Myosin Light Chain–activating Phosphorylation Sites Are Required for Oogenesis in Drosophila

Pascale Jordan and Roger Karess

Centre de Génétique Moleculaire, 91198 Gif-sur-Yvette, France

Abstract. The Drosophila *spaghetti squash* (*sqh*) gene encodes the regulatory myosin light chain (RMLC) of nonmuscle myosin II. Biochemical analysis of vertebrate nonmuscle and smooth muscle myosin II has established that phosphorylation of certain amino acids of the RMLC greatly increases the actin-dependent myosin ATPase and motor activity of myosin in vitro. We have assessed the in vivo importance of these sites, which in Drosophila correspond to serine-21 and threonine-20, by creating a series of transgenes in which these specific amino acids were altered. The phenotypes of the transgenes were examined in an otherwise null mutant background during oocyte development in Drosophila females.

Germ line cystoblasts entirely lacking a functional *sqh* gene show severe defects in proliferation and cytokinesis. The ring canals, cytoplasmic bridges linking the oocyte to the nurse cells in the egg chamber, are abnormal, suggesting a role of myosin II in their establishment or maintenance. In addition, numerous aggregates of myosin heavy chain accumulate in the *sqh* null cells. Mutant *sqh* transgene *sqh*-A20, A21 in which both serine-21 and threonine-20 have been replaced by alanines behaves in most respects identically to the null allele in this system, with the exception that no heavy chain aggregates are found. In contrast, expression of *sqh*-A21, in which only the primary phosphorylation target serine-21 site is altered, partially restores functionality to germ line myosin II, allowing cystoblast division and oocyte development, albeit with some cytokinesis failure, defects in the rapid cytoplasmic transport from nurse cells to cytoplasm characteristic of late stage oogenesis, and some damaged ring canals. Substituting a glutamate for the serine-21 (mutant *sqh*-E21) allows oogenesis to be completed with minimal defects, producing eggs that can develop normally to produce fertile adults. Flies expressing *sqh*-A20, in which only the secondary phosphorylation site is absent, appear to be entirely wild type. Taken together, this genetic evidence argues that phosphorylation at serine-21 is critical to RMLC function in activating myosin II in vivo, but that the function can be partially provided by phosphorylation at threonine-20.

Whether involving intracellular vesicle transport or changes in cell shape and migration occurring during growth and development, cell motility is a reflection of cytoskeleton dynamics and interactions with molecular motors. Although many such motors have now been identified for both the microtubule and actin filament cytoskeletons, it is not yet known how individual motor molecules are regulated in vivo to carry out their specific tasks.

The classical nonmuscle (cytoplasmic) myosin, (referred to hereafter as myosin II), has recently been demonstrated to participate in many cellular movements and developmental events beyond its well-established role in cytokinesis. Dictyostelium cells lacking the gene for the myosin heavy chain fail to complete the morphogenetic changes required to form a mature fruiting body (13). Analysis of Drosophila mutants in the gene *zipper* (*zip*) (65) encoding the myosin II heavy chain and in the *spaghetti squash* (*sqh*) gene (25) encoding the myosin II regulatory light chain, has shown that myosin II is required for tissue movements during embryogenesis, proper imaginal disk development, cell migration, (15) syncytial nuclear migration, and various aspects of oogenesis (15, 61).

Myosin II consists of a pair of myosin heavy chains (MHCs)[1] carrying the globular motor domain and the α-helical tail, and a pair each of the essential and regulatory light chains (EMLCs and RMLCs, respectively; reviewed in 32). The state of phosphorylation of the RMLC nor-

Address all correspondence to Dr. Roger Karess, Centre de Génétique Moleculaire, Ave de la Terrasse, 91198 Gif-sur-Yvette, France. Tel.: (33-1) 69-82-32-25; Fax: (33-1) 69-82-31-50. E-mail: karess@cgm.cnrs-gif.fr

1. *Abbreviations used in this paper*: hts, huli-tai-shau; MHC, myosin heavy chain; MLCK, myosin light chain kinase; RMLC, regulatory myosin light chain.

mally regulates myosin activity (reviewed in 47, 52, 55). Phosphorylation by the enzyme myosin light chain kinase (MLCK) of specific RMLC residues increases the actin-activated ATPase activity and the in vitro motility of pure myosin. In vivo, phosphorylation of the RMLC by MLCK is sufficient to induce contraction in smooth muscles (23). The major site of this activating phosphorylation in vertebrate RMLC is serine-19 (40) with the adjacent threonine-18 being a secondary target (22). An alignment between the Drosophila RMLC and vertebrate smooth muscle RMLC reveals that the two proteins are very similar (81% amino acid identity; reference 25), especially around these important serine and threonine residues (see Fig. 1). In Drosophila, the corresponding positions are serine-21 and threonine-20 (25).

Despite the abundant biochemical evidence that light chain phosphorylation regulates myosin activity, it has not been shown in vivo that the various myosin II–mediated events in nonmuscle cells require this phosphorylation. Indeed, it has recently been reported that the principal activating phosphorylation site of Dictyostelium RMLC is of only minor importance to myosin function in vivo (39). However, the details of Dictyostelium myosin II regulation differ slightly from those of metazoan myosin II (reviewed in reference 20), and the light chain primary structure is somewhat divergent.

In this study, we examine the importance of myosin II and specifically the presumed sites of RMLC phosphorylation in germline-derived cells during Drosophila oogenesis. This is a well-studied system demonstrating developmentally regulated cell motility and cell shape changes, aspects of which have already been shown to depend on a proper actin-myosin network (11, 15, 61). (For reviews of oogenesis and a definition of the 14 morphological stages, see references 36, 48.)

Drosophila oogenesis begins when a single germ-line stem cell divides in the germarium to produce a cystoblast daughter cell that undergoes four further mitotic divisions to yield a cluster of 16 cystocytes, which is then surrounded by a monolayer of somatically derived follicle cells to form a cyst, or egg chamber. Cytokinesis in the cystoblast divisions is by design incomplete, and thus the 16 cells remain interconnected by a series of cytoplasmic bridges that eventually develop into elaborate structures called ring canals (45). One of the first two cystoblasts to form becomes the oocyte, while the remaining 15 cells differentiate into nurse cells. Nurse cells supply the majority of the cytoplasmic components to the oocyte by transport through the ring canals. For most of oogenesis, up to stage 10, this is a slow process, depending largely on microtubule-dependent motors (19, 59). During stage 11, however, the entire remaining cytoplasmic contents of the nurse cells are transferred to the oocyte within 30 min, in a step known as rapid transport, or "dumping" (19). Dumping requires an intact actin cytoskeleton, as shown by drug studies (19) and mutant analysis (7, 10, 57, 63). It also requires germline myosin II (61).

We describe here the germline phenotypes of specific mutations in the RMLC. We find that germinal stem cells in clones lacking the gene encoding RMLC quickly become incapable of cytokinesis and sustained division. Germ cells expressing RMLC with an alanine at position 21, the site corresponding to the activating phosphorylation, can keep dividing, but they display a consistent, though relatively mild, reduction in the efficiency of cystoblast cytokinesis. Nevertheless, such mutants can complete oogenesis and lay eggs that initiate development. When, however, serine-21 and the adjacent threonine-20 are both replaced by alanines, the phenotype is nearly identical to that seen in an RMLC null mutant germ line. Substituting a glutamic acid residue for serine-21 substantially restores functionality to the RMLC during these events. These observations provide strong genetic arguments that, in Drosophila, serine-21 (or threonine-20) of RMLC needs to be phosphorylated for myosin function in vivo, and suggest that different myosin-mediated events may require different minimal levels of myosin activity.

Materials and Methods

Fly Stocks

Flies were raised on standard corn meal Drosophila medium at 25°C. The markers and chromosomes are described in reference 34, except as noted. The isolation and cloning of sqh^1 is described in reference 25. In the null allele sqh^{AX3}, most of the *sqh* transcription unit encoding the RMLC had been removed by a 5-kb deletion. Its isolation will be described in detail elsewhere. The chromosome carrying sqh^{AX3} is marked with *y*, which allows hemizygous or homozygous mutant sqh^{AX3} larvae to be identified by their yellow mouth hooks. The stocks of *FRT-101* and ovo^{D1} *FRT-101*/Y; $hs\text{-}flp^{38}$ used to generate germline clones (9) were obtained from Dr. N. Perrimon. Wild-type controls used either *FRT-101* or $y\ w^{67}$, obtained from the Bloomington stock center.

Mutant sqh Constructs

The mutant RMLCs (Fig. 1) were constructed by PCR using as template DNA the Bluescript SK+ vector (Stratagene Inc., La Jolla, CA) containing a 0.75-kb EcoR1 fragment of genomic sqh^+ (described in reference 25). The mutagenizing primers were (with the altered codon in bold type) for *sqh*-A21: 5′ AAG AAG CGC GCC CAA CGC GCC ACC **GCG** AAT GTG TTC; for *sqh*-E21: 5′ AAG AAG CGC GCC CAA CGC GCC ACC **GAG** AAT GTG TTC; for *sqh*-A20, A21: 5′ AAG AAG CGC GCC CAA CGC GCC **GCG GCG** AAT GTG TTC; for *sqh*-A20: 5′ AAG AAG CGC GCC CAA CGC GCC **GCG** TCC AAT GTG TTC. The second primer for each reaction was the universal sequencing primer. The resulting PCR products digested with BssHII and KpnI (a site in the Bluescript polylinker) were used to replace the corresonding fragment in the wild-type 0.75-kb EcoRI fragment. This fragment was subsequently assembled into an intact *sqh* gene controlled by its natural promoter. The carboxy terminal end of this *sqh* gene was engineered to contain a 10 amino acid extension encoding the FLAG epitope (Eastman Kodak Co., Rochester, NY). The tag served as an electrophoretic marker allowing the protein expressed by the transgene to be distinguished from the endogenous RMLC. The peptide tail has no effect on the activity of an otherwise wild-type RMLC, as assayed by its ability to fully rescue the sqh^{AX3} null allele. Details of this construction will be described elsewhere.

The altered *sqh* genes were cloned into the polylinker of the P transformation vector CasPer (44), which carries in addition the selectable marker *mini-white*$^+$, and introduced into the germ line of $y\ w^{67}$ flies by standard methods (2), using the helper plasmid pUC-hs-Δ2-3 (38) as a source of transposase. Transformants carrying the mutant *sqh* transgenes on chromosomes 2 or 3 were selected. Extracts of transgenic flies or larvae were prepared using the method described in reference 65, and examined by Western blot to verify that the transgenes were expressing the altered RMLC. The samples were run on 13% polyacrylamide gels transferred to polyvinyldifluoride membranes, and then incubated with our rabbit anti–Drosophila RMLC antiserum at 1/200 dilution, a secondary peroxidase-conjugated antibody at 1/15,000 dilution (Jackson ImmunoResearch Laboratories Inc., Avondale, PA) and the ECL detection kit (Amersham Corp., Arlington Heights, IL).

