were then mapped to their respective domains by Western blotting against stalk and stalk/tail fragments (Fig. 4).

Immunoperoxidase Staining of Sea Urchin Embryos

Gametes were collected by intracoelomic injection with 0.56 M KCl; eggs were stored in sea water (SW) at 15°C and sperm were stored "dry" in a sealed tube on ice. Eggs were dejellied by passing seven times through a 105–150- μ m mesh Nitex screen and three washes with SW. Dilute dejellied eggs were fertilized in SW containing 10 mM PABA (pH 8.0) by adding ~ 1 ml of dilute sperm (1 drop/50 ml) per 500 ml of egg suspension. They were washed and resuspended in >10 vol aerated sea water containing 10 mM PABA and allowed to develop to the desired stage with gentle stirring at 15°C.

If MT depolymerization was desired, 10 μ g/ml of nocodazole was added (as a 10 mg/ml stock in DMSO) 15 min before harvesting the embryos. Controls of 0.1% DMSO showed no differences from untreated embryos. Embryos near the desired stage were washed in calcium-free sea water (CFSW) containing 10 mM PABA and 5 mM EGTA then passed through a 105–150- μ m Nitex screen to remove fertilization membranes one to seven times until >95% were freed. Eggs or embryos were collected by settling or gentle hand centrifugation into a low volume and loaded dropwise onto multiwell slides that had been previously flamed, coated with poly-L-lysine (1.2 mg/ml in dH₂O), air-dried and cooled to 15°C. After 1–3 min for attachment, these slides were then dipped into wash buffer (1 M glucose and 10 mM Tris-HCl, pH 8.0) to remove salts and treated appropriately. Cells to be detergent extracted were placed into lysis buffer (25 mM Pipes, pH 6.9, 6 mM EGTA, 1 mM MgSO₄, 20% glycerol, and 1% NP-40 [vol/vol], plus 1 mM DTT, 1 mg/ml TAME-HCl, 100 μ M PMSF, 100 μ g/ml SBTI, and 10 μ g/ml each leupeptin, pepstatin, and aprotinin) for 15 min at room temperature. Cells to be fixed were immersed in fixative (90% methanol plus 50 mM EGTA, pH 6.0 [10 mM EGTA for blastula or later stages to prevent deciliation]) at –20°C for 1–72 h. No staining differences were noted in cells left in fixative at –20°C for up to 3 d. Each experiment was repeated at least three times in a given species. No significant differences were noted in the staining patterns of the different species of sea urchins used.

Antibody solutions were made up in sterile filtered PBS containing 10% normal goat serum. The data presented were obtained using a mixture com-

posed of equal amounts of three mouse monoclonal anti-sea urchin kinesin heavy chain antibodies (SUK 2, 4, and 5; 200 $\mu\text{g/ml}$ total; Ingold et al., 1988). Essentially the same results were obtained using each of the monoclonal anti-kinesins alone (at 200 $\mu\text{g/ml}$), as well as affinity-purified polyclonal antibodies (Scholey et al., 1985; Fig. 4; immunostaining not shown here). For cytoplasmic membrane staining, sea urchin calsequestrin antiserum (Henson et al., 1989) was used (1:100 to 333). For tubulin staining, a mouse monoclonal anti- β -tubulin ascites (Klymkowsky, M., University of Colorado Boulder, CO) was used at 1:1,000 to 5,000 (Leslie et al., 1987). Nonspecific mouse monoclonal IgG₁ antibody (MOPC21, 200 $\mu\text{g/ml}$; e.g., Fig. 6) and rabbit or mouse preimmune sera (1:100–333) were used as negative controls and displayed no staining in any experiment.

Slides of fixed eggs or embryos were washed three times for 10 min in PBS at room temperature and each well was covered with 15 μl of primary antibody. Slides were incubated in humidified chambers for either 1–3 h at 37°C or overnight at 4°C, washed three times for 10 min in PBS, then similarly incubated in the appropriate affinity-purified secondary goat antiserum (1:20), washed, and incubated in the appropriate peroxidase-conjugated anti-peroxidase (PAP) tertiary antibody (1:40). These slides were again washed three times for 10 min in PBS, then three times for 5 min in TBS, developed at room temperature in TBS containing 0.5 mg/ml 4-chloro-1-naphthol, 20% methanol, and 0.025% H₂O₂, quenched in cold dH₂O, mounted in 90% glycerol, and observed with brightfield illumination using a 40 \times Plan-achromatic objective on a Zeiss IM-35 microscope.

Immunofluorescent Staining of Calsequestrin in Frozen Sections of Embryos

S. purpuratus embryos were prepared, labeled, and photographed as described previously (Henson et al., 1989). Briefly, embryos were fixed in 3% formaldehyde, 0.1% glutaraldehyde in CFSW, embedded in gelatin, and frozen in liquid freon. Semithin (0.5–1.0 μm) frozen sections were cut on a Reichert ultracycromicrotome and labeled with calsequestrin antiserum

(1:300) followed by rhodamine-conjugated goat anti-rabbit IgG secondary antibody (1:200) containing 1 $\mu\text{g/ml}$ Hoechst 33258 (to stain chromatin).

Immunofluorescent Staining of Kinesin in Coelomocytes

S. droebachiensis coelomocytes were collected and isolated according to Edds (1977). Cells were allowed to settle onto poly-L-lysine-coated coverslips and then fixed and incubated with kinesin antibodies as described for the embryos. The secondary antibody used was rhodamine-conjugated goat anti-mouse IgG (1:50) which had been preabsorbed with an acetone powder extract of *S. purpuratus* eggs. For extraction experiments, cells were treated for 5 min with 0.5% Triton X-100 in a MT-stabilizing buffer (0.1 M Pipes, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 5 mM EGTA) before fixation in methanol.

Photography

Representative cells, gels, and blots were photographed using an Olympus OM-2S camera with Plus-X pan film at ASA 400. Black and white images were developed with Diafine developer and printer on Polycontrast III RC paper developed in Dektol developer.

Results

The Sequence of the Sea Urchin Kinesin Heavy Chain

Northern blotting and cDNA sequencing reveal that a 3093 nucleotide open reading frame on a 10 kb maternal mRNA encodes the sea urchin kinesin heavy chain. The protein sequence of this heavy chain was deduced from the nucleotide sequence of a cloned 4.8 kb cDNA, pKHC5, plus overlap-

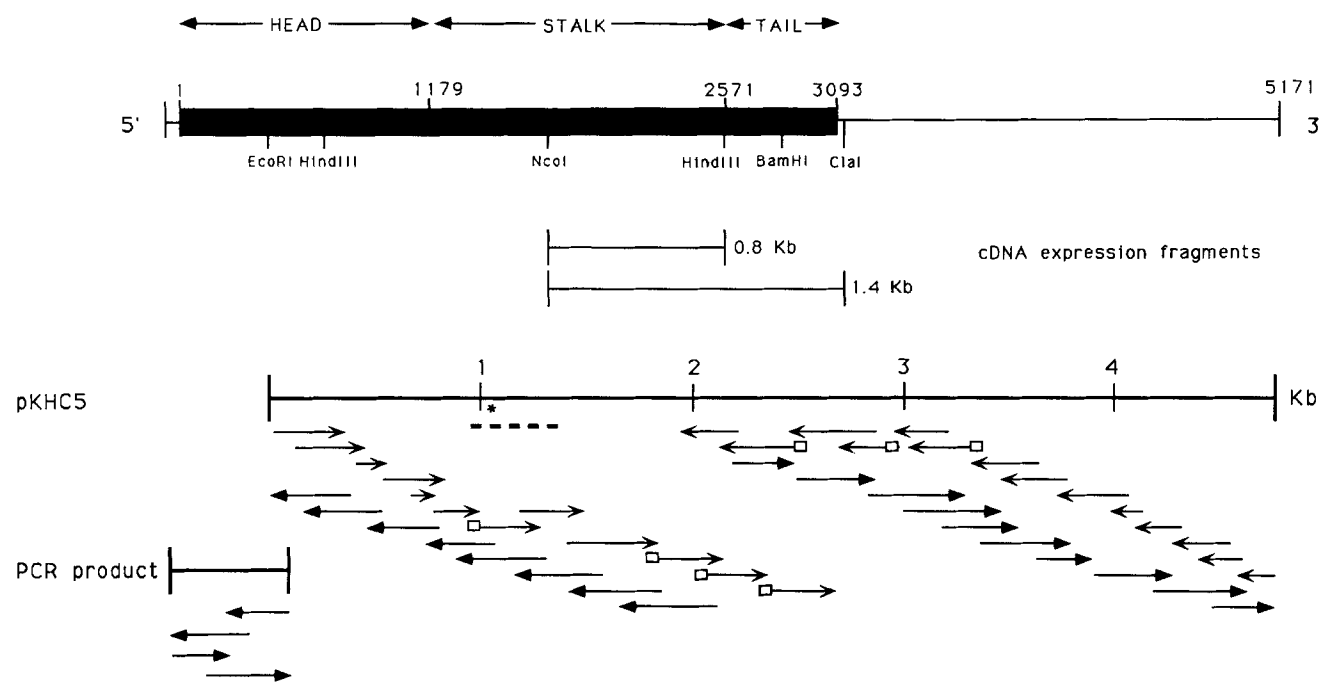


Figure 1. Map of the sea urchin kinesin heavy chain cDNAs and sequencing strategy. Our data suggest that the 3,093-nt open reading frame (black box) is flanked by ~500-nt upstream and 6-kb downstream untranslated sequence on the 10-kb mRNA (not shown). The figure displays the segments encoding the head, stalk, and tail, plus the cDNA fragments that were expressed in transformed bacteria to obtain stalk/tail antibodies (Figs. 3 and 4). The 4.8-kb clone, pKHC5, was sequenced by the dideoxy chain termination method using Exo III/mung bean nuclease-generated nested deletions and universal primers (arrows). The boxes indicate synthetic oligonucleotide primers used in sequencing the gaps between the nested deletions. Confirmatory sequence was obtained using complementary oligodeoxynucleotide primers. The dotted line indicates the PCR product that was used to resolve the artifactual stop codon designated by the asterisk. To obtain the 5' end region of the heavy chain cDNA, a specific kinesin cDNA pool was amplified through two rounds of anchored PCR with nested primers. Asymmetric PCR, followed by dideoxy sequencing (of stretches indicated by the arrows), was used to complete the sequence of the open reading frame that encodes kinesin heavy chain.