Generation of Germline Clones

The FLP-DFS system (9) was used to generate homozygous sqh^{AX3} germline clones either alone or in the presence of one of the different mutant *sqh* transgenes P[w^+, *sqh*-A21], P[w^+, *sqh*-E21], or P[w^+, *sqh*-A20, A21] inserted on an autosome. *y w* sqh^{AX3} sn^3 FRT^{101}/FM7c females (with or without a homozygous transgene) were crossed to *w* ovo^{D1} FRT^{101}/Y;*IIhs-FLP*^{38} males. 24-h pulses of eggs from this cross were allowed to develop to first, second, or third instar larvae, as needed, and then heat shocked at 37°C for 2 h in an air incubator. In the non-FM7 individuals, heat shock–induced expression of the FLP recombinanse leads to mitotic recombination between the two FRT sties on the X chromosome homologs. A fraction of the cells thus become homozygous *y w* sqh^{AX3} ovo^+/*y w* sqh^{AX3} ovo^+, while all others remain ovo^{D1}. If the genome carries one of the mutant *sqh* transgenes, the sole form of RMLC expressed in the induced clones will be the mutant form. The successful induction of mitotic recombination in the somatic cells of these flies could be easily monitored by the frequent appearance of *y sn* bristles on the thorax and the abdomen. The ovo^{D1} mutation causes dominant sterility by blocking oogenesis at a very early stage (stage 4–5). Any egg chambers developing beyond this stage have lost the ovo^{D1} allele and therefore are genetically sqh^{AX3}/sqh^{AX3}, or sqh^{AX3}/sqh^{AX3}; P[*sqh*-A21], or sqh^{AX3}/sqh^{AX3}; P[*sqh*-E21] or sqh^{AX3}/sqh^{AX3}; P[*sqh*-A20, A21]. For each construct, at least two independently derived transgenic lines were tested. The results being the same, the data for the different lines were pooled.

For examining embryogenesis, putative clone-bearing (non-FM7) females were crossed to *y* w^{67}/Y; P[sqh^+] males. The eggs laid from the germline clones are referred to in the text as *sqh*-A21 or *sqh*-E21 to indicate the genetic origin of the egg cytoplasm. Their zygotic genotype depends on the fertilizing sperm, of course, which in this case is always sqh^+.

Ovo^{D1} Perduring Effects on Cystocyte Division

In the course of this study, we observed that ~5% of all the egg chambers generate with the ovo^{D1} system contain more than the normal 16 cells (see Table I and Fig. 3, *G–J*), suggesting that a fifth round of cystoblast division had occurred. This has also been reported for the sqh^1 allele (61). This effect was independent of the genotype being examined, which indicated that it was an artifact related to the clonal induction itself. Since ovo^{D1} is a dominant mutation, producing a protein with antimorphic activity (42), it was possible that perdurance of ovo^{D1} product in genetically *sqh* mutant clones might be responsible. Different alleles of *ovo* are known to disrupt cystoblast division during oogenesis (42) and in ovo^{D1} ovaries, egg chambers with >15 nurse cells have been observed (6).

To test for a possible perduring effect of the ovo^{D1} protein on cystocyte division, +/+ (wild-type) germline clones generated from ovo^{D1} /+ females were examined (Table I). The majority of the wild-type clones generated in these conditions were normal, but some 4% of the +/+ egg chambers derived from ovo^{D1} /+ individuals had apparently sustained a fifth round of cystoblast division, resulting in >25 nurse cells and nuclei, and five ring canals linking nurse cells to the oocyte (Fig. 3, *I* and *J*). In addition, 6% of the egg chambers contained <15 nurse cells. These deviations from wild-type nurse cell number were relatively rare (10% in total), and the effect was diminished in older clones (data not shown). Thus, the class of egg chambers with >16 nurse cells common to all the *sqh* alleles in Table I (and to sqh^1) is in fact not part of the *sqh* mutant phenotype. However, the other classes cannot be explained by an effect of ovo^{D1} and therefore are consequences of the *sqh* alleles themselves. Binuclear nurse cells were never seen in the wild-type germline clones, and the myosin heavy chain aggregates are also specific to the sqh^{AX3} mutant clones (see Fig. 7, *H* and *I*), while the frequency of hypotrophic chambers is far below that seen with the *sqh* mutants (Table I).

These results indicate that ovo^{D1} can have an important perduring effect on cystocyte division. Others who use the ovo^{D1} technique for examining germline phenotypes of different mutations should keep in mind this potential artifact.

Immunocytochemistry

Ovaries were dissected in PBS, pH 7, to release the egg chambers. They were fixed 10 min in PBS, 0.3% Triton X-100, 4% formaldehyde, and then rinsed three times in PBT (PBS with 0.3% Triton X-100 and 5% BSA). The antiphosphotyrosine monoclonal antibody (Sigma Chemical Co., St. Louis, MO) was used at 1:1,000 dilution in PBT. The tissue culture supernatants containing monoclonal antibodies to kelch and huli-tai-shau (hts) were gifts from Dr. Lynn Cooley and used at 1:4 dilution. The ovaries were incubated 2 h at room temperature, and then overnight at 4°C. Two different anti–myosin heavy chain antibodies were used. One, a gift from Dan Kiehart (30) was used at a 1:100; the other, generated in our lab (raised against a portion of the α-helical tail) was used at 1/2,000 dilution in PBT. The ovaries were incubated overnight at 4°C. Bound antibodies were detected using a goat anti–rabbit or anti–mouse IgG secondary antibody (as appropriate) conjugated to fluorescein or rhodamine, (Jackson Immunoresearch Laboratories Inc.), diluted 1/100. For actin staining, tissues were incubated for 2 h at room temperature with 0.2 μM rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR). Because ring canals stain very intensely with both phalloidin or antiphosphotyrosine, a double label with either of these probes and the anti–MHC risked giving a false positive signal in the MHC channel. To avoid this potential cross-channel contamination when assessing the MHC content of the ring canals, oocytes were stained with the anti–MHC serum alone. For DNA staining, fixed egg chambers were incubated 5 min in 1 μg/ml DAPI (4, 6-diamidino-2-phenylindole) in PBT. Samples were routinely mounted in Citifluor (UKC Chemical Laboratory, Canterbury, UK) and viewed using dry lenses at 20 and 40× magnification.

Microscopy

Phase-contrast and fluorescent images were viewed using a Microphot microscope (Nikon Inc., Melville, NY) fitted with rhodamine, fluorescein, and UV channels for fluorescence microscopy. Confocal images were obtained on a Molecular Dynamics, Inc. (Sunnyvale, CA) confocal unit attached to a Nikon Optiphot microscope. Images were collected, and processed with Adobe Photoshop, adjusting contrast and brightness. Normarsky images were obtained on a Leitz DMRB (Leica Inc., Deerfield, IL) microscope.

Counting Nurse Cells

To reliably determine the number of nurse cells, we counted not physical cells but the number of ring canals, as suggested by reference 45. Ring canals are the structures derived from the incomplete cytokinesis of the cystoblast divisions. The number of ring canals therefore reflects the number of cells. In wild-type egg chambers, there are 15 ring canals linking 16 cells, and their distribution reflects the lineage of the cells from the original cystoblast. The first two cells retain four canals, the next two retain three canals, the next four retain two canals, and the remaining eight cells have just one canal. As described in reference 45, immunostaining of ovaries with antiphosphotyrosine antibodies shows strong staining of all 15 ring canals, as well as a lower level staining on membranes and in cytoplasm. For each egg chamber, we can thus determine the number of cells (equaling the number of ring canals plus one), and the number of nurse cell nuclei stained with DAPI.

Results

Cystocyte Proliferation and Oogenesis in sqh Null Mutant Egg Chambers

sqh^{AX3} is a null allele of the *sqh* gene in which a 5-kb deletion has removed most of the *sqh* transcription unit and all of the coding sequences the RMLC (Jordan, P., and R. Karess, manuscript in preparation). Most mutant sqh^{AX3} individuals die during the first larval instar and have no detectable RMLC by Western blot (Fig. 1, *bottom*). Using the method of Chou and Perrimon (9), which combines the high frequency inducible mitotic recombination of the yeast FRT-FLP recombinase system with the dominant female sterile technique for identifying germline clones (43), we generated sqh^{AX3}/sqh^{AX3} homozygous germline clones in sqh^+ ovo^{D1}/sqh^{AX3} ovo^+ heterozygous females (see *Materials and Methods*). These clones allowed us to examine the requirement of germline RMLC during oogenesis.

The ovariole is an assembly line in which the most distal (posterior) egg chambers are the oldest and, therefore, the most developed. Within each ovariole is a germarium

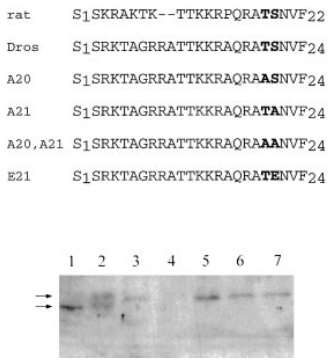

Figure 1. (*top*) The amino terminal sequence of the regulatory myosin light chains of rat aortic smooth muscle myosin (54), Drosophila nonmuscle myosin (25), and the mutants used in this study. The sites of phosphorylation by MLCK and the altered acids are in bold. The phosphorylation of serine-19 of vertebrate RMLC is required for myosin II activity in vitro (see *Introduction*). The spacing and number of the neighboring basic residues known to be required for recognition and phosphorylation by purified MLCK (27, 28) are perfectly conserved between Drosophila and vertebrate RMLC. Moreover, bacterially expressed Drosophila MLCK can phosphorylate chicken smooth muscle RMLC at the activating site (31). We assume therefore that serine-21 is the major site of activating phosphorylation in Drosophila. (*bottom*) Western blot of modified RMLCs encoded by mutant transgenes, using a rabbit anti–Drosophila RMLC antiserum. The lower arrow points to the endogenous wild-type 20 kD RMLC; the upper arrow to the altered RMLCs encoded by the different transgenes, which contain a tag of 10 additional amino acids at the carboxy terminus, and thus migrate slightly slower (see *Materials and Methods*). (*1*) Wild type; (*2*) wild type expressing the transgene *sqh*-A20, A21; (*3*) the null allele sqh^{AX3} expressing the transgene *sqh*-A20, A21; (*4*) sqh^{AX3} alone; (*5*) sqh^{AX3} expressing the transgene *sqh*-E21; (*6*) sqh^{AX3} expressing transgene *sqh*-A21; (*7*) sqh^{AX3} expressing transgene *sqh*-A20. Samples 1, 2, 5, 6, and 7 are extract equivalents of single third instar larvae. Samples 3 and 4 each contain ~20 first instar larvae, since these genotypes do not survive beyond first instar.

containing two or three stem cells, each of which can divide to produce a cystoblast, which then undergoes four further divisions to generate the 16 cystocytes of the egg chamber. Induction of germline sqh^{AX3} clones will cause one or more of these stem cells to become genetically sqh^{AX3}, while the other stem cells remain ovo^{D1} and display the ovo^{D1} phenotype of oogenesis arrest before stage 5 (6).

Clones were induced by heat shock at different stages of development, from first instar to early pupal. However, no clones could be identified in individuals heat treated as first instar larvae (data not shown). Since during the larval period germ cells divide every 24–36 h (62), the failure to obtain germline clones suggests that sqh^{AX3} germ cells possess only a limited capacity to divide, a capacity that is lost upon exhaustion of the available RMLC synthesized before the induction of the homozygous mutant clone, a phenomenon known as perdurance. sqh^{AX3} clones induced later, and dissected 2 h posteclosion, did produce a few highly abnormal developing egg chambers (Table I and Fig. 2). In such clone-bearing ovarioles, very few egg chambers had the normal complement of 15 nurse cells plus oocytes. Indeed, 64% of the egg chambers had fewer than half the normal number of nurse cells and, most strikingly, 24% of the egg chambers were comprised of just one or two large cells (Fig. 2, *A–D*), apparently nurse cells, with large nuclei. Most egg chambers (60%) had at least one, and often several, clearly binuclear or multinuclear nurse cells (for example, Fig. 2, *E–G*), indicating failure of cytokinesis. The most advanced sqh^{AX3} egg chambers attained the size and the elongated shape expected for stage 11–12; in which, however, the oocyte occupied an abnormally small portion of the volume, and chorion deposition around both oocyte and nurse cells had occurred (Fig. 2, *A* and *B*). These chambers apparently do not develop further because no recognizable later stages were observed and no eggs were laid. Anterior to these chambers were usually found a string of smaller egg chambers with few cells, of a size corresponding to stages 3–4. When stage 8–10 egg chambers were observed, they appeared to have relatively "normal" overall morphology, containing an oocyte and

Table I. sqh Mutants Affect Nurse Cell Number and Cytokinesis

Germline genotype	Total egg chambers scored	Normal	>15 nurse cells	<15 nurse cells	<14 nurse cells	<7 nurse cells	Exactly 7 binucleate nurse cells (three divisons)	Egg chambers with at least one multinucleate nurse cell‡
y w FRT§	81	73	3	5	2	2	0	0
		90%	3.7%	6%	2.5%	2.5%		
sqh^{AX3}	144	4	6	134	131	92	ND	103
		3%	4%	93%	90%	64%		60%
sqh^{AX3}; *sqh*-A20,A21	112	9	7	96	89	58	ND	55
		8%	6%	86%	80%	52%		50%
sqh^{AX3}; *sqh*-A21								
Young clones‖	337	68	19	254	185	1	31	225
		20%	6%	75%	55%	0.3%	9%	67%
Old clones‖	113	3	0	109	103	0	30	105
		2.5%		96%	91%		26%	93%
Total	450	71	22	363	288	1	61	330
		15%	5%	80%	64%	0.2%	13%	73%
sqh^{AX3}; *sqh*-E21	157	124	10	23	0	0	0	14
		80%	5%	15%				9%

*This class appears to be due to perduring effects of the ovo^{D1} protein (see text).
‡The extent of multinucleate nurse cells is understated in the sqh^{AX3} and *sqh*-A20, A21 figures, since the majority of the larger egg chambers had several multinucleate (often trinucleate) nurse cells, while a large fraction of egg chambers containing only one or two nurse cells tended to be mononucleate.
§Wild-type clones induced in *y w FRT*/ovo^{D1} *FRT* females.
‖Young clones are those induced in 3rd instar larvae or early pupae and dissected 7 d after clone induction; old clones were induced in first instar larvae and dissected 10–11 d after clone induction.

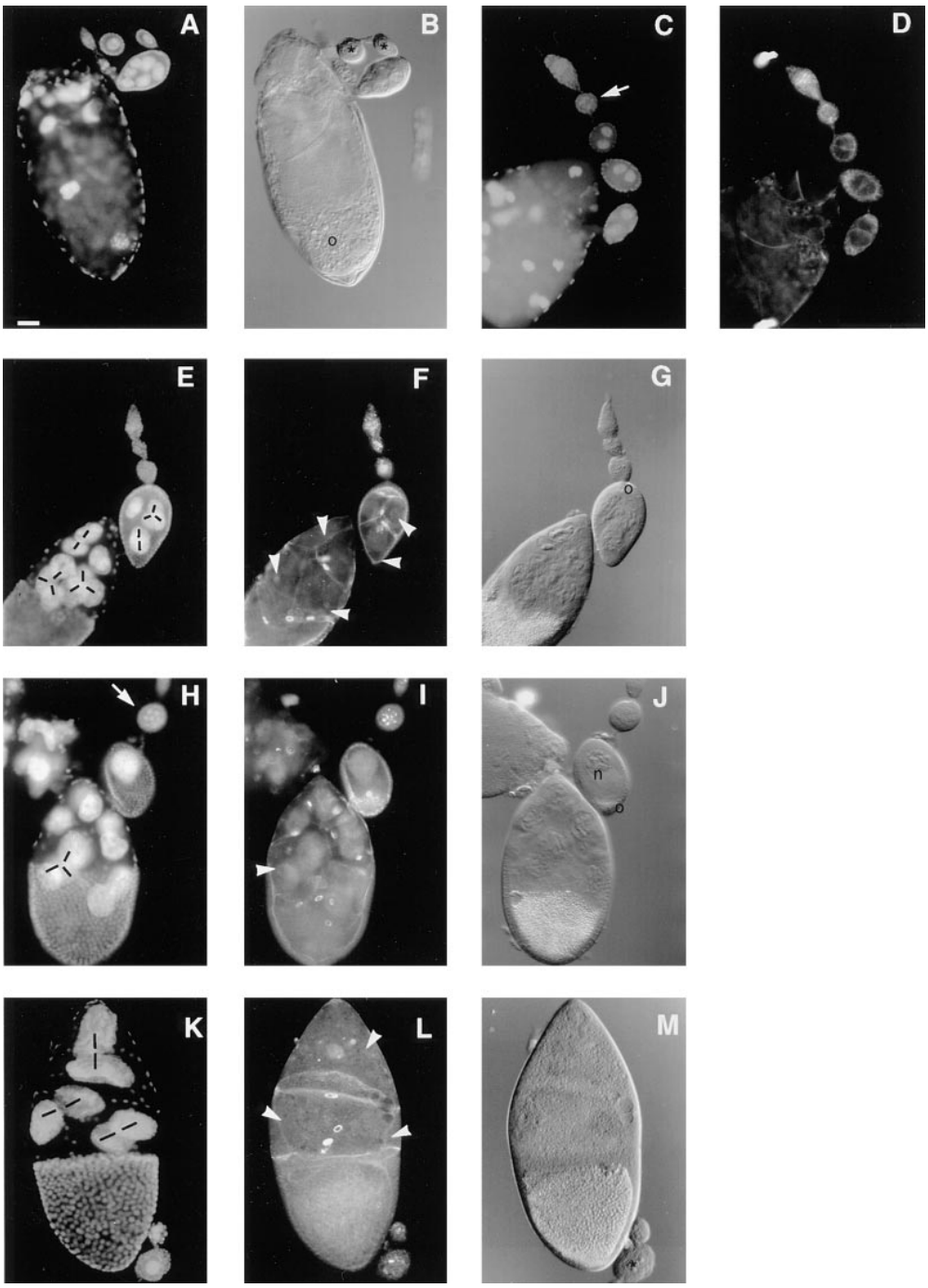

Figure 2. sqh^{AX3} and *sqh*-A20, A21 germ line egg chambers show similar defects in cystoblast proliferation and cytokinesis. (*A–J*) sqh^{AX3} egg chambers; (*K–M*) a *sqh*-A20, A21 egg chamber. (*A*, *C*, *E*, *H*, *K*) DAPI-staining of DNA. (*B*, *G*, *J*, *M*) Normarsky optics. (*D*) Phalloidin staining of F-actin. (*F*, *I*, *L*) Antiphosphotyrosine staining to reveal ring canals. In each example, the younger chambers usually contain only a few nurse cells, usually just one or two, indicated by stars. The small multicellular chambers indicated by arrows are probably derived from genetically ovo^{D1} stem cells (see text). In *B*, note the small volume occupied by the oocyte (*o*) in the single late-stage chamber. In *E–M*, multinuclear nurse cells are apparent (some are indicated with white arrowheads in *F*, *I*, and *L*), indicating cytokinesis failure. Nuclei sharing a single cell are marked with short lines in *E*, *H*, and *K*. In *E–G*, the central egg chamber has reversed polarity, with the oocyte positioned anteriorly, towards the germarium. In *H–J*, the central egg chamber is composed of a single nurse cell (*n*) and a single oocyte (*o*), accumulating yolk. In *K–M*, a single late stage egg chamber consists of three binuclear nurse cells. For wild type, see Fig. 3, *A* and *B*. Anterior is up in all frames. Bar, 35 μm.

well-organized nurse cells despite the reduction in nurse cell number. A few two-cell egg chambers even contained a differentiated oocyte (Fig. 2, *H–J*) linked by a single ring canal to a single polyploid nurse cell. In 5% of the egg chambers, polarity with respect to the germarium was reversed. That is, the oocyte was found at the anterior of the egg chamber (proximal to the germarium, Fig. 2, *E–G*). The follicles cells (somatically derived and therefore sqh^{+}) always migrated normally to cover the oocyte regardless of its location within the chamber. In ovaries of older females (dissected >5 d posteclosion), there was no evidence of new egg chamber production; only old, degenerating egg chambers and ovo^{D1} chambers could be found (not shown), again suggesting that the supply of perduring RMLC had been exhausted.

Interspersed with the sqh^{AX3} egg chambers containing only one or two nuclei were occasional early chambers with near-normal numbers of cells and nuclei (Fig. 2, *C* and *H*). These are most likely to be genetically ovo^{D1} cysts derived from a germ cell of the same germarium, but which had not sustained a mitotic crossover. A small fraction of the egg chambers contained more than 15 nurse cells (Table I). These and similar egg chambers found in the other *sqh* alleles examined (see Fig. 3, *G–J*), we believe, are related to a perduring effect of ovo^{D1}-expressing transgene (see *Materials and Methods*).