1	MAD....PAE	CNIKVVCRVR	PMNATEQN.T	SHICTKF...	ISEEQVQIGG	KLNMDFRIFK	PNTTQEEVYN	KAARQIVKDV	LDGYNGTIFA	YGQTSSGKTF	(1-92)
2	MSAEREIPAE	DSIKVYCRFR	PLNDSEEKAG	SKFVYKFPNM	VEENCISIAQ	KYVLFDKVFK	PNASQEKVYN	EAAKSIIVTV	LAGYNGTIFA	YGQTSSGKTH	
3	M.....DVASE	CNIKVICRVR	PLNEAEERAG	SKFILKFPT.	..DDISIASIAG	KYVVFDFKVLK	PNVSQEVVYN	VGAKPIVADV	LSGCNGTIFA	YGQTSSGKTH	
KHC	M.....paE	cNIKVVCRVR	Pin..Ee.ag	Skf..Kfp..	..e..isIaG	Kv..Fdkvfk	PN.sQE.VYN	.aak.IV.DV	L.GyNGTIFA	YGQTSSGKTH	
1	TMEGVMGNPQ	YMGIIIPRIVQ	DIFNHIYQMD	ESLEFHIKVS	YFEIYMDRIR	DLLDVSKTNL	SVHEDKNRVP	FVKGATERFA	SSPEEVMVDI	EEGKSNRHIA	(93-192)
2	TMEGVIGDSV	KQGIIPRIVN	DIFNHIYAME	VNLEFHIKVS	YFEIYMDKIR	DLLDVSKVNL	SVHEDKNRVP	FVKGATERFV	SSPEDVFEVI	EEGKSNRHIA	
3	TMEGVLDPKS	MHGIIIPRIVQ	DIFNHIYQMD	ENLEFHIKIS	YFEIYLDKIR	DLLDVTKTNL	AVHEDKNRVP	FVKGATERFV	SSPEEYMEVI	DEGKSNRHVA	
KHC	TMEGV.g.p.	..GIIIPRIVq	DIFNhiY.Md	enLEFHIkVs	YfEIYmDKIR	DLLDVsKtNL	sVHEDKNRVP	FVKGATERFv	SSPEeVmeVI	eEGKsNRHIA	
1	VTNMNEHSSR	SHSIFLIQVK	QENMETKKKL	SGKLYLVDLA	GSEKYSKTGA	EGTVLDEAKN	INKSLSALGN	VISALADGKK	SHIPPYRDSKM	TRILQESLGG	(193-292)
2	VTNMNEHSSR	SHSVFLINVK	QENLENQKKL	SGKLYLVDLA	GSEKYSKTGA	EGTVLDEAKN	INKSLSALGN	VISALADGKK	THIPPYRDSKL	TRILQESLGG	
3	VTNMNEHSSR	SHSVFLINVK	QENVETQKKL	SGKLYLVDLA	GSEKYSKTGA	EGAVLDEAKN	INKSLSALGN	VISALADGKK	SHVPPYRDSKL	TRILQESLGG	
KHC	VTNMNEHSSR	SHSVfLIInVq	QEN..m.q	SGfLYLVDLA	GSEKYSKTGA	EgTvLDEAKN	INkSLsALGN	ViSALADGkK	sHiPPYrDSkT	TRILQESLGG	
1	NARTTIVICC	SPSSFNESSE	KSTLMFGQRA	KTIKNTVTYN	MELTAEFWRN	RYEKEKEKNG	RLKAQLLILE	NELQRWRAGE	SVPVKEQGNK	NDEILKEMMK	(293-392)
2	NARTTIVICC	SPASFNESSE	KSTLDFGRRR	KTIVKNVVCVN	EELTAEFWRK	RYEKEKEKNA	RLKGKVEKLE	TELARWRAGE	TVKAEQIINM	ED..LMEAST	
3	NARTTIVICC	SPASVNESET	KSTLLFGQRA	KTIVKNVVCVN	EELTAEFWRK	RYEKEKEKRV	KLKATMAKLE	AELQRWRAGE	AVSVEEQVDL	KE.....	
KHC	NARTTIVICC	SPaSFNESEt	KSTL.fGqRa	KTiKNTvV.N	eELTAeFwR	RYeKeKeKn.	RLKa...kLE	.ELqRWRaGe	.V.veEQ.n.	.d..l.e...	
1	PK.....QMTVH	VSEEEKNKWE	EKVLYEQL	DEKDEIDNQ	SRLTEKLNQ	MLEQEELLSS	MORDYELLQS	QMGRLEAENA	(393-469)
2	PNLEVEAAQT	AAAEALAAQ	RTALANMSAS	VAVNEQARLE	TECERLYOQL	DDKDEEINQ	SOYAEQLKEQ	VMEQEELIAN	ARREYETLQS	EMARIQOENE	
3	...DVPASP	ATSTTSLAGGLIAS	MNEGDRTOLE	EERLKYOQL	DDKDEEINQ	SQLEIKLKEQ	MMEQEDLIAQ	SRRDYENLQS	DMSRIQADNE	
KHC	p...v.a...la...m.as	v.e.e...le	eE..kLYqQL	DdKd.EInnQ	SqL.EkLkEq	mMEqEeLia.	.rRdYe.LQs	.M.RiqaeNe	
1	AAKEEAKVEL	QALEEMAVNY	DEKSKEVEDK	NRMNETLSEE	VNEKMTALHT	TSTELQKLQE	LEQHRRRIT	EMMASLLKDL	GEIGTAL..G	GNAADMKNPV	(470-567)
2	SAKEEVKEVL	QALEELTVNY	DQKSQEIQNK	NKDIDALNEE	LQKQSLVNS	ASTELQQLKD	MSSHQKRIT	EMLTNLLKDL	GEVQQAIPAG	ESSIDLKMSA	
3	SAKEDVKEVL	QALEELAVNY	DQKSQEVEDK	NKENENLSEE	LQKQSLVNS	LQNELQQLKD	SMHHRKRIT	DMMINLLKDL	GDIGTIV..G	GNAAEIKPTA	
KHC	sAKeEvKevL	QALeElaVny	DqKsQeVedk	Nk.nE.LsEE	lNqK.s.lN.	.sTeLqLkD	.s.HqrkRiT	eMm.nLLkDL	GeiGta...G	gnaad.Kp.a	
1	...ENIEKVD	EFTMARLFV	SKMKTEVKT	SQRCKILEAS	NAENETKIRT	SEDELDSCRM	TIQQHEAKMK	SLSENIRETE	GKKRHLEDSL	DMLNEEIVKL	(568-664)
2	LAGTDASKVE	EFTMARLFI	SKMKTEAKNI	AQRCSNMETQ	QADSNKKISE	YEKDLGEYRL	LISQHEARMK	SLQESMREAE	NKKRLEEQI	DSLREECAL	
3	GSGE...KIE	EFTVARLYI	SKMKSEVKT	VSRNQLENT	QQDNFKIET	HEKDLNCKL	LIQQHEAKMA	SLQEAIKDSE	NKKRMLDSE	NKKRMLDSE	
KHC	..ge...kve	EefTMarLfI	SKMKtEvKt.	.qRc..lE..	qadn.kKI.t	.EkdL..crI	lIqQHEAKMK	SLqE.ire.E	nKKR.Led..	DsLnEE.aKL	
1	RAAEEIRLTD	QEDKKREED	KMQSATEMQA	SMSEQMESHR	DAHQKQLANL	RTEINEKEHQ	MEELKDVNR	MTLQHEKLQL	DYEKLIIEEA	EKAAKLRELS	(665-764)
2	KAEE..HVSA	VNAEEQ..R	...AEELRS	MFDSDQDEL	EAHTRQVSEL	RDEIAAQOHE	MDMEKDVHQ	LLLAAHQMTA	DYEVKQEDA	EKSSELQNI	
3	KAQEOMHLAA	LSEREKETSQ	...ASETRE	VLEKQMEMHR	EQHQKQLQSL	RDEISEKQAT	VDNLKKDNQR	LSLALEKLQA	DYDKLKQEEV	EKAAKLADLS	
KHC	kAaE..hI.aeke...A.E.r.QMe.hr	eahqkQl..L	RdEi.eKqh.	mdeIKDvNr	l.LahekIqa	DyEkIkqEea	EkaakL..Is	
1	QQFDRREQAK	QDLKGLEEV	AKELQTLHNL	RKLFVSDLQN	RVKKALE.GG	DRDDSDGGSQ	AQKQKISFLE	NNLEQLTKVH	KQLVRDNADL	RCELPKLERR	(765-863)
2	LTNERREQAR	KDLKGLDVT	AKELQTLHNL	RKLFVQDLQ	RIRKNV..VN	EESDEDGGS	AQKQKISFLE	NNLDQLTKVH	KQLVRDNADL	RCELPKLEKR	
3	LQIDRREQAK	QDLKGLEEV	AKELQTLHNL	RKLFVQDLQN	KVKKSCSKTE	EDEDTGGA	AQKQKISFLE	NNLEQLTKVH	KQLVRDNADL	RCELPKLEKR	
KHC	lq.dRREQAK	qDLKGLeeTv	AKELQTLHNL	RkLfvQdLqN	rvkK.....	eeded.GGs.	AQKQKISFLE	NNLeQLTKVH	qQLvRDNADL	RCELPKLEKR	
1	LRATSERVKA	LEMSLKETKE	GAMDRDKRYQ	QEVDRIEAV	RQRNFAKRG	SAQIAKAIIRA	G.HPPSPGG	STGIRGGGYS	GIRGGGSPVI	RPPSHGSPPE	(864-962)
2	LRCTMERVKA	LETALKEAKE	GAMDRDKRYQ	YEVDRIEAV	RQKHLGRRG	QAQIAKPIRS	GQ.....G	ATAIRGGG..	AVGGSPPLA	VNPVNS	
3	LRATMERVKS	LESALKDAKE	GAMDRDKRYQ	YEVDRIEAV	RQKHLARRG	AAQIAKPIR	GQHSVSPAQ	AAAIRGGG..	GLSQNGPMT	STPIRMAPES	
KHC	LraTMeRVKa	LE.aLkEaKe	GAMDRDKRYQ	.YEVDRIEAV	RQkNlarRG.	.AQIAkPIR.	Gqh...sp.g	a.aIRGGG..	g..g.gp...	..P....pe.	
1	ISHNNSFEKS	LNPDAENME	KKANKRLPKL	PPGGNLTES	DIAAMKARSK	ARNNTPGKAP	LTTSGEQGS				(963-1031)
2				
3	KA.....				
KHC				

Figure 2. Comparison of the deduced amino acid sequence of the sea urchin kinesin heavy chain (1) to those of *Drosophila* (2) and squid (3). Based on this comparison we obtained the kinesin heavy chain consensus sequence where upper and lower case letters indicate identities between three and two sequences, respectively. The sequences were compared and aligned using the UWGCG BESTFIT and LINEUP programs. The numbers (right) refer to amino acids in the deduced sea urchin sequence, whose nucleotide sequence is available from EMBL/GenBank/DDBL under accession number X56844.

ping cDNAs that were amplified by PCR using pKHC5-specific primers (Figs. 1, 2; Northern and nucleotide sequence presented for review but not shown here).