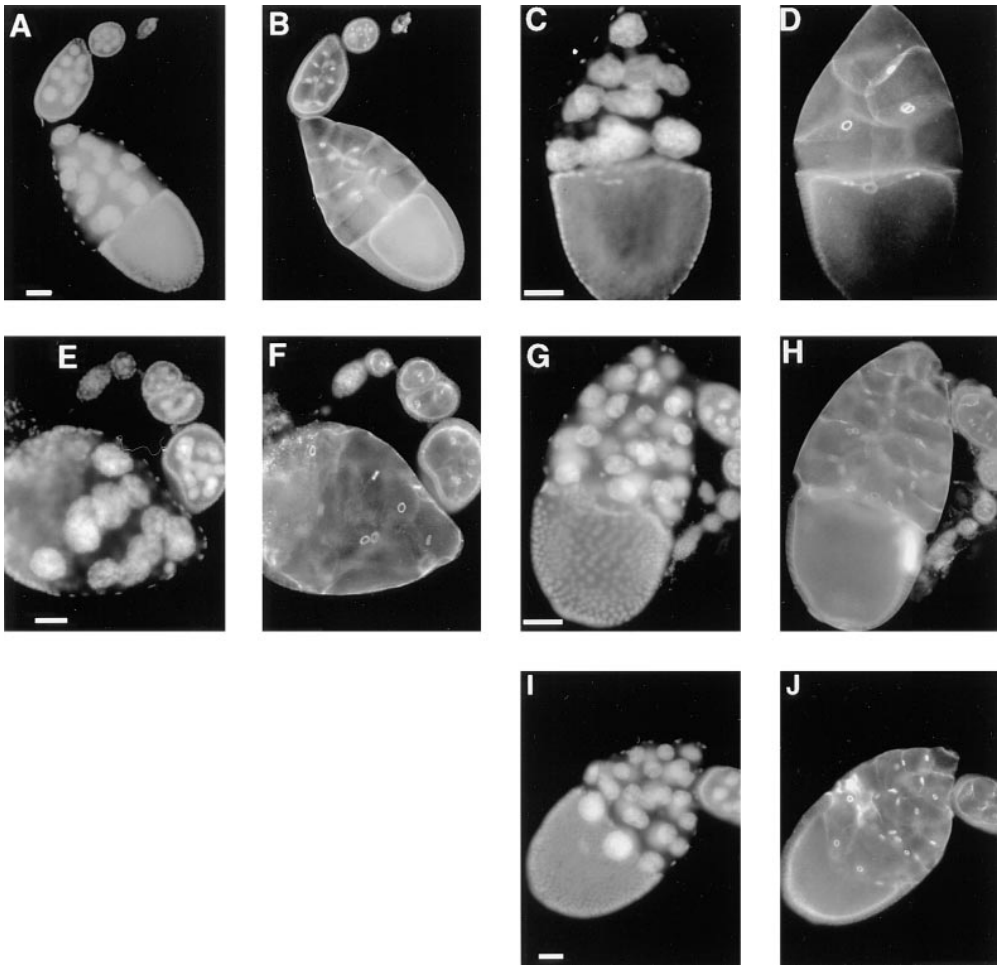

Figure 3. Egg chambers expressing RMLC-A21 show mild defects in cytokinesis. (*A* and *B*) Wild-type egg chambers, stages 4, 7, and 10. (*C* and *D*) A stage 10 *sqh*-A21 egg chamber containing seven binuclear nurse cells (not all visible in the photo), probably resulting from a uniform failure of the fourth round of cystoblast cytokinesis. (*E* and *F*) A *sqh*-A21 egg chamber with ~14 nuclei and 10 cells. (*G* and *H*) An egg chamber from a *sqh*-A21 clone containing approximately twice the normal number of nurse cells and nuclei. (*I* and *J*) A similar hypertrophic egg chamber from a wild-type clone induced in an ovo^{D1}/+ heterozygote female. Such egg chambers probably result from the perduring effects of the neomorphic OVO^{D1} mutant protein (see *Materials and Methods*). (*A*, *C*, *E*, *G*, *I*) DAPI staining of DNA. (*B*, *D*, *F*, *H*, *J*) antiphosphotyrosine staining to reveal ring canals. Anterior is up in all frames (except *E* and *F* where anterior is to the left). Bar, 35 μm.

Establishing sqh Mutant-transformed Lines

To test the in vivo importance of the activating phosphorylation sites for myosin function, we constructed four different modified RMLCs by directed mutagenesis (Fig. 1, *top*). In the first, both threonine-20 and serine-21 (equivalent to T18 and S19 of vertebrate RMLC) were replaced by alanines (transgene *sqh*-A20, A21, encoding protein RMLC-A20, A21). A second construct (*sqh*-A20) eliminated only T20, the secondary site of MLCK phosphorylation. In the third construct (*sqh*-A21), only S21, the primary target of MLCK phosphorylation, was substituted by alanine. Finally, in *sqh*-E21, the serine was substituted by a glutamate, which, because of its charge and shape, can sometimes mimic phosphorylated serine (17, 24, 37, 51). Each construct was used to establish several transgenic lines by P element-mediated germline transformation, and by the appropriate crosses, the transgenes were introduced into flies of genotype sqh^{AX3}.

Expression of the altered RMLCs encoded by the different transgenes was confirmed by Western blot (Fig. 1, *bottom*). Because the transgenes each carried an additional 10 amino acids at the COOH terminus (see *Materials and Methods*), the endogenous wild-type RMLC and the altered RMLCs encoded by the transgenes could be distinguished by their electrophoretic mobility (Fig. 1, *bottom*, *lower* and *upper arrows*, respectively). All the transgenic RMLCs are expressed at roughly similar levels. The endogenous wild-type RMLC, migrating slightly faster (Fig. 1, lanes *1* and *2*), is not detectable in the sqh^{AX3} mutant background (Fig. 1, lanes *3–7*).

Serine-21 but Not Threonine-20 Is Required for Normal Myosin Function

In the initial complementation tests between the mutated transgenes and the null sqh^{AX3}, it was found that the mutant transgene *sqh*-A20 alone among the four constructs tested could fully rescue the lethal phenotype of the null mutation, allowing the establishment of a Drosophila line in which all of the RMLC was of the mutant form. Such flies being entirely wild type, we conclude that T20 is not normally essential to RMLC function in Drosophila, neither in oogenesis nor in any other aspect of development. In contrast, none of the other transgenes was capable of complete rescue. *sqh*-A21 allowed development of otherwise sqh^{AX3} individuals just to the early pupal stage, *sqh*-E21 to the late pupal stage, and *sqh*-A20, A21 died as first instar larvae, the same lethal stage as seen for sqh^{AX3} alone. These results suggested that serine-21 is essential for normal myosin function. (A full description of the zygotic phenotypes will be published elsewhere, Jordan, P., and R. Karess, manuscript in preparation.)

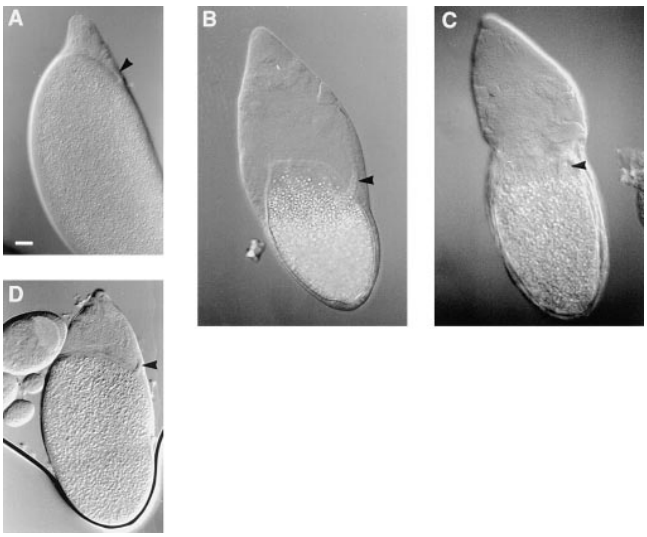

Figure 4. Failure of rapid cytoplasmic transport in egg chambers expressing RMLC-A21 or RMLC-E21. (*A*) Wild-type stage 13 egg chamber. (*B* and *C*) Two stage 13 egg chambers in *sqh*-A21 germline clones. Note the large fraction of cytoplasm retained in the nurse cells. (*D*) A stage 13 *sqh*-E21 egg chamber. In general, dumping is less severely affected in such clones. Arrowheads indicate growing dorsal appendages, a landmark for stage 13. Anterior is up in all figures. Normarsky optics. Bar, 70 μm.

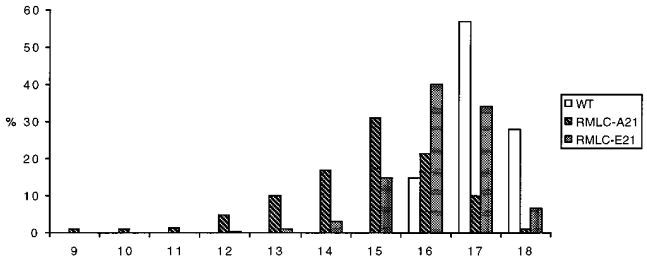

Figure 5. Distribution of egg length in wild type and *sqh* mutant germline clones. Abscissa, length of egg in arbitrary units. Ordinate, percent of total eggs laid. Eggs counted: wild type, $n = 265$; RMLC-A21, $n = 905$; and for RMLC-E21, $n = 1{,}197$.

Activating Phosphorylation Sites Are Required for Proper Oogenesis

To examine the need for the activating phosphorylations during oogenesis, we induced germline clones of sqh^{AX3} in the presence of each of the *sqh* transgenes encoding the modified RMLC proteins. Thus, except for perduring wild-type myosin, these germline clones should contain exclusively the mutant form of RMLC.

Germline clones expressing RMLC-A20, A21 proved to have phenotypes nearly indistinguishable from those of sqh^{AX3} (compare Fig. 2, *A–J* and *K–M*). The egg chambers displayed a similar range of nurse cell number and suffered similarly from apparent failures of cystocyte division (*Fig. 2, K–M*, and Table I), and again the oocyte was frequently abnormally small. In addition, early induction of the clones (during the first larval instar) led to a similar failure to obtain identifiable egg chambers distinguishable from the background of ovo^{D1}, suggesting that these mutant clones, like sqh^{AX3}, rapidly become unable to proliferate. One feature of *sqh*-A20, A21 clones, however, was different from sqh^{AX3}: the distribution of myosin heavy chains (see below). Thus, RMLC-A20, A21 retains very little, if any, functionality, suggesting that phosphorylation of either T20 or S21 (or both) are required for myosin activity in vivo.

Unlike the sqh^{AX3} and *sqh*-A20, A21 germline clones, which rapidly lost their capacity to generate cystoblasts, mutant egg chambers expressing the transgene *sqh*-A21 could be induced by heat shock at any postembryonic developmental stage, and such germ cells continued to produce egg chambers for up to 2 wk. Moreover, flies bearing *sqh*-A21 clones laid eggs, some of which even initiated embryogenesis (see below). The morphology of *sqh*-A21 egg chambers was grossly normal, with a well-differentiated oocyte and accompanying nurse cells. However, 73% of the egg chambers had at least one binuclear cell, indicating an elevated frequency of cytokinesis failure (Fig. 3, *C–H*, and Table I). Cells containing more than two nuclei were rare. 13% of the chambers consisted of exactly seven binuclear nurse cells and one oocyte (Fig. 3, *C* and *D*), strongly suggesting that all the cystoblasts had failed the fourth round of cytokinesis (Table I). 80% of the *sqh*-A21 egg chambers contained <15 nurse cells, and half had <15 nurse cell nuclei. This reduction presumably reflects either nuclear fusion within a binuclear cell or mitotic failure during cystoblast division or both (Fig. 3, *E* and *F*). As with sqh^{AX3} and *sqh*-A20, A21 cones, there was some evidence for perdurance of wild-type sqh^{+} activity, since older *sqh*-A21 clones tended to have a slightly more severe phenotype than younger clones (Table I).