The amino acid sequence of the heavy chain is 1,031 residues long with an estimated molecular mass of 117,390 D and a pI of 6.62. As reported previously for *Drosophila* and squid kinesins, the sea urchin kinesin heavy chain appears to be organized into three structural domains (Yang et al., 1989; Kosik et al., 1990); the NH₂-terminal head domain (residues 1-393; predicted $M_r = 44,457$, and pI ≈ 8.08 , containing consensus sequences for binding ATP [amino acids 84-91] or GTP [amino acids 84-91, 230-233, 381-384] and the SUK4 epitope [amino acids 312-382]); the stalk (residues 394-857, predicted $M_r = 54,193$, and pI = 4.83); and the COOH-terminal tail (residues 858-1031, predicted $M_r = 18,776$, and pI = 11.53).

The comparison of the sea urchin, *Drosophila*, and squid kinesin heavy chains (Fig. 2) reveals a high degree of sequence similarity throughout the length of these three polypeptides. For example, the degree of similarity and identity,

respectively, between the fly and sea urchin sequences are 78 and 64% overall; 84 and 76% in the head; 78 and 66% in the tail; and finally 73 and 53% in the stalk. We note that the sea urchin heavy chain has an additional stretch of 70 amino acids at the COOH-terminal end.

Comparisons between the sea urchin kinesin heavy chain and several kinesin-like proteins, including *bimC* (Enos and Morris, 1990), *nod* (Zhang et al., 1990), *KAR3* (Meluh and Rose, 1990), and *ncd* (Endow et al., 1990; McDonald and Goldstein, 1990) revealed sequence similarity through regions corresponding to the motor domains, but outside the motor domains there is no evidence of any sequence similarity (dotplots submitted for review but not shown here).

Preparation of Domain-specific Antikinesins

Three monoclonal antibodies to sea urchin kinesin, SUK4, 6, and 7, were previously found to bind to the kinesin heads (Ingold et al., 1988; Scholey et al., 1989). We have now mapped the epitopes of our other mAbs and we have affinity-

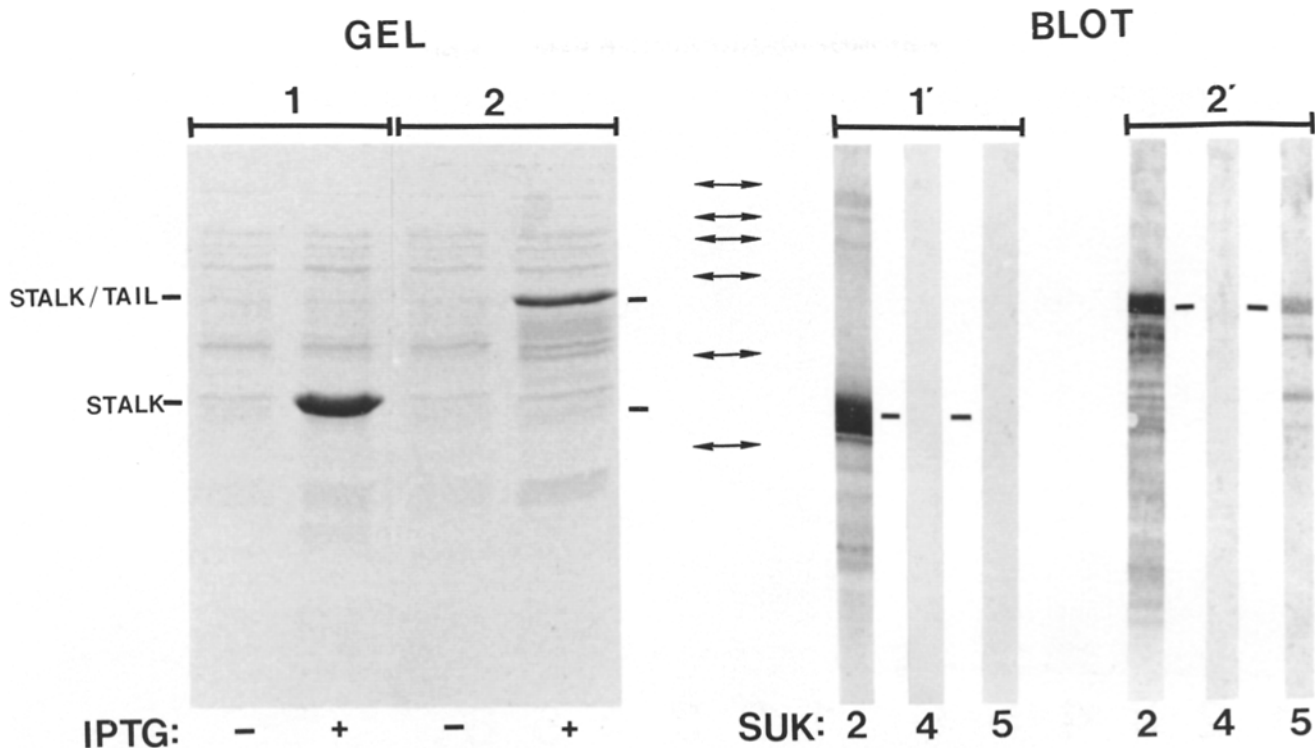


Figure 3. Mapping the binding sites of monoclonal antibodies to structural domains of the kinesin heavy chain. (**GEL**) Coomassie blue-stained SDS-PAGE showing aliquots of cultures of bacteria transformed with T7 vectors containing kinesin cDNA inserts (Fig. 1) before (-) and after (+) induction of expression of stalk (1) and stalk/tail (2) subfragments of the sea urchin kinesin heavy chain. After induction, the kinesin polypeptides are the most prominent bands in these cultures, as indicated on the left of the gel. (**BLOT**) Duplicate gels of the expressed proteins were transferred to nitrocellulose filters (stalk = 1'; stalk/tail = 2') and probed with three monoclonal IgGs, SUK2, SUK4, and SUK5 (Ingold et al., 1988). As expected, SUK4 which binds to the kinesin heads reacts with neither fragment; SUK2 binds to both fragments showing that it is a stalk binding antibody; SUK5 binds only to the fragments containing the tail sequences, showing that it is a tail binding antibody (See Fig. 4 also). The double-headed horizontal arrows show molecular mass markers of 205, 116, 97, 66, 45, and 29 kD.

purified polyclonal antibodies to the stalk and tail domains of the heavy chain. This work involved the preparation of bacterially expressed subfragments of the kinesin heavy chain (Figs. 1, 3, and 4); based on SDS gel analysis (Fig. 3) the molecular masses of these stalk and stalk/tail fragments were estimated to be 31 and 51 kD, which agrees reasonably

well with the molecular masses of 33,762 and 51,717 D predicted from the deduced protein sequence.

The stalk and stalk/tail fragments were first used to map the binding of monoclonal antibodies, SUK 1-7 (Ingold et al., 1988), to subdomains of the heavy chain by immunoblotting (Fig. 3). In the example shown, the head-binding mono-

ANTIBODY:	BINDING-DOMAIN:			BINDING-REGION: (AMINO-ACIDS)
	HEAD	STALK	TAIL	
Mab SUK 2		+		578-858
Mab SUK 4	+			1-393 (312-382*)
Mab SUK 5			+	859-1031
Mixed SUK 2,4,5	+	+	+	1-1031
P α SUK _{HST}	+	+	+	1-1031
P α SUK _{ST}		+	+	578-1031
P α SUK _S		+		578-858

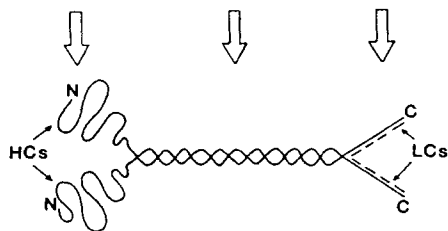
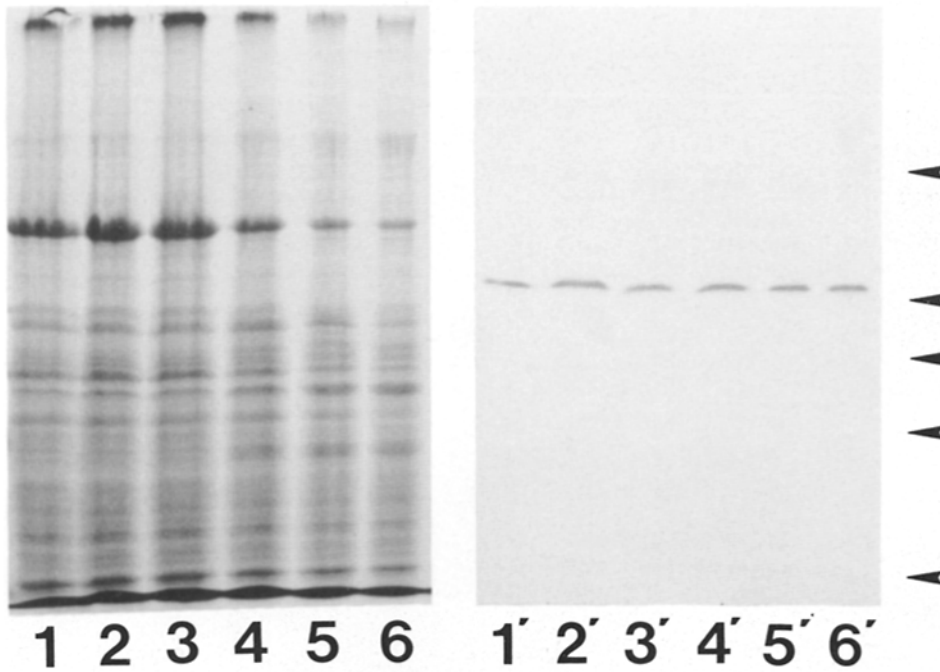
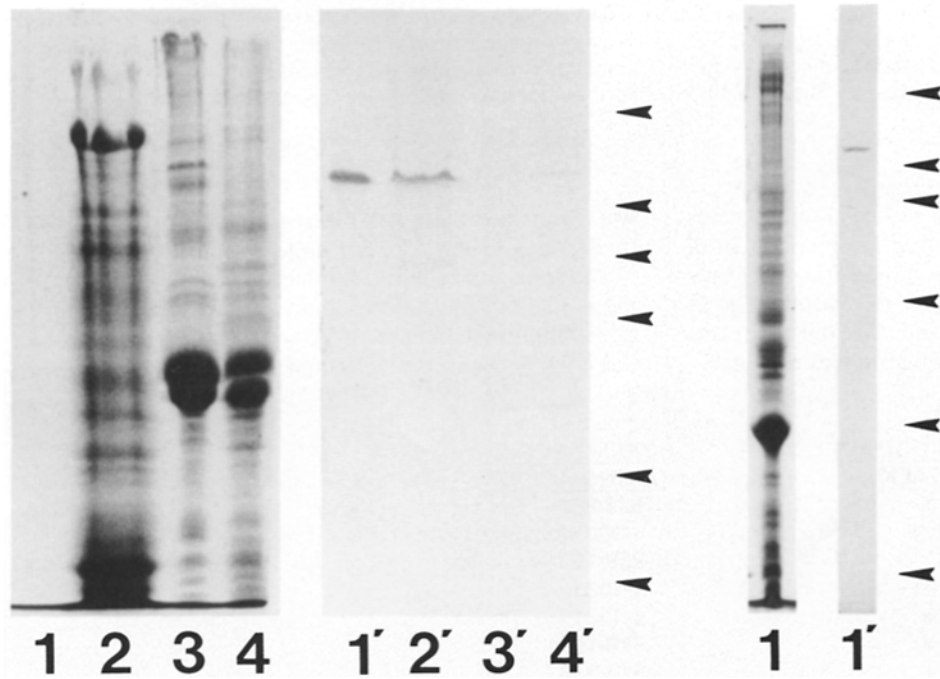