Before stage 10, the general morphology of the *sqh*-A21 egg chambers was relatively normal, despite the variations in nurse cell number mentioned above. However, in many older *sqh*-A21 egg chambers, substantial nurse cell cytoplasm remained untransferred even by stage 13. (Fig. 4, *B* and *C*; compared with wild-type stage 13 oocyte, *A*). Normally, by stage 11–12, the entire cytoplasmic content of the nurse cells is transferred to the oocyte in a process called fast transport or "dumping" (11). Thus, in those *sqh*-A21 clonal egg chambers, fast transport apparently did not occur or was incomplete. Nevertheless, the oocytes often finished development, and some eggs were oviposited. Approximately 70% of the laid eggs were smaller than normal (Fig. 5). This probably represents an underestimate of the dumpless phenotype since the most severely effected oocytes appeared not to be oviposited, and were seen only upon dissection.

Approximately 23% of the eggs laid by *sqh*-A21 clone-bearing females, fertilized by males homozygous for a sqh^{+} transgene (and thus zygotically wild type), began development (based on the production of cuticle), but these embryos never hatched (Table II). Normally, the first three mitotic divisions after fertilization occur in the anterior third of the egg. During the next four division cycles, the cloud of nuclei expands towards the posterior in a process referred to as "axial expansion," which depends on a functional actin network (3, 21, 58, 67). Subsequently, the nuclei migrate outwards towards the cortex during mitoses 8 and 9, and uniformly populate the blastoderm surface by cycle 10 in a microtubule-dependent process (16, 26). Nu-

Table II. Viability of Individuals Derived from sqh Germline Clones

Genotype of germline	Number of eggs tested	Percentage of eggs initiating development	Percentage of eggs hatching	Percentage of larvae producing adults
wild-type (yw^{67})	338	ND	90.3	77.5
sqh-A21	986	23.5	0	0
sqh-E21	1,556	60.4	41.2	46

Clone-bearing females were mated with wild-type males homozygous for a sqh^+ transgene; the embryos all being thus genotypically sqh^+.

clear migration in *sqh*-A21 eggs, by contrast, was often isotropic, producing a spherical cloud of nuclei, with the result that within the ellipsoid of the egg, many nuclei quickly reach the subcortical surface at the anterior of the egg, while the posterior end of the egg remained devoid of nuclei (Fig. 6, *C* and *D*). Even at later time points, the nuclei in *sqh*-A21 embryos rarely reached the posterior end of the egg. Such a phenotype resembles that seen in sqh^1 clonally derived eggs (61), and eggs treated with cytochalasin (21). Thus, the myosin involved in executing axial expansion is not properly functioning in the *sqh*-A21 clones.

In summary, the phenotype of mutant *sqh*-A21 revealed defects in a number of myosin-dependent processes. However, compared to the sqh^{AX3} null mutation and *sqh*-A20, A21, this transgenic allele clearly retained considerable activity: it permitted cystoblast generation and oogenesis to continue for many days, even producing eggs capable of initiating embryogenesis; and it provided a level of myosin activity sufficient to execute cytokinesis, if unreliably.

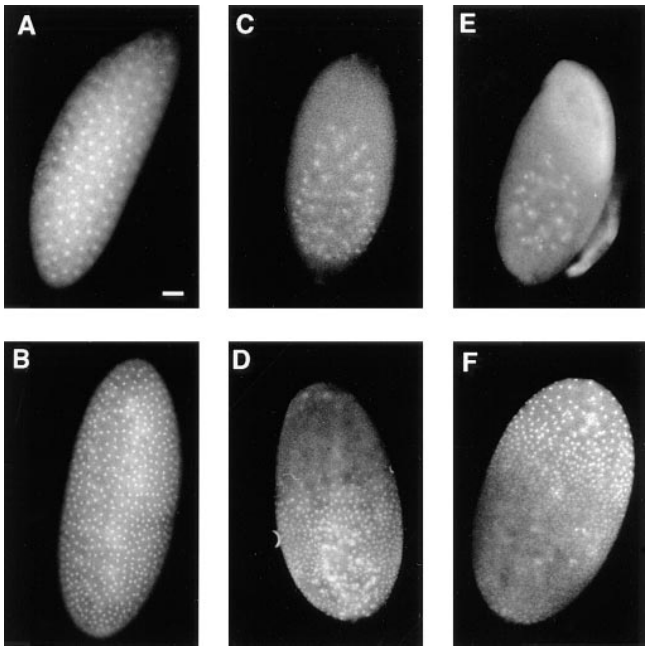

Figure 6. Axial expansion of syncytial nuclei in embryos derived from *sqh* mutant clones. (*A* and *B*) Wild-type embryos. (*C* and *D*) Two embryos derived from *sqh*-A21 germline clones. (*E* and *F*) Two embryos derived from *sqh*-E21 germline clones. In *sqh*-A21 embryos, the posterior end rarely fills with nuclei (*D*). *sqh*-E21 embryos are also defective in axial expansion, but usually such embryos eventually populate the entire subcortical surface with nuclei (*F*). Nuclei are stained with DAPI. Bar, 70 μm.

Glutamate Substitution at Position 21 Has a Relatively Benign Effect on Myosin Function in Oogenesis

If in the above studies RMLC-A21 failed to function normally because it could not be adequately phosphorylated, then substituting an acidic amino acid residue at position 21 might conceivably mimic the electrostatic interactions normally involving phosphoserine, and thus confer greater functionality to the light chain. Therefore *sqh*-E21 was generated and expressed in sqh^{AX3} germline clones. In fact, *sqh*-E21 egg chambers were considerably healthier than *sqh*-A21. Approximately 80% of such egg chambers appeared to be entirely wild type. Some 15% contained fewer than the expected 15 nurse cells, but there were always more than 12, and only a few cysts (9%) had any binuclear cells, indicating that mitosis and cytokinesis failure during cystoblast division was relatively rare (Table I).

sqh-E21 egg chambers presented a weak dumpless phenotype. Stage 13 egg chambers containing significant amounts of nurse cell cytoplasm were relatively common (Fig. 4 *D*), but the average size of oviposited eggs was larger than in *sqh*-A21 chambers (Fig. 5), with 80% of them indistinguishable in length from wild type. This result indicates that the rapid cytoplasmic transport of stage 11 is reduced, but is nonetheless more efficient than in *sqh*-A21 egg chambers.

Approximately 60% of the *sqh*-E21 eggs initiated development when fertilized by sperm carrying a wild-type *sqh* transgene. Axial expansion was slightly retarded in these eggs (Fig. 6 *E*), but compensatory divisions apparently filled the syncytial blastoderm, since in late time points nuclear distribution appeared close to normal (Fig. 6 *F*). Of the developing embryos, 41% hatched. Of these larvae, 46% reached fertile adulthood, independent of the zygotic presence of the *sqh*-E21 transgene. Thus, maternally supplied myosin II containing RMLC-E21 functions well enough to often complete all aspects of early embryogenesis before the onset of zygotic RMLC expression.

Actin- and Myosin-containing Structures in sqh Mutant Clones

Myosin II was localized in egg chambers by using a polyclonal antibody raised against a portion of the myosin heavy chain tail. In wild-type stage 10 egg chambers, the myosin staining is associated primarily with the subcortical actin, but not with the transverse actin filaments, nor with the ring canals (Fig. 7; reference 61, but see also reference 15). The follicle cells, which completely envelope the egg chamber, are also strongly staining.

In sqh^{AX3} egg chambers, the anti–MHC antibody stained both the cortex and a heterogeneous population of particles similar to those observed in sqh^1 and sqh^2 mutant cells (15, 61), where they were interpreted to be nonfunctional aggregates of myosin II, a consequence of lacking regulatory light chain. Most of these particles were found at the surface of the nurse cells, not associated with actin (Fig. 7 *B*). In contrast, *sqh*-A21 (Fig. *G*), *sqh*-E21 (data not shown), and most importantly *sqh*-A20, A21 (Fig. 7, *E* and *F*) egg chambers showed only the cortical staining and displayed none of the MHC-containing aggregates found in sqh^{AX3}. Thus, even though in all other examined aspects the phenotype of *sqh*-A20, A21 resembles that of sqh^{AX3},