Figure 4. Summary of domain specificity of the kinesin heavy chain antibodies that were used to immunolocalize kinesin in mitotic cells. *, transposed from binding region on *Drosophila* kinesin (Scholey et al., 1989). Mab, mouse monoclonal antibody; P α , affinity-purified rabbit polyclonal antibody; SUK, sea urchin kinesin heavy chain; H, head; S, stalk; T, tail region of heavy chain.

A



B



C

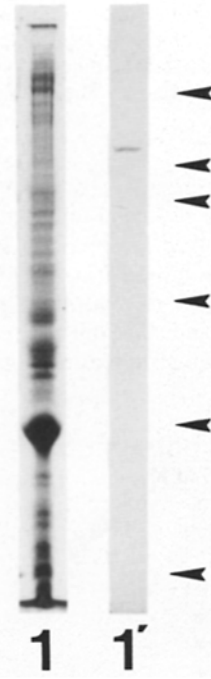


Figure 5. Coomassie blue-stained SDS gels (*left*) and monoclonal antikinesin-stained immunoblots (*right*) of sea urchin fractions. Blots probed with mixed mAbs SUK2, 4, and 5. (*A*) Synchronously developing first (lanes 1 and 1') second (2, 2'), and third (3, 3') metaphase cells plus early (4, 4') mid (5, 5'), and late (6, 6') blastula stage embryos. Each lane contained equal volumes of embryos indicating that the amount of kinesin per embryo remained approximately constant. (*B*) Sea urchin kinesin standard (1, 1'), whole egg homogenate (2, 2'), sperm flagellae (3, 3'), and blastula-stage cilia (4, 4'). Kinesin was not readily detected in either flagellae (*B*, lane 3) or cilia (*B*, lane 4). (*C*) Coelomocyte homogenate. Arrowheads indicate molecular mass standards (205, 116, 97, 66, 45, and 29 kD, *top to bottom*).

clonal, SUK4, binds neither fragment, as expected (Fig. 3, lanes 1'4 and 2'4), SUK2 binds both fragments (lanes 1'2 and 2'2), whereas SUK5 binds only to the fragment containing the tail as well as the stalk sequences (binds in lane 2'5 but not in lane 1'5). The ladder of polypeptides reacting with the stalk/tail antibodies is thought to result from proteolysis and

aggregation of a small fraction of the expressed proteins. We also used the bacterially expressed fragments to blot-affinity purify stalk and stalk/tail specific polyclonal antibodies from our kinesin heavy chain antisera (see Materials and Methods). Thus we have produced antibodies to the three structural domains of kinesin (Fig. 4). These include monoclonal

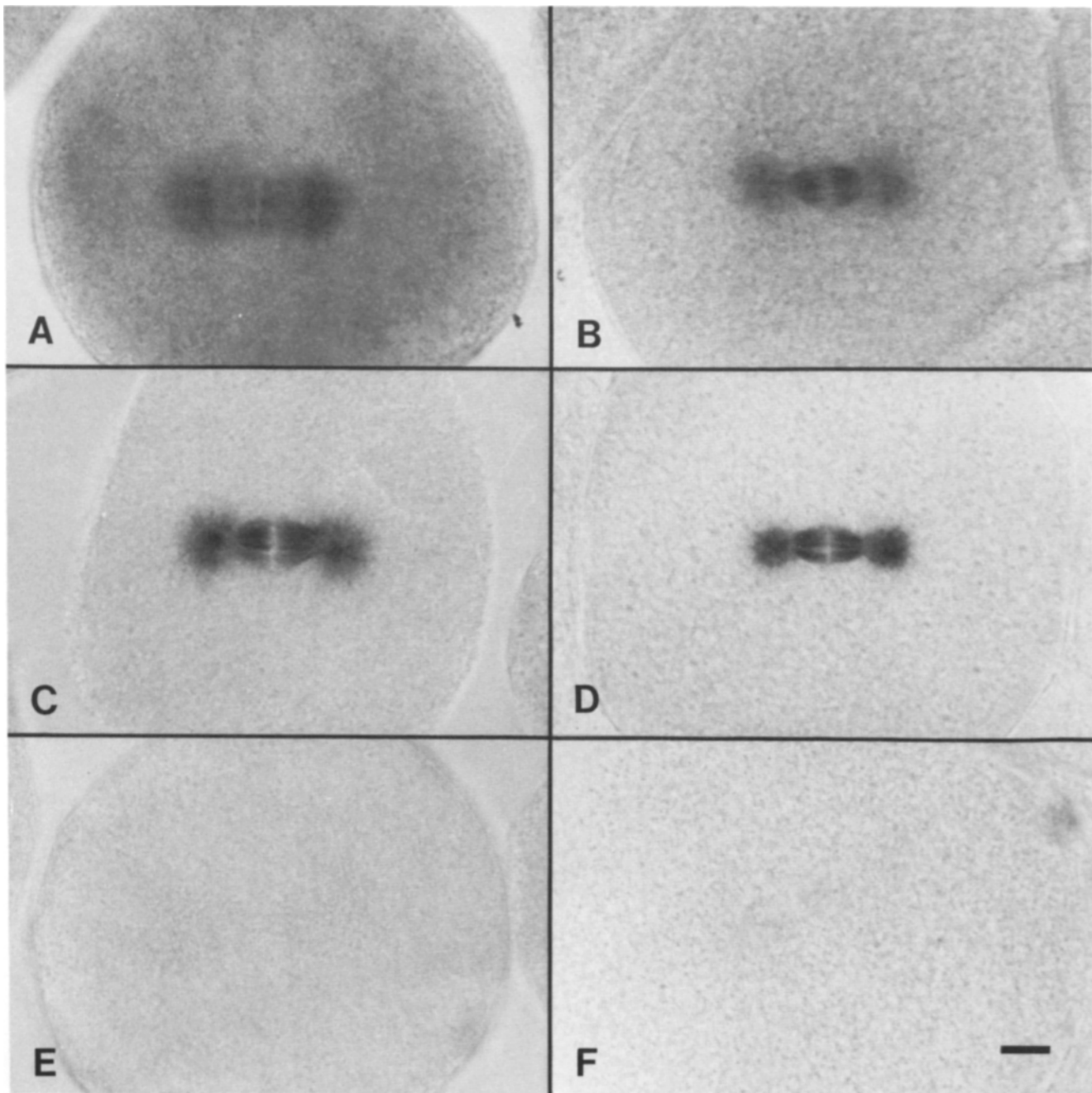


Figure 6. Antikinesin, antitubulin and control antibody staining of first metaphase blastomeres. Cells were fixed without (*A*, *C*, and *E*) or with NP-40 lysis (*B*, *D*, and *F*) then probed with antikinesin (*A* and *B*), antitubulin (*C* and *D*), or nonspecific control (*E* and *F*) monoclonal antibodies. In this example, a mixture of mAbs SUK2, SUK4, and SUK5 were used (*A* and *B*), but identical MA staining was obtained using the other antikinesins listed in Fig. 4. Note that detergent lysis removes most of the cytoplasmic antikinesin staining but enhances its fibrous character in the MA. Bar, 10 μm .

and polyclonal antibodies that bind to the nonconserved stalk and tail domains and consequently distinguish between kinesin and kinesin-like proteins.

Immunoblotting Sea Urchin Fractions with the Antikinesins

The antikinesins used for immunolocalizations reacted specifically with the kinesin heavy chain ($M_r = 130$ kD which is greater than the estimate of 117 kD based on sequencing)

on immunoblots of homogenates of synchronously developing cleavage-stage embryos (Fig. 5 *A*) and terminally differentiated coelomocytes (Fig. 5 *C*). In contrast, under conditions where kinesin was readily detected on blots of whole egg or embryo homogenates, neither flagella nor cilia displayed an appreciable reactivity with antikinesins (Fig. 5 *B*, lanes 3 and 4). A faint band was detectable in cilia (Fig. 5 *B*, lane 4) but this is likely to be due to cellular contamination in the preparation.

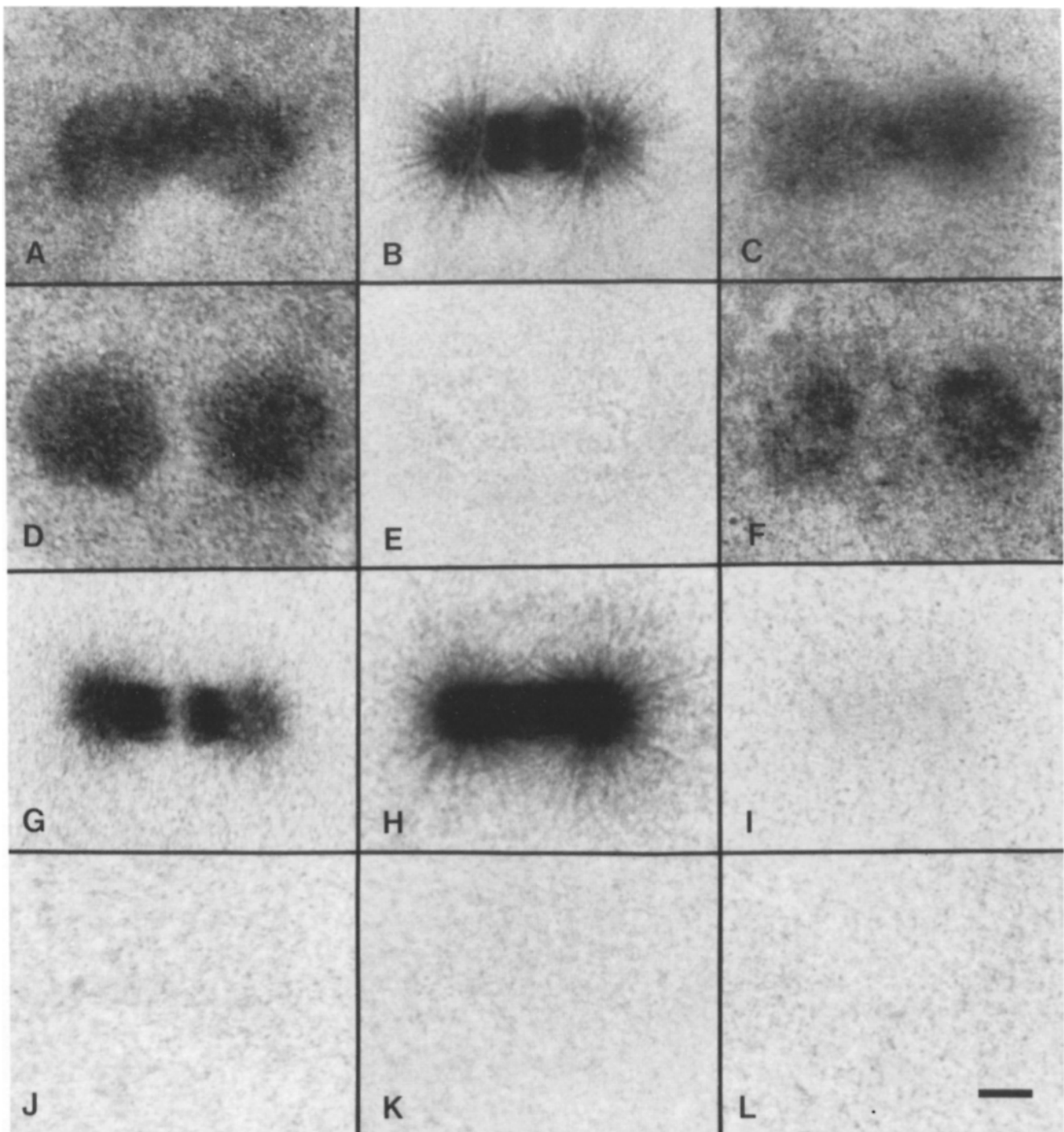


Figure 7. Effects of microtubule depolymerization and/or detergent lysis on MA staining for kinesin, tubulin, and calsequestrin. First metaphase embryos were fixed (*A*, *B*, and *C*), nocodazole treated and fixed (*D*, *E*, and *F*), detergent lysed then fixed (*G*, *H*, and *I*), or nocodazole-treated and detergent lysed then fixed (*J*, *K*, and *L*), then probed with monoclonal antikinesin (*A*, *D*, *G*, and *J*), antitubulin (*B*, *E*, *H*, and *K*), or anticalsequestrin (*C*, *F*, *I*, and *L*). Kinesin (*A*) and calsequestrin (*C*) are both concentrated into the MA, though there clearly is diffuse cytoplasmic staining as well. The pattern of staining for kinesin (*D*) and calsequestrin (*F*) is altered by MT depolymerization, but remains clearly visible in the region of the MT-depleted MA. Detergent extraction of membranes removes calsequestrin as expected (*I*) whereas antikinesin staining of the MA becomes more fibrous (*G*). However, sequentially depolymerizing MTs and detergent-extracting cells before fixation results in the loss of kinesin (*J*) and calsequestrin (*L*), as well as tubulin (*K*). Bar, 10 μm .

Immunolocalization of Kinesin to the Mitotic Apparatus

Methanol-fixed unlysed (Fig. 6, *A*, *C*, and *E*) and detergent-lysed (Fig. 6, *B*, *D*, and *F*) first metaphase embryos were probed with domain-specific anti-kinesins (Fig. 6, *A* and *B*),

anti-tubulin (Fig. 6, *C* and *D*), and a nonspecific control antibody (Fig. 6, *E* and *F*). PAP (peroxidase anti-peroxidase) staining was used rather than fluorescence to circumvent the high background produced by autofluorescence of whole eggs and early embryos.

Kinesin appeared to be more concentrated in the MA than

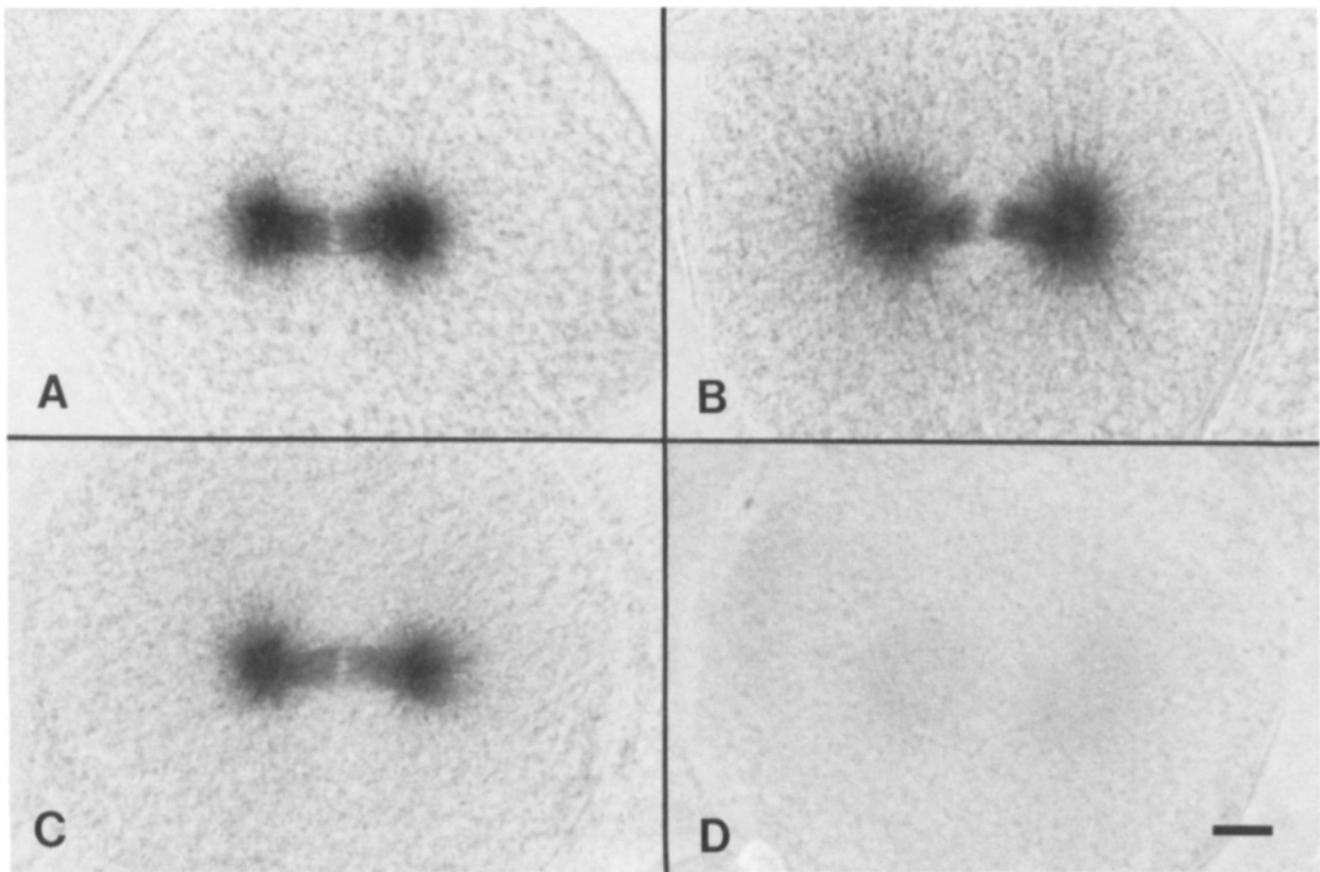


Figure 8. Nucleotide sensitivity of kinesin staining of MAs in detergent-extracted cells. First metaphase embryos were detergent lysed in the presence of 10 mM MgSO₄ (A), 10 U/ml Mg · Apyrase (B), 10 mM Mg · AMP-PCP (C) or 10 mM Mg · ATP (D). Compared to controls (A), Mg · AMP-PCP had no effect on MA staining (C), while depletion of nucleotides by apyrase (B) enhanced MA staining somewhat. Mg · ATP, the optimal nucleotide substrate for kinesin, totally abolished kinesin localization to the MA (D). The addition of nucleotides had no effect on antitubulin staining (not shown). Bar, 10 μm.

the surrounding cytoplasm of fixed embryos (Fig. 6 A), possibly because it associates with membranes that accumulate in these MAs. After NP-40 lysis, the anti-kinesin staining of the cytoplasm was decreased, and became more “fibrous” in the MA (Fig. 6 B), indicating that kinesin-bound membranes were extracted by the detergent, leaving behind kinesin that was associated with MA MTs (compare Fig. 6, B and D).