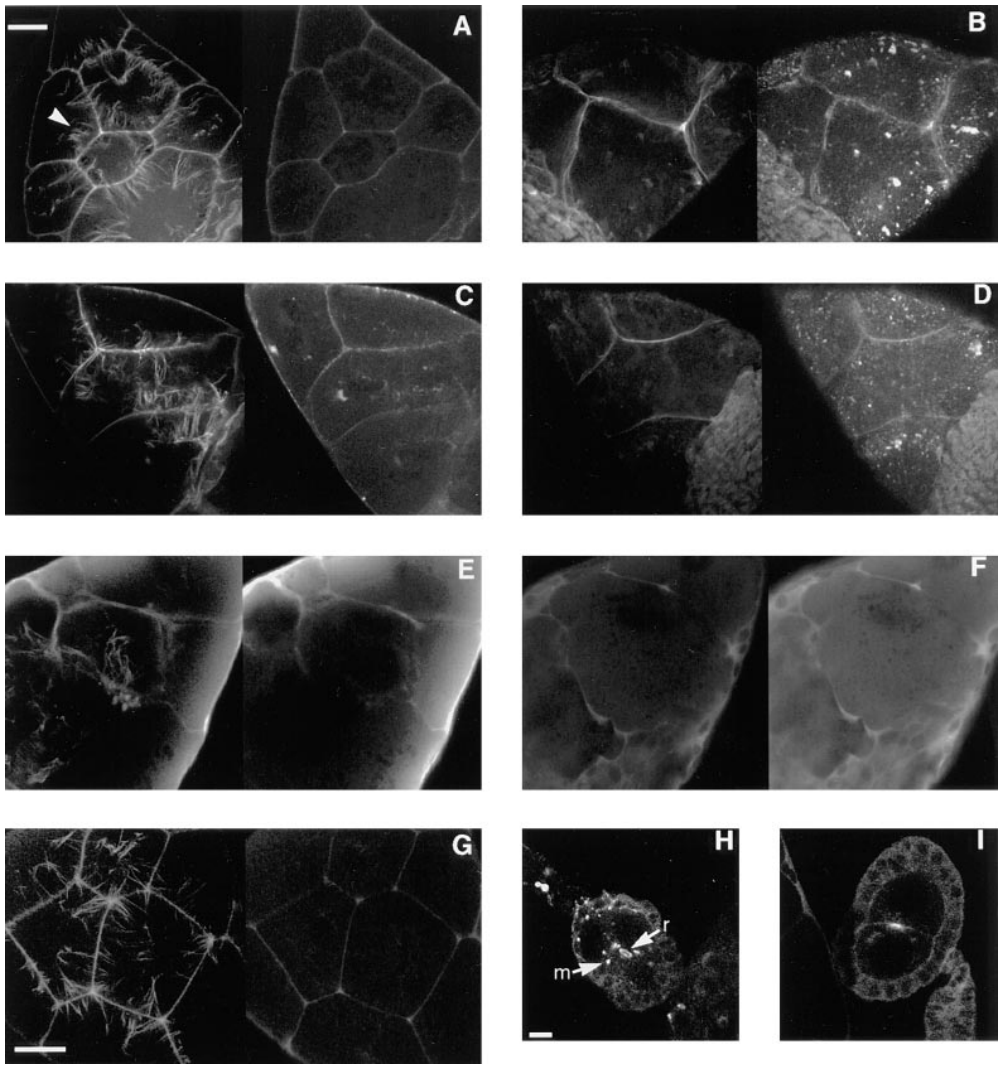

Figure 7. Actin and myosin distribution in *sqh* mutant egg chambers. Confocal images of F-actin (*left*) and myosin heavy chain (*right*) for each pair (*A–G*). (*A*) Wild type, medial view through the nurse cells. Myosin is found primarily in the subcortical cytoskeleton. (*B–D*) Two examples of sqh^{AX3} egg chambers. (*B* and *D*) Surface optical sections; (*C*) medial section. Myosin aggregates are prominent in the subcortical region. The transverse actin filament bundles are present but somewhat disorganized. (*E* and *F*) A *sqh*-A20, A21 egg chamber in medial (*E*) and surface (*F*) views, respectively. No myosin aggregates are visible. (*G*) A *sqh*-A21 egg chamber. Both actin and myosin staining appear normal. (*H* and *I*) Early stage two-cell cysts from a sqh^{AX3} clone (*H*) and a similar two-cell cyst from a sqh^{+} ouo^{D1} ovariole (*I*), stained simultaneously with anti–MHC and antiphosphotyrosine to label ring canals. The myosin aggregates are present in *I*, but absent from *J*. The ring canals seen in *H* and *I* are visible because of cross-channel contamination from the antiphosphotyrosine signal. Actin was stained with rhodamine-phalloidin; myosin with anti–MHC antibody, and fluorescein with secondary antibody. Bar, 50 μm in *A–G*, 10 μm in *H* and *I*.

the doubly mutant light chain can rescue the myosin aggregation phenotype associated with the total or near total absence of RMLC. The myosin particles also allowed us to distinguish two-cell egg chambers found in sqh^{AX3} clones (Fig. 7 *H*) from similar two-cell chambers present in the "background" of genetically sqh^{+} ovo^{D1} ovarioles (Fig. 7 *I*).

Before stage 10B, the major F-actin-containing structures in the nurse cells of wild-type eggs are the intracellular ring canals and the subcortical actin associated with the plasma membranes (19, 60). Starting at stage 10B, thick transverse microfilament bundles assemble forming a "halo" around the nurse cell nuclei and extending towards the plasma membranes (19), apparently serving to anchor the nuclei (10). When visualized with rhodamine phalloidin, the actin filament bundles all appear essentially normal in *sqh*-A21 (Fig. 7 *G*), and *sqh*-E21 (data not shown) egg chambers. Since only the first egg chambers formed after induction of the sqh^{AX3} or *sqh*-A20, A21 stem cell can reach something close to stage 10B, the transverse microfilament bundles were more difficult to observe in these genotypes. Filament bundles were nevertheless present (Fig. 7, *C* and *E*), although they sometimes lacked the perinuclear organization of the wild type. In some cells, the nuclei appeared to hug the posterior surface of the nurse cells, as if they were not properly anchored (not shown).

Ring canals are structures derived from the contractile rings of cytokinesis that gave rise to the nurse cells and the oocyte. They are primarily composed of actin, but a number of other protein components have been identified, including the products of the *hts* gene (45), the *kelch* gene (63), and at least one protein rich in phosphotyrosine found in both the inner and outer rings of the canal (45). A wild-type ring canal in a stage 9–10 egg chamber takes the form of a near perfect short hollow cylinder, when stained with antiphosphotyrosine antibody (Fig. 8, *A–C*). The wall

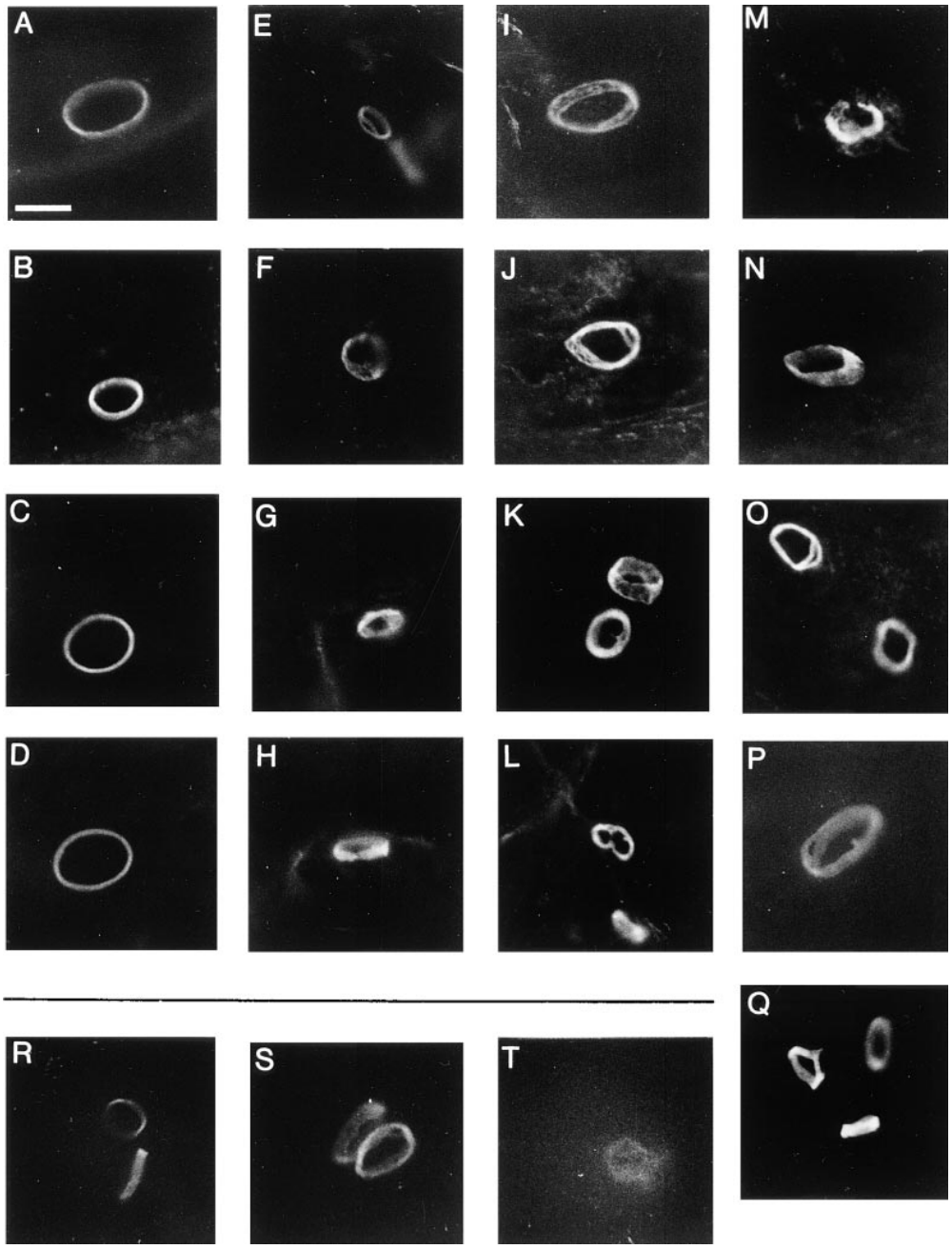

Figure 8. Ring canals are damaged in *sqh* mutant egg chambers. (*A–C*, and *R*) Wild type; *D–F*, *S*, and *T*) *sqh*-A21; (*G–H*) *sqh*-E21; (*I–L*) *sqh*-A20, A21; (*M–Q*) sqh^{AX3}. Mutant ring canals were often stretched, with the fibrous material appearing less compact. Some resembled DNA "replication intermediates," in which the canal material appeared partially torn in half. Although the same range of defects could be found in egg chambers of mutant for any of the four alleles, the frequency of abnormalities was much greater for sqh^{AX3} and *sqh*-A20, A21. The majority of canals in *sqh*-E21 and *sqh*-A21 appeared normal (as in *D*) (see Table III). Ring canals above the line (*A–Q*) are labeled with antiphosphotyrosine. *R* and *S* are labeled with anti–hts; *T* is labeled with anti–kelch. Bar, 5 μm.

of the ring appears relatively uniform by conventional fluorescent microscopy.

In sqh^{AX3} and *sqh*-A20, A21 egg chambers, a majority of the ring canals were deformed (Table III). Most common were canals where the cylinder wall appeared stretched at one or more points (Fig. 8, *K–N*, *P*, and *Q*). In many rings, the walls appeared wrinkled and the height of the cylinder was greater than in wild type (e.g., Fig. 8, *K* and *N*). This seemed to be accompanied by a fraying of the cylinder wall, as a result of which individual fiber bundles could be resolved (Fig. 8, *I–K*, and *N*). At times, the walls were partially torn in half, giving a "DNA-replication intermediate" aspect to the ring canals (Fig. 8, *J*, *O*, and *P*). Some rings were associated with diffuse jets of phosphotyrosine-containing material not seen in wild type (Fig. 8 *M*). Occasionally, a ring would become diffuse and broad (as in the *sqh*-E21 canal of Fig. 8 *G*), although in general the canals did not appear occluded when stained with phosphotyrosine or actin (not shown).

Many of these abnormalities were also found in the egg chambers of the weaker alleles, *sqh*-A21 (Fig. 8, *E* and *F*), and *sqh*-E21 (Fig. 8, *G* and *H*), although the majority of canals in these mutants appeared normal (Table III, and Fig. 8 *D*). In general, fraction of abnormal canals corresponded to the relative severity of the alleles based on other phenotypes. However, the extremely damaged canals (such as Fig. 8 *G*) and the "replication intermediate" forms (e.g., Fig. 8, *E* and *F*) were only rarely seen in the weaker alleles. In more advanced egg chambers (stage 9 and beyond), the ring canals had a greater tendency to ap-

Table III. Ring Canal Abnormalities in sqh Mutant Egg Chambers

	sqh-E21		*sqh*-A21			
	Below stage 9	Stage 9-10	Below stage 9	Stage 9-10	sqh^{AX3}	*sqh*-A20,A21
Total chambers examined	1,360	846	499	382	550	707
Percent normal	88	80	86.5	66	39	24
Percent abnormal	12	20	13.5	34	61	76

pear damaged (Table III). Abnormal ring canals appeared to still contain both kelch (Fig. 8 *T*) and hts (Fig. 8 *S*) proteins, and again revealed a diffuseness and broadening of the ring wall. Since both of these proteins are added after the initial formation of the canal (45), their presence on *sqh* mutant ring canals suggests that at least this aspect of ring maturation had occurred properly.