Several other detergent treatments (0.02% saponin, 0.2% Brij-58, and 1% Triton X-100; Hollenbeck, 1989) gave similar results to 1% NP-40. Only 1% SDS (which completely dissolved the embryos) was able to effectively extract the kinesin staining of spindle MTs in these cells (data not shown). We also observed similar results using aldehyde fixatives in place of cold methanol. Essentially identical staining patterns were obtained in these experiments with all the antikinesins listed in Fig. 4, whereas nonspecific control antibodies showed no staining of cells fixed without (Fig. 6 E) or with detergent lysis (Fig. 6 F).

Effects of MT Disassembly and Detergent Extraction of Membranes on the Localization of Kinesin and Calsequestrin in Mitotic Cells

We compared the distribution of kinesin, tubulin, and the luminal ER protein, calsequestrin (Henson et al., 1989; 1990), in mitotic cells treated to disrupt the organization of MA membranes and MTs (Fig. 7). Calsequestrin, like kine-

sin, was concentrated in the MAs of methanol-fixed blastomeres, but with a less fibrous distribution than that displayed by tubulin (compare Fig. 7, A, B, and C), and there was diffuse cytoplasmic staining as well.

Treatment of embryos with 10 μg/ml nocodazole before fixation (Fig. 7, D, E, and F) resulted in the complete loss of antitubulin staining of the MA (Fig. 7 E), accompanied by the disorganization, but not loss, of both kinesin (Fig. 7 D) and calsequestrin (Fig. 7 F) in the region of the previously intact MA (prolonging the incubations with nocodazole much beyond 30 min resulted in the complete dispersion of kinesin and calsequestrin).

Detergent lysis to extract membranes before fixation (Fig. 7, G, H, and I), completely removed calsequestrin as expected (Fig. 7 I), and enhanced the fibrous quality of anti-kinesin staining (Fig. 7 G) which then more closely resembled anti-tubulin staining (Fig. 7 H). However, when metaphase embryos were first subjected to MT depolymerization and then detergent lysis before fixation (Fig. 7, J, K, and L), anti-kinesin staining of the MT-depleted (Fig. 7 K) and endomembrane-depleted (Fig. 7 L) MA was completely abolished (Fig. 7 J). (When the treatment order was reversed [i.e., detergent lysis followed by MT depolymerization] this concentration of nocodazole proved unable to completely depolymerize MTs within the 30 min time frame studied [data not shown]).

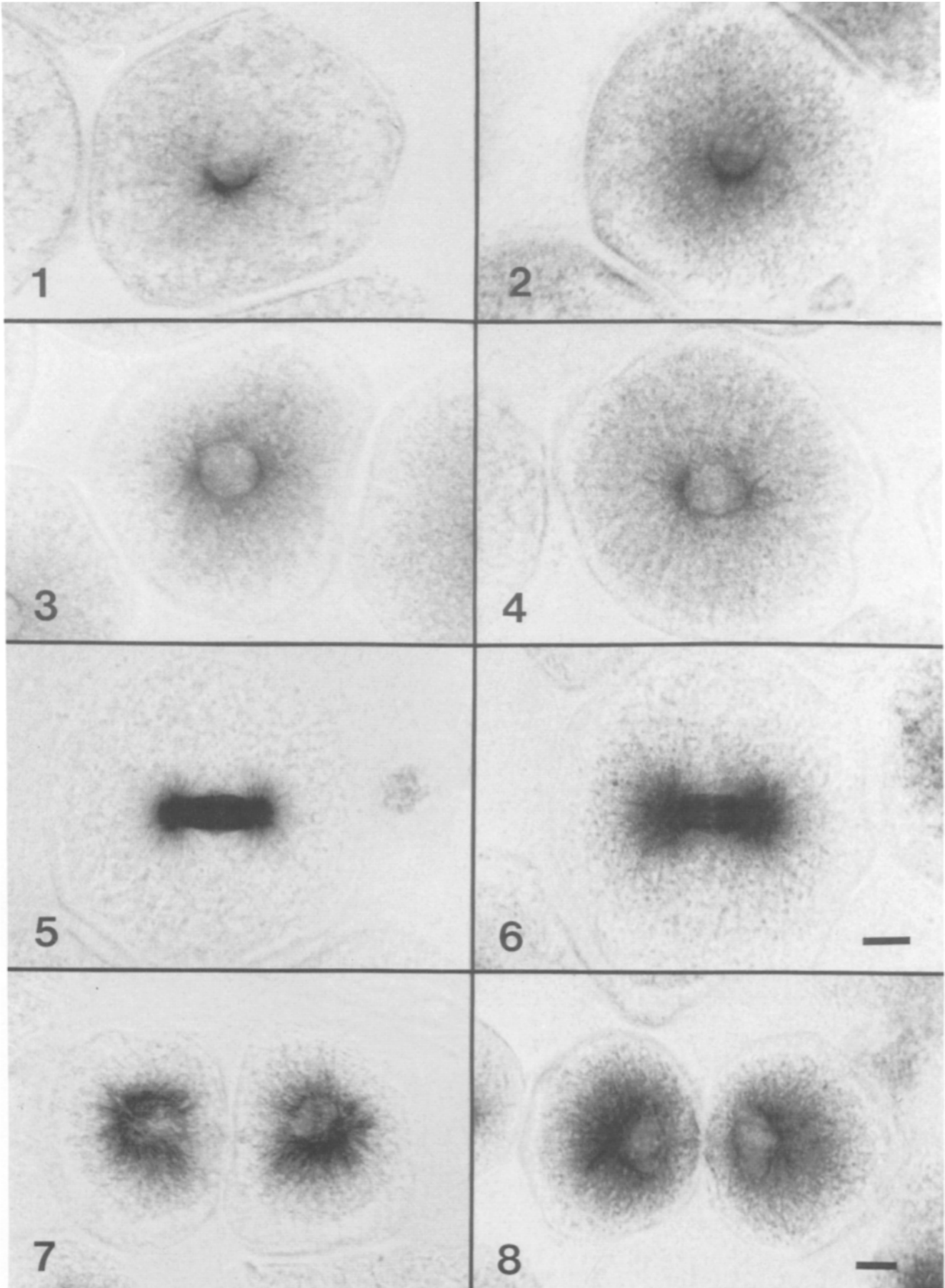
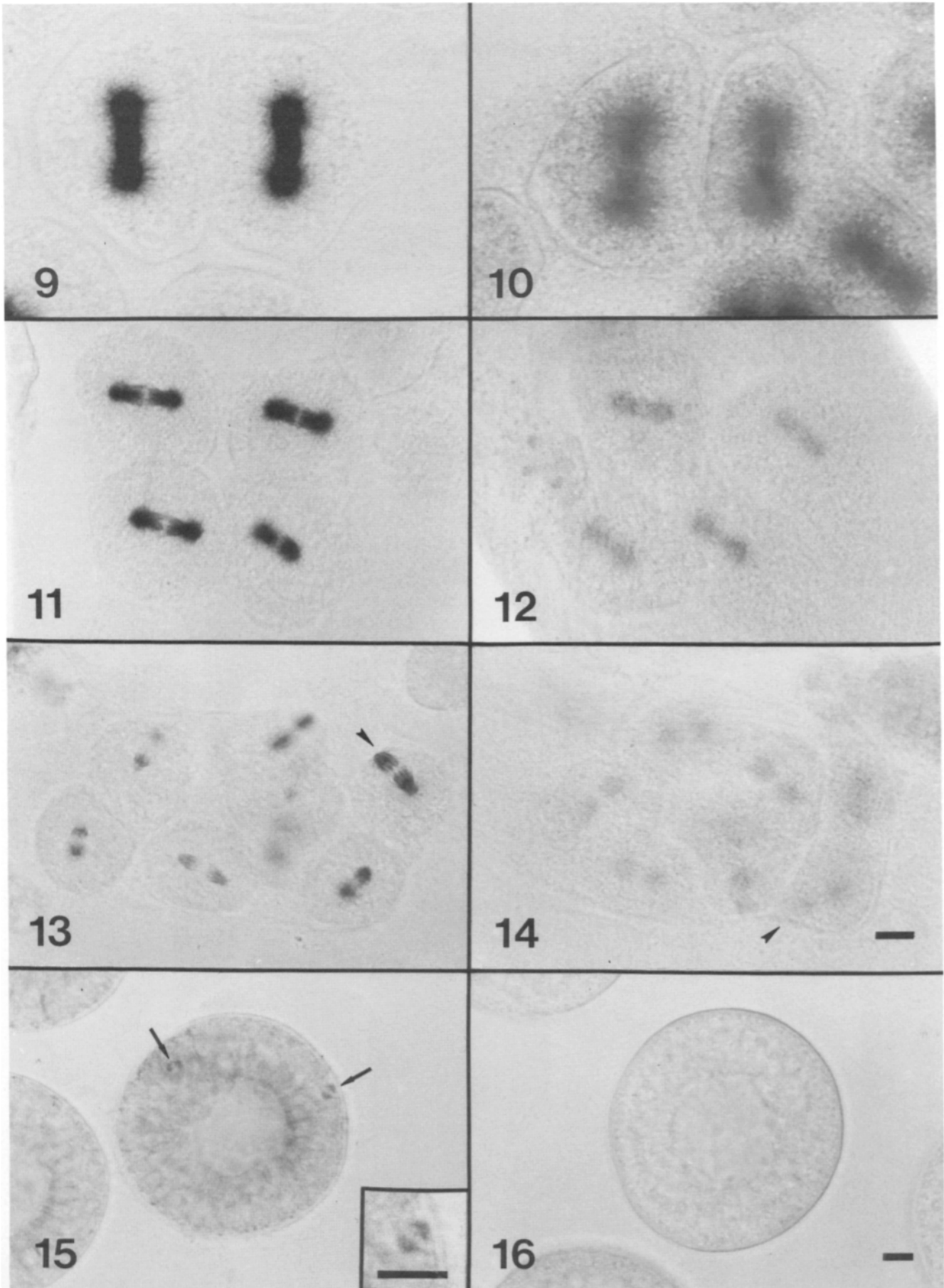


Figure 9. Comparison of kinesin (*right*) and tubulin (*left*) localization during cleavage-stage development. Aliquots of synchronously developing embryos at the following stages were stained with antikinesin (*even numbers*) or antitubulin (*odd numbers*); sperm aster (1, 2), disaster (3, 4) first metaphase, (5, 6), second interphase (7, 8), second metaphase (9, 10), third metaphase (11, 12), fourth metaphase



(13, 14), and hatching blastulae (15, 16). All but the blastulae were lysed before fixation. Note the gradual loss of intensity of antikinesin staining and the presence of acentric spindles in the asymmetric fourth division (13, 14; arrowheads). By the blastula stage, diminutive spindles are clearly present (15, arrows, and inset), which do not seem to stain for kinesin (16). Bars, 10 μ m.

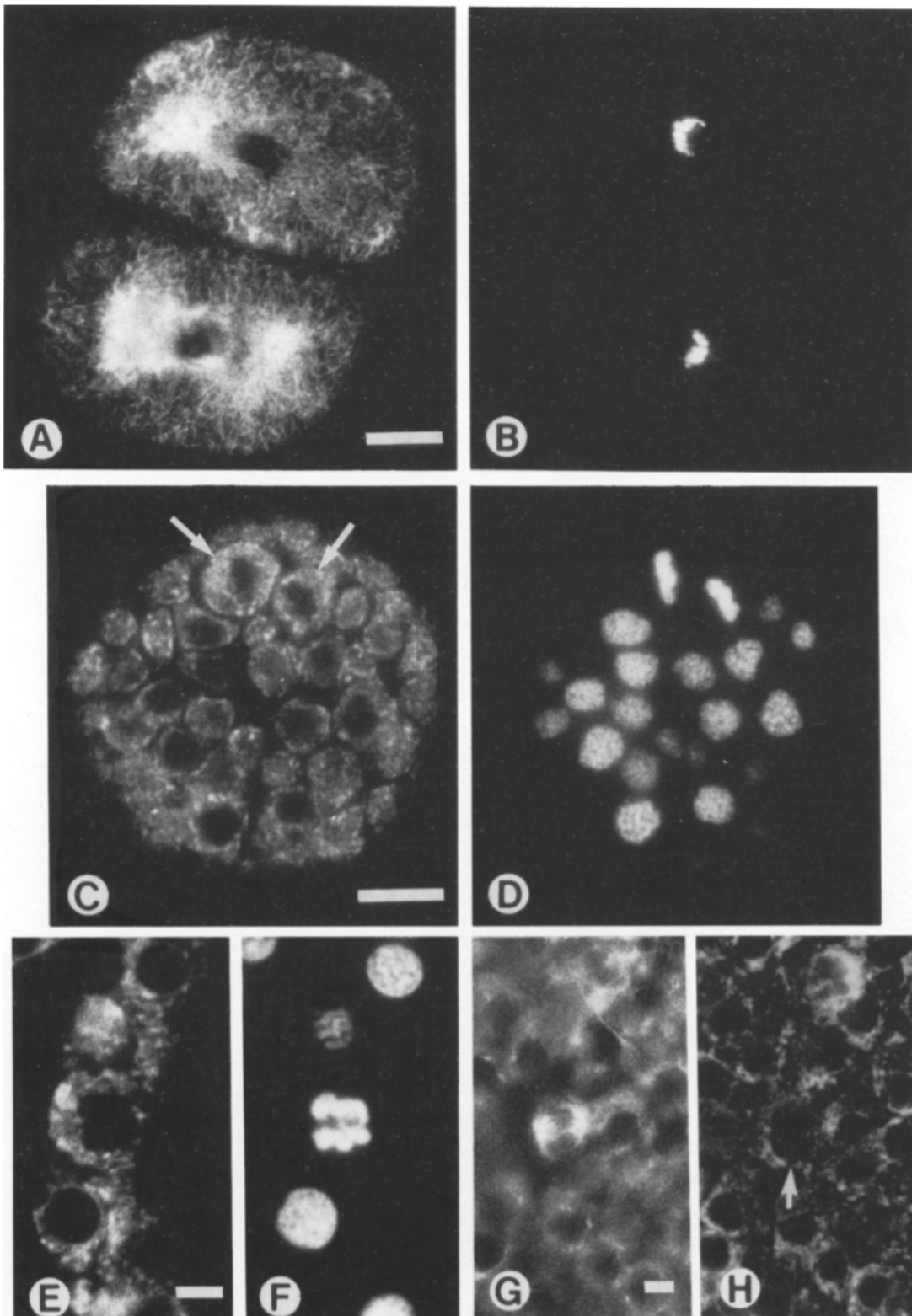


Figure 10. Immunofluorescent localization of sea urchin calsequestrin (*A*, *C*, and *E*) and Hoechst dye staining of chromatin (*B*, *D*, and *F*) in frozen sections of embryos. Calsequestrin showed a clear concentration in the MAs of second division embryos (*A*), but not in dividing cells from blastula (*arrows* in *C*) or gastrula (*E*) embryos. Mitotic cells can be identified by the presence of Hoechst-stained condensed chromosomes (*B*, *D*, and *F*). Whole-mount, methanol-fixed gastrula embryo double labeled with anti-tubulin (*G*) and anti-calsequestrin (*H*) indicates that calsequestrin-containing membranes are actually excluded from spindles (*H*, *arrow*). Bars: (*A*) 10 μm ; (*C*) 5 μm ; (*E* and *G*) 2 μm .

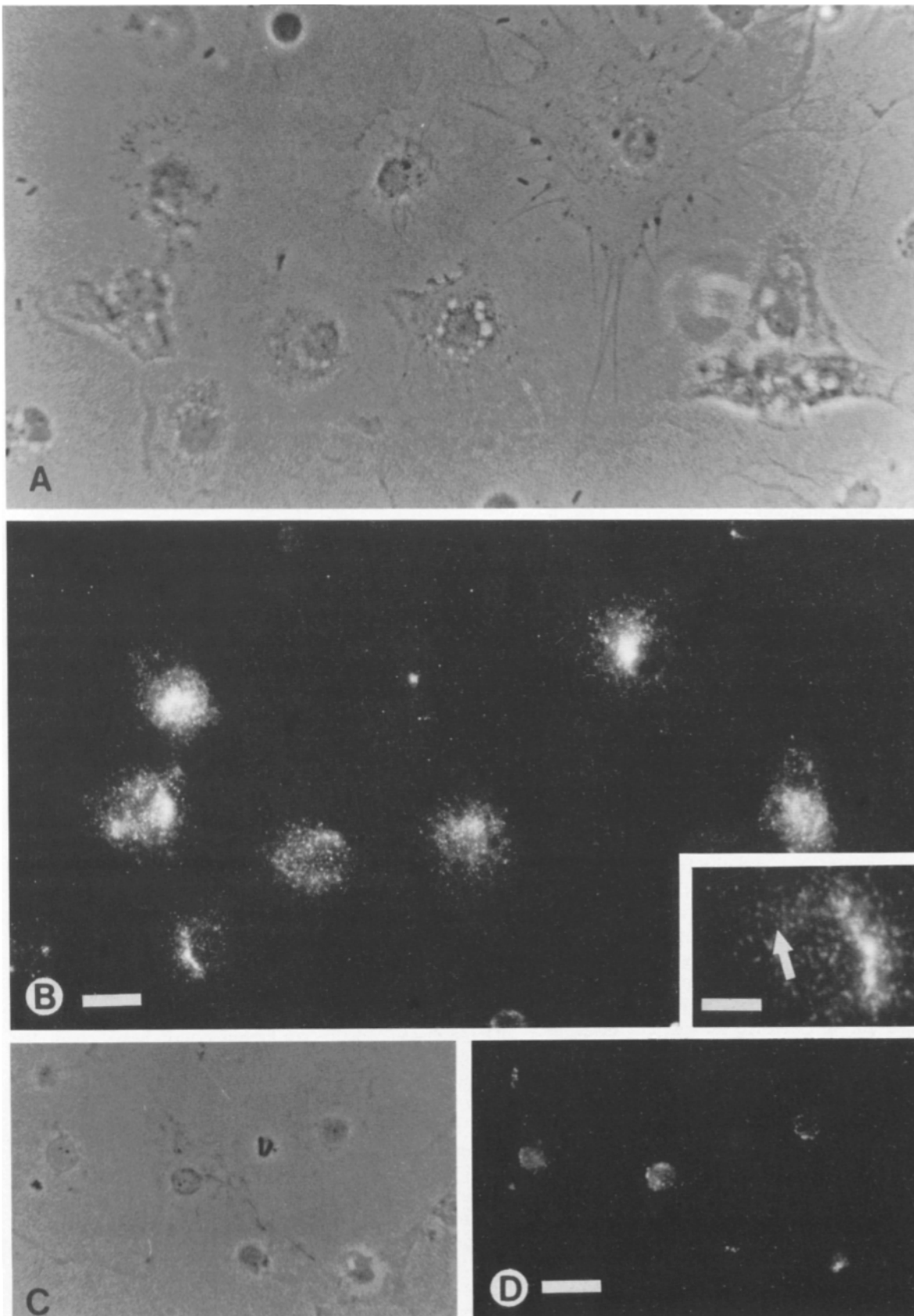


Figure 11. Immunofluorescent localization of kinesin in coelomocytes. Comparison of phase-contrast (*A*) and fluorescent (*B*) images revealed that kinesin is associated with vesicle-like particles concentrated around the nucleus and absent from the cortex of the cell. The stained structures occasionally appeared to align in linear arrays (*arrow* in *inset*). The particulate cytoplasmic staining was abolished in cells which were extracted before fixation with Triton (*C* and *D*). The detergent treatment did result in staining of the nucleus and/or nuclear associated structures (*D*). Bars: (*B* and *D*) 10 μm ; (*inset*) 5 μm .

Our experiments suggest that kinesin cross-links membranes to MTs in the MA, so that when MTs are depolymerized, kinesin remains associated with MA membranes (Fig. 7 D), if intracellular membranes are extracted kinesin remains bound to MTs (Fig. 7 G), and removal of both MTs and membranes results in the complete extraction of kinesin from the MA (Fig. 7 J).

Kinesin Displays Nucleotide-sensitive Binding to the MA in Detergent-lysed Cells

Anti-kinesin staining was not noticeably affected when the detergent buffer used to lyse metaphase embryos contained MgSO₄ (Fig. 8 A) or Mg-AMP-PCP (Fig. 8 C) in concentrations that have little effect on kinesin-driven motility (Cohn et al., 1989). In contrast, depletion of endogenous nucleotides using apyrase, which stabilizes kinesin-MT interactions (Scholey et al., 1985), enhanced kinesin staining of the MA (Fig. 8 B), whereas the addition of Mg-ATP, which dissociates kinesin from MTs, completely eliminated kinesin staining of the MA (Fig. 8 D). Mg-ADP also dissociates kinesin from MTs *in vitro* and similarly depleted antikinesin staining of the MA (data not shown). None of these treatments removed the anti-tubulin staining of the MAs in these cells (data not shown). These results indicate that the kinesin remaining after detergent extraction of MA membranes is associated with MTs by nucleotide-sensitive bonds, presumably through the motor domains.