Discussion

The principal finding of this study is that the presumed sites of activating phosphorylation on the RMLC of Drosophila cytoplasmic myosin II are indeed critical to myosin function in vivo. If only the primary phosphorylation site (serine-21) is removed, myosin activity is reduced but not eliminated. Substituting a glutamate for this serine substantially restores activity. If both the major and minor phosphorylation sites are removed, the phenotype is nearly indistinguishable from that of a true null mutation. We have used the various mutant RMLCs to examine more carefully the requirements of myosin II activity during Drosophila oogenesis.

RMLC Phosphorylation Sites Are Required for Myosin Activity

Numerous biochemical studies have established that phosphorylation of the activating serine of RMLC is required for significant actin-activated APTase activity and in vitro motor activity of purified myosin II (reviewed in references 47, 52). Moreover changes in RMLC phosphorylation in vivo correlates with many events in nonmuscle cells, such as cytokinesis (64) stimulated exocytosis (8), and platelet activation (1, 12). However, there has been little direct testing of the requirement for RMLC phosphorylation in vivo in nonmuscle cells. Indeed, this question has only been directly addressed genetically for Dictyostelium myosin II, where Ostrow et al. (39) came to the surprising conclusion that RMLC phosphorylation was largely dispensable for Dictyostelium viability and development. In particular, they found that cells expressing only a nonphosphorylatable form of RMLC were capable of cytokinesis, although at a slightly reduced efficiency.

We have taken a genetic approach similar to that of Ostrow et al. (39) to evaluate the importance of RMLC phosphorylation for specific events in Drosophila oogenesis. However, Drosophila does not lend itself to biochemical characterization of the mutant myosins as does Dictyostelium. Thus, while we can discuss the genetic activity of the light chain mutations, we can only surmise the corresponding biochemical activity. Nevertheless, the fact that the Drosophila nonmuscle MHC (29) and RMLC (25) primary structures are so similar to those of their vertebrate homologues, and that Drosophila MLCK has been shown to phosphorylate vertebrate nonmuscle RMLC (31) on the residues corresponding to T20 and S21, makes it very likely that Drosophila nonmuscle myosin II responds to phosphorylation in a manner essentially similar to that of vertebrate nonmuscle myosin II.

The four *sqh* mutant transgenes tested here can be arranged in an allelic series of increasing severity: sqh^{+} = *sqh*-A20 > *sqh*-E21 > *sqh*-A21 > *sqh*-A20, A21 ≈ sqh^{AX3}. In addition, sqh^{AX3} germline clones display an increasingly severe phenotype as the residual preinduction wild-type RMLC is exhausted in the stem cell. From the failure to detect sqh^{AX3} clones induced in first instar larvae, we conclude that the most severe phenotype is probably stem cell lethality, or at least inability to divide. The most severely affected sqh^{AX3} egg chambers still distinguishable from the ouo^{D1} background are comprised of just one or two cells with a poorly formed ring canal and brightly staining MHC-containing particles.

The *sqh*-A20, A21 mutant behaves in most respects identically to the null sqh^{AX3}. This suggests that a light chain lacking both of the two potential activating phosphorylation sites is incapable of activating myosin. Although it might be argued that this particular transgene is failing to complement sqh^{AX3} for artifactual reasons, such as poor expression or instability of the modified protein, or because of reduced affinity for the heavy chain, we believe this is unlikely. Firstly, the protein RMLC-A20, A21 is easily detected in Western blots, both in wild-type and in sqh^{AX3} backgrounds (Fig. 1, *bottom*, lanes *2* and *3*), at levels comparable to those of the other transgenes and the endogenous RMLC. Secondly, biochemical studies have shown that MHC with an unoccupied light chain binding site (the IQ domain) tends to aggregate in vitro (56), but that substantial alterations to the structure of the RMLC are still compatible with binding to the MHC (for example, see references 46, 56). The carboxy half of RMLC alone is capable of binding, and in doing so suppresses the aggregation of MHC (56). Nor have in vitro analyses of nonphosphorylatable mutant forms of RMLC revealed any reduced affinity for MHC (5, 51). Finally, and most compellingly, the phenotype of the transgene *sqh*-A20, A21 does differ in one crucial respect from the phenotype of sqh^{AX3}: the myosin heavy chain does not form in vivo aggregates. This last observation argues that not only is RMLC-A20, A21 expressed, but it is binding to its correct site on the heavy chain IQ domain.

Thus, unlike the situation in sqh^{AX3}, sqh^{1} (25), or sqh^{2} (15), the phenotype of *sqh*-A20, A21 cannot be explained by a simple precipitation of the myosin motor in inactive clumps. As well as demonstrating that the mutant RMLC A20, A21 must be binding the MHC in vivo, these results argue strongly that phosphorylation on at least one of the

activating sites is indeed essential for myosin activity in vivo, unlike the situation in Dictyostelium.

Yet *sqh*-A20 is entirely wild type, and *sqh*-A21, though clearly mutant, retains considerable activity by comparison to *sqh*-A20, A21 since the mutant clones continue to proliferate (with some failure of cytokinesis) and generate oocytes. In vertebrate smooth muscle cells, phosphorylation at T18 of RMLC is rare in vivo under physiological conditions (52). Diphosphorylation at T18 and S19 does, however, strongly correlate with the rate of stimulated exocytosis in a basophilic cell line (8), and thus may participate in specific myosin-dependent cellular events. However, it would appear that in Drosophila at least this particular phosphorylation site is normally dispensable. The substantial difference in phenotype between *sqh*-A21 and *sqh*-A20, A21 is most simply explained by assuming that in the RMLC-A21 protein the threonine at position 20 has become critical to myosin function, most likely by becoming the sole target of phosphorylation by MLCK.

The relatively weak phenotype of the *sqh*-E21 egg chambers, as well the normal development of many of the embryos derived from those clones, shows that this light chain variant functions substantially better than *sqh*-A21. It seems plausible that both RMLC-E21 and RMLC-A21 can be phosphorylated in vivo at threonine-20. Perhaps, then, the improved activity of RMLC-E21 is due to the presence of two sets of negative charges at the two adjacent positions, which better approximates the state of a wild-type RMLC phosphorylated on serine-21. The biochemical studies of mutated vertebrate RMLCs support this interpretation. RMLC-E19, in which glutamate substituted for serine at position 19 (the site of the activating phosphorylation in vertebrate light chain) had no significant actin-activated ATPase activity or motor activity on its own (51). Once phosphorylated at the adjacent threonine-18, however, this mutant light chain conferred near-wild-type velocity to myosin in the motility assay (51) and high actin-activated ATPase activity (24). In contrast, myosin containing RMLC-A19 phosphorylated on threonine-18 had relatively little actin-activated ATPase activity; although, surprisingly, it functioned in in vitro motility assays nearly as well as wild type (5).

Germline Requirements of Myosin II

Edwards and Kiehart (15) have recently reported the critical importance of myosin II for the migrations and cell shape changes of the somatically derived follicle cells that envelope the egg chamber, synthesize the chorionic structures of the egg, and provide essential signals for oocyte development. We have focused on myosin's role in the germline-derived nurse cells and the oocyte.

Based on the phenotypes described here and elsewhere (15, 61), it is clear that myosin activity is required for the proliferative capacity of the stem cells, for cystoblast cytokinesis, for the rapid cytoplasmic transport at stage 10, for ring canal integrity, and axial migration of nuclei in the early embryo. However, some of these events appear to have a more stringent requirement for myosin activity than others. Only the strongest mutant alleles, sqh^{AX3} and *sqh*-A20, A21, lead to stem cell death or arrest. The rapid phase of cytoplasmic transport is severely disrupted by *sqh*-A21, but cytokinesis is only mildly affected.

The sqh^{AX3} and *sqh*-A20, A21 germline egg chambers nearly always contain fewer than the full complement of 16 cells. In each ovariole, it is only the first egg chamber produced (the one with potentially the most perduring wild-type RMLC) that might contain 10 or more cystocytes. Younger cysts often contain only one or two cells, and when the clone induction is performed before second instar larvae no sqh^{AX3} clones can be identified. This increasingly severe phenotype is most simply explained by the dilution of perduring preinduction wild-type myosin in the stem cells, which then must cease division altogether if induction is sufficiently early.

The sqh^{AX3} nurse cells of younger clones (before stem cell division ceased) were very often multinuclear, indicating a failure of cytokinesis, but in addition the total number of nurse cell nuclei in sqh^{AX3} egg chambers was nearly always less than the normal 15. The most extreme egg chambers comprised of just one or two nurse cells, for example, rarely had more than four nuclei in total. This may indicate that such sqh^{AX3} cystoblasts cannot sustain four cycles of mitosis, and would be consistent with the apparent failure of clones induced in early larval life to proliferate and generate recognizable egg chambers. However, it is also possible that two daughter nuclei found within a single cystoblast might fuse, as can occur in the mitotically active somatic tissues of sqh^{1} larvae (25). Thus the observed reduction in the number of nuclei in sqh^{AX3} egg chambers may reflect some combination of the two mechanisms.

It is interesting that many aspects of oogenesis continue in the sqh^{AX3} and *sqh*-A20, A21 egg chambers. In cysts containing more than four cells, an oocyte is always present. Although cysts comprised of only two or four cells often had no oocyte, there were some striking examples of egg chambers consisting of only two cells, where one was a well differentiated oocyte with yolk accumulation and the other a nurse cell with a single large nucleus (Fig. 2, *I–K*). Such egg chambers resemble those of insects such as *Chironomous*, in which the egg chamber is naturally composed of just a single nurse cell and the oocyte (described in reference 14). This suggests that myosin II has no essential role in oocyte differentiation, and in addition that differentiation of the oocyte does not depend on the number of cells in the cyst. The phenotype of mutations in the *hu li tai shao* gene bear a superficial resemblance to sqh^{AX3}. In *hts*, cysts also contain less than a full complement of nurse cells and in addition frequently lack an oocyte (33, 66). The hts proteins are components of both ring canals and the fusome, a structure believed to be involved in oocyte specification, and in some alleles of *hts*, the majority of egg chambers with <15 nurse cells also lack an oocyte. By contrast, only the most severely affected sqh^{AX3} egg chambers have no oocyte.