Kinesin Localization during Cleavage-stage Development

Although the amount of kinesin per embryo remained approximately constant during development of the fertilized egg into the blastula (Fig. 5 A), the subcellular distribution of kinesin changed. The unfertilized egg stained uniformly for kinesin throughout the cytoplasm in fixed cells, but showed no kinesin staining when cells were detergent extracted (data not shown). Staining of synchronously developing embryos with antikinesin and anti-tubulin (Fig. 9) revealed that during mitosis and interphase of the first two cell cycles, kinesin was concentrated into MT arrays. However, this association of kinesin with MTs gradually faded with subsequent divisions up to the blastula stage. Fig. 9, panels 15 and 16 display anastral spindles that stain with antitubulin (magnified in *inset*) but no concentration of kinesin in these MAs was detected during extensive microscopic observations. The antikinesin did stain the blastula cytoplasm clearly above background, suggesting that the kinesin remains diffuse in the cytoplasm and no longer accumulates in the MA (not shown).

Anti-kinesin staining was further applied to embryos developing to pluteus larval stage (data not shown); generally a diffuse distribution of kinesin was observed, although SUK2 (but none of the other antikinesins) apparently bound to a cross-reactive extracellular matrix antigen that gave rise to a transient concentration of staining in the region of the blastopore during development of the archenteron (not shown).

Immunofluorescent Localization of Calsequestrin in Cleavage-stage Embryos

Semi-thin frozen sections of embryos were stained with anti-

serum against calsequestrin (Henson et al., 1989, 1990), in order to compare the distribution of ER-derived membranes with the pattern of kinesin localization during embryogenesis. Like kinesin, calsequestrin showed a clear concentration in the MAs of embryos undergoing second division from the two-cell to the four-cell stage (Fig. 10, A and B). As expected for this ER marker, the staining pattern appeared vesicular in the astral regions and as a tubuloreticular network in the remainder of the blastomere cytoplasm. Staining of sections of blastula embryos also indicated the presence of a calsequestrin-containing cytoplasmic reticulum. However, consistent with the kinesin localization patterns, there appeared to be no concentration of calsequestrin-stained elements in the spindle region of dividing blastula-stage blastomeres, identified by the presence of condensed chromosomes (Fig. 10, C and D). This lack of MA-associated calsequestrin staining was also evident in frozen sections of gastrula embryos (Fig. 10, E and F), and in methanol-fixed, whole-mount gastrula embryos (Fig. 10, G and H) processed similarly to the kinesin-labeled embryos.

Immunofluorescent Localization of Kinesin in Coelomocytes

We labeled phagocytic amoebocytes from the coelomic cavity of adult sea urchins with anti-kinesin, in order to examine the localization of kinesin in a terminally differentiated, non-mitotic cell type. Anti-kinesin staining of coelomocytes revealed a juxtannuclear distribution of punctate, vesicle-like particles (Fig. 11, A and B). The staining did not extend into the actin-rich cortex of the cells, consistent with the known distribution of MTs (Edds, 1984). In some cells, the vesicles appeared to align in linear arrays (Fig. 11 B, *inset*). The cytoplasmic staining pattern was abolished in cells treated, pre-fixation, with Triton under MT-stabilizing conditions (Fig. 11, C and D), suggesting that the kinesin is associated with membrane-bound structures. The nucleus appeared to stain in detergent extracted (Fig. 11 D) but not unextracted (Fig. 11 B) cells. Control staining with secondary antibody alone showed no specific staining (data not shown).

Discussion

In this report we have described the isolation and analysis of cDNAs that encode the sea urchin kinesin heavy chain, the mapping of antibody epitopes on the encoded proteins, and the immunolocalization of kinesin in sea urchin cells. The results obtained suggest that the protein that we previously localized to sea urchin MAs using polyclonal kinesin antibodies is a conventional, membrane-bound kinesin, rather than a kinesin-like protein.

The predicted sequence and tripartite structure of the sea urchin kinesin heavy chain are characteristic of conventional kinesins (Yang et al., 1989; Kosik et al., 1990). Furthermore, the predicted size (117,390 D) and structure of the heavy chain are in reasonable agreement with the results of our previous studies of the protein itself (Scholey et al., 1985, 1989; Johnson et al., 1990). In particular, the predicted molecular mass of the amino-terminal motor domain is very close to the value of 45 kD obtained from studies using limited proteolysis and the decoration of kinesin with

function-blocking antibodies (Ingold et al., 1988; Scholey et al., 1989). This motor domain contains an ATP-binding consensus sequence which forms element I of a G-protein consensus sequence. The presence of element II and III GTP-binding sequences as well (Dever et al., 1987; Obar et al., 1990) may be relevant to the observation that sea urchin kinesin can use GTP to fuel MT movement (K_m for GTP = 1.9 mM and for ATP = 63 μ M; Cohn et al., 1989). The COOH-terminal tail is very basic, a feature which may be important for interactions of the sea urchin kinesin heavy chain with light chains (Hirokawa et al., 1989) and components of the transported cargo (e.g., acidic protein or phospholipid constituents of membranes).

By manipulating and expressing kinesin heavy chain cDNAs, we obtained fragments of the corresponding polypeptide that were used to map monoclonal antibody epitopes to the head, stalk, and tail domains of the heavy chain (Scholey et al., 1989; this report). In addition, the expressed stalk and tail fragments were used as affinity ligands for purifying polyclonal antibodies specific for the stalk and tail domains. While some antibodies to the conserved motor domain of kinesin might cross-react with kinesin-like proteins in the MA, there is no reason to expect antibodies to the non-conserved stalk/tail domains to do so, yet antibodies to the head, stalk, and tail domains displayed identical staining patterns in immunolocalization experiments. Therefore it seems unlikely that a cross-reactive kinesin-like protein of the type thought to participate in cell division can account for the MA staining that we observe, and we conclude that kinesin itself is concentrated in the sea urchin MA.

Based on work in other systems, kinesin, as distinct from a kinesin-like protein, is proposed to serve as a membrane motor (see Introduction). Therefore, the accumulation of kinesin-bound ER-derived membranes in MAs of the sea urchin early embryo provides a plausible explanation for the observation that antikinesins stain these MAs. This idea is supported by our studies of the localization of kinesin in mitotic blastomeres treated so as to disrupt MTs and calyculin-containing MA-membranes, and in lysed cells treated so as to perturb nucleotides (Figs. 7 and 8); in these preparations the distribution of kinesin changed in a manner consistent with it cross-linking membranes (presumably bound at the stalk or tail domains) to MTs, which bind by ATP-sensitive bonds to the kinesin motor domains, allowing the mechanoenzyme to carry its membranous cargo along the MT.

In order to minimize the effects of the *in vitro* modification of the MA in these experiments (Figs. 7 and 8), the depolymerization of MTs and the manipulations of the nucleotide concentrations were performed at or before the time of lysis. The results obtained differ from those obtained previously using mitotic cytoskeletons prepared by detergent lysis and multiple buffer washes, where subsequent ATP-treatment or cold calcium-induced MT depolymerization did not remove antikinesin staining (Leslie et al., 1987; results confirmed using mAbs by B. D. Wright). It appears that the MAs undergo time-dependent changes during lysis, washing, and storage of the cytoskeletons; thus, for example, MTs become resistant to nocodazole-induced depolymerization and the kinesin in the cytoskeletons displays a loss of solubility and ATP sensitivity of MT binding. We consider it likely that the properties displayed by kinesin in the present study (Figs. 7

and 8) more closely reflect the behavior of kinesin in living cells.

Comparisons with calyculin indicate that the distribution of kinesin changes during normal development in a manner consistent with it being a membrane-associated MT motor; both kinesin and calyculin-containing membranes are concentrated in the MA of early blastomeres, but in later blastomeres they display a diffuse localization. Furthermore, it is striking that in sea urchin coelomocytes, kinesin is localized to detergent-sensitive perinuclear particles that were sometimes found in linear arrays. These structures are similar to those stained by antikinesins in the cytoplasm of cultured cells as described by Pfister et al. (1989), and are proposed to be kinesin-bound vesicles capable of interacting with MT tracks.

While the function of kinesin-driven vesicle transport in sea urchins has not been demonstrated, it is easy to imagine that in coelomocytes, kinesin could transport secretory pathway vesicles from the perinuclear ER/Golgi network out to the cell periphery, for example to provide new cell membrane and extracellular components as these cells undergo the shape changes that are characteristic of the petaloid/filopodial transformation (Edds, 1977, 1984). In the sea urchin MA, kinesin-bound membranes may regulate calcium levels or serve structural roles (Hepler, 1989), and kinesin-driven membrane transport may be involved in establishing the position of the cleavage furrow (Vallee et al., 1990). We further speculate that the MAs of early sea urchin embryos may serve to store kinesin-bound, ER-derived vesicles that are actively dispersed as development proceeds; these vesicles may deliver exported macromolecules and new cell membrane to the cell surface, or become recruited into the cytoplasmic endomembrane system of blastomeres in the developing embryo (Henson et al., 1989, 1990).

It should be emphasized that our results do not eliminate the possibility that kinesin participates in chromosome movement, nor do they address the role of kinesin-like proteins in the sea urchin MA. While we have not yet directly visualized the binding of kinesin to MA membranes, our results do support the hypothesis that kinesin functions as a MT-based vesicle motor in the sea urchin MA as well as in the interphase cytoplasm. They further suggest that in other cells, MAs that do not stain with antikinesin (Pfister et al., 1989; Hollenbeck, 1989) contain relatively few kinesin-bound vesicles. It will now be important to further characterize the interactions between kinesin and membranes and to determine the physiological functions of kinesin-bound membranes in the sea urchin MA and cytoplasm, using antibody microinjections, membrane binding assays, and immunoelectron microscopy.

The cloning project emerged from a collaboration between J. M. Scholey, Bill Saxton, and Larry Goldstein. We thank them plus the following persons for advice, discussions, and reagents: Mitch Sogin, Hille Elwood, Paul Schneeman, Laurie and Jim Bamburg, Kip Sluder, Roger Leslie, David Begg, Ben Kaminer, Mike Klymkowsky, Matthew Suffness of the National Cancer Institute for kindly supplying the necessary taxol, Ron Vale, Michael Smith, and members of the Scholey lab.

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