The volume occupied by the oocyte in sqh^{AX3} and *sqh*-A20, A21 egg chambers before stage 10 is often considerably smaller than wild type, indicating a possible defect in the slow phase of cytoplasmic transport to the oocyte. Slow transport before stage 7 depends on microtubules and microtubule-dependent motors, and is insensitive to cytochalasin (19), (reviewed in reference 11). Recently,

Bohrmann and Biber (4), using video-enhanced microscopy, reported that some particle transport towards the oocyte during early vitellogenesis (stages 7–10A) is dependent on an actin–myosin network. It is possible that such transport is affected in the *sqh* mutant clones, indicating a role for myosin II. However, the fact that 70% of the ring canals are visibly damage in sqh^{AX3} egg chambers may contribute to the phenotype by impeding the flow of cytoplasmic components from the nurse cells.

In 5% of the sqh^{AX3} and *sqh*-A20, A21 egg chambers, the oocyte is found at the anterior end (proximal to the germarium), instead of the normal posterior. The reversed polarity is not due to a change in oocyte specification, since the oocyte is still invariably one of the first cells born in the cyst; that is, one of the two cells containing the largest number of ring canals. Normally, within the earliest cysts of the germarium, oocyte is first found at the anterior-most position of the cyst. However, by the time the cyst has left the germarium, the oocyte has already rotated to the posterior size. How this rotation is achieved is unknown. Other mutants showing occasional mislocation of the oocyte include *armadillo* (41) and *spindle-C* (18). In these mutants, however, the oocyte can also be occasionally found in intermediate positions within the egg chamber, something never seen in the *sqh* mutants.

Myosin Phosphorylation and Cytokinesis

Nearly every *sqh*-A21 egg chamber had sustained at least three rounds of cytokinesis, and then failed at least once during the fourth cystoblast mitosis, producing between 7 and 14 nurse cells plus the oocyte. This was true regardless of the time of induction of the *sqh*-A21 clones, indicating that it was not due to perdurance of the wild-type RMLC synthesized in the stem cell before induction of the homozygous mutant. When cytokinesis does fail, the result is a binuclear nurse cell. In the 13% of the egg chambers containing exactly seven nurse cells and the oocyte, each of the nurse cells was binuclear, suggesting that the fourth round of cystoblast cytokinesis had uniformly failed. If so, then the oocyte too ought to be binuclear. The DAPI staining method used here does not always permit visualization of the oocyte nucleus, but we have confirmed that occasional binuclear oocytes do form and even begin development (Jordan, P., R. Karess, and S. Roth, manuscript in preparation).

Thus, it seems that *sqh*-A21 is only mildly defective in cytokinesis, particularly by contrast to the severe cytokinesis defects observed in somatic and germline cells of the genotype sqh^{1}, in which a small amount of wild-type RMLC is expressed (25, 61). We conclude that phosphorylation at serine-21 is required for reliable cytokinesis, but that in its absence, the phosphorylation at threonine-20 of RMLC-A21 still activates adequate myosin II activity for cytokinesis to be successfully executed, albeit with reduced fidelity.

Myosin Phosphorylation and Rapid Cytoplasmic Transport

Myosin II is the molecular motor required for the fast phase of cytoplasm transport ("dumping") from nurse cells to oocyte that occurs at stage 11 (61). More specifically, as shown here, efficient dumping requires the presence of serine-21 in the RMLC. As had been observed for sqh^{1} clones (61), the actin network in the *sqh*-A21 and *sqh*-E21 germline clones is essentially normal, in contrast to the effects in other classes of dumpless mutants, like *chickadee*, *singed*, or *quail*. For those mutants, failure in cytoplasmic transport has its origin in the obstruction of the ring canals by untethered nurse cell nuclei (7, 10, 35, 63). The dumping problem in *sqh*-A21 germline clones is by contrast the consequence of inadequate myosin II activity. These results are consistent with the hypothesis mentioned in reference 61 that dumping is triggered by a signal, perhaps activating the calmodulin-dependent MLCK responsible for RMLC phosphorylation on serine-21, which in turn causes nurse cells to contract.

It is possible, since some *sqh*-A21 ring canals are visibly damaged, that canal obstruction contributes to the dumpless phenotype. However, this is probably only a minor contribution to the overall phenotype because (*a*) the majority of the ring canals appear completely normal (for example, Fig. 8 *D*), whereas the dumpless phenotype is severe and (*b*) even the damaged canals rarely show evidence of channel obstruction. However, more complete examination of the ring canal ultrastructure will be necessary to confirm this.

Myosin Distribution and Actin Cytoskeleton

In a normal egg chamber, myosin II colocalizes with the subcortical actin cytoskeleton (15, 61), at least in post-stage 6–7 egg chambers. In germline clones when the RMLC is absent or greatly reduced, much of the immunostaining with antibody against myosin heavy chain labels the myosin aggregates, but some cortical staining is still evident. When the RMLC is present but mutated at both phosphorylation sites (*sqh*-A20, A21), there is no myosin aggregation and apparently no myosin activity, and yet at least some myosin can still be found in the cortex. These two observations suggest that myosin need not be activated by light chain phosphorylation to be localized to the membrane. It is generally assumed that only active myosin, in the form of bipolar filaments capable of translocating on actin cables, can localize to the cytoskeleton. However, there is accumulating evidence that myosin heavy chain contains other means of associating with the cytoskeleton besides its actin-binding domain. For example, Drosophila MHC can specifically bind p127, a cytoskeleton-associated protein product of the *l*(2)*gl* gene (49, 50), and such a liason could conceivably mediate myosin's subcortical localization independently of the state of RMLC phosphorylation.

At least in the somewhat more developed sqh^{AX3} and *sqh*-A20, A21 egg chamber (Fig. 8), the actin cytoskeleton of nurse cells seems to be present and near normal, especially the subcortical cytoskeleton associated with myosin. More specifically, the transverse filament bundles of stage 10 still assemble, although they appear somewhat disorganized and no longer surround the nurse cell nuclei. Such a perturbation of the actin bundles was also reported by Edwards and Kiehart (15) with their sqh^{2} mutant. Exactly why the bundles are in disarray is not clear. Perhaps there is a small requirement for myosin II to recruit the fila-

ments around the nuclei, or else the sites in the plasma membrane cytoskeleton that normally anchor the bundles may not be properly functioning.

Axial Nuclear Migration and Early Embryogenesis

Axial migration is the movement of the expanding cloud of cleavage nuclei from an anterio-central position to a more uniform distribution along the length of the egg. This process depends on actin (20) and myosin II (61) and it has been proposed that the nuclei are propelled by cycles of solation and contraction linked to the mitotic cycle (58).

The fact that *sqh*-A21 clonally derived eggs display defects in axial expansion confirms the importance of myosin II in this process, and further suggests that the solation–contraction cycle may be induced at least in part by cycles of phosphorylation of the RMLC. The *sqh*-E21 clonally derived eggs have much milder axial migration defects, consistent with the generally weaker phenotype of this allele. In addition, the eggs appear to be capable of compensating for the retarded arrival of nuclei to the posterior surface That 1/3 of *sqh*-E21 eggs fertilized by sperm carrying a wild-type *sqh* allele can develop to normal fertile adults indicates that RMLC-E21 functions sufficiently well for all the myosin II activity required maternally for development. By contrast, none of the 25% of *sqh*-A21 embryos, which begin development and survive to the larval stage even when their zygotic genome contains a wild-type copy of the *sqh* gene.

Myosin II and Ring Canals

The aberrant ring canals of *sqh* mutant nurse cells strongly suggest a role for myosin II in ring canal assembly or maintenance. Electron microscopy of normal mature ring canals reveals two distinct substructures: an electron opaque inner ring, and an electron dense outer ring (45). Actin appears to be the major constituent of ring canals, which also contain the products of the *kelch* (45, 63) and *hu-li tai shao* (45, 66) genes, and a set of proteins phosphorylated on tyrosine (45). The ultrastructural study of Tilney et al. (53) revealed that the actin filaments in mature rings are arranged in bundles and then woven together.

Surprisingly, the available evidence pointing to a role for myosin II in ring canals has been inconclusive. It was reported by Edwards and Kiehart (15) that anti–MHC labels mature wild-type ring canals, whereas we have been unable to detect any myosin in ring canals using either our own antibody or the Kiehart antibody (reference 61, and this study). In their examination of the allele sqh^2, (described as a null or near null, reference 15) and our own earlier study of the weaker allele sqh^1 (61), no evidence for a change in ring canal structure was found. Both of these studies relied on actin staining to visualize the ring canals, while the present study uses antiphosphotyrosine, which is a relatively specific label for the ring canals, and may have allowed us to detect the aberrations. In any case, it would appear that myosin II is unlikely to be a major component of the mature ring canal, and a very small amount of myosin II activity is sufficient to largely preserve its integrity since even in the sqh^{AX3} cells some 39% of the ring canals still appeared quite normal, at least by light microscopy.

Since ring canals are built upon the residual contractile ring structure, one can easily imagine that, where the contractile ring is not entirely normal (though still capable of cleaving a given *sqh* cell) because of inadequate functional myosin II, the ring canal would by consequence be damaged as well. In this scenario, the canal could be "properly" assembled, but upon an improperly formed scaffold, resulting in the observed phenotype. This model is consistent with our failure to detect myosin II in mature canals, and with the observed presence of the kelch and hts proteins, which are added after the initial formation of the ring canal (45, 63). It would predict that ring canal defects should first be visible in the earliest cysts, even in the germarium. Alternatively, the imperfect canals may reflect an absence of rigidity or cross-linking due directly to a lack of functional myosin II, which is indeed present in normal canals despite our failure to detect it. Ring canal diameter and actin content greatly increase between stages 3 and 10 (45, 53). If myosin II is actively required for this growth, one might predict that the defects in the RMLC mutants should become more severe as the egg chambers and ring canals grow. We attempted to address this question in Table III, where early and late stage ring canals were compared. Although there is a tendency for the ring canals of more advanced egg chambers to be more damaged, this may be due simply to the fact that they are larger, and thus abnormalities are easier to spot. More careful examination of early stage mutant ring canals, as well as ultrastructural studies, will be necessary to better understand the defects and perhaps allow these two models to be distinguished.

We thank Annie Glatigny and Janine Crochet for their invaluable help at certain key moments. We thank Lynn Cooley and Dan Kiehart for their antibodies, and for their valuable discussions over the years. We thank Linda Sperling, Maryvonne Mevel-Ninio, and Amanda Simcox for their comments on the manuscript. Thanks to Julie Fradelezi and Elio Sucena for their construction of two transgenes used here, and S. Kulkarni for the *sqh* antibody.

This work was supported in part by an award from the Muscular Dystrophy Association and grant DCB-9019680 from the National Science Foundation. P. Jordan was supported by Le Ministère Nationale de l'Enseignement Superieure, and l'Association pour la Recherche sur le Cancer (Paris, France).

Received for publication 30 May 1997 and in revised form 7 October 1997.

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