Genetic dissection of sperm individualization in Drosophila melanogaster

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Accepted 6 March; published on WWW 22 April 1998

SUMMARY

The morphogenesis of spermatids generally takes place within a syncytium, in which all spermatid nuclei descended from a primary spermatocyte remain connected via an extensive network of cytoplasmic bridges. A late step in sperm maturation therefore requires the physical resolution of the syncytium, or cyst, into individual cells, a process sometimes referred to as sperm individualization. Despite the identification of specialized machinery involved in the individualization of Drosophila spermatids (Tokuyasu, K. T., Peacock, W. J. and Hardy, R. W. (1972) Z. Zellforsch 124, 479-506), and of many Drosophila genes mutable to male-sterile phenotypes, little is known of the mechanisms by which this extensive remodeling of the cyst is accomplished. Here, the identification of a major cytoskeletal component of the individualization complex as actin is confirmed with a simple fluorescence assay. Using rhodamine-phalloidin as a probe, the individualization complex is readily visualized forming around bundles of spermatid nuclei at one end of highly elongated cysts, then translocating along the length of the cysts. The structure of the individualization complex in a male-sterile clathrin heavy chain (Chc) mutant is observed to be reduced or disrupted relative to wild-type, consistent with the individualization-deficient phenotype of this mutant. Using the fluorescence assay, a sampling of male-sterile mutant phenotypes in which spermatogenesis proceeds to the assembly of highly elongated cysts distinguishes at least four different phenotypic classes: (1) mutations (narking class) that block or significantly retard the assembly of the actin-based individualization complex around the nuclear bundle, (2) mutations (dud class) in which the individualization complex assembles in/around the nuclear bundle, but fails to translocate down the cyst, (3) mutations (mulet class) that allow the assembly of a morphologically normal individualization complex around the nuclear bundle, but result in a breakdown in the complex after it begins to translocate down the cyst, and (4) mutations (purity of essence class) that allow the assembly of a motile but morphologically altered or reduced individualization complex. Individualization also fails in a number of mutants with altered nuclear shape, consistent with the hypothesis that spermatid nuclei provide a physical scaffolding for the assembly of the individualization complex. Genetic analysis suggests that a substantial number of additional loci with phenotypes distinguishable with this assay remain to be identified. The large proportion of male-sterile mutations resulting in a late block to spermatogenesis, in which highly elongated cysts fail to be individualized, suggest a substantial susceptibility of this process to a broad range of cellular perturbations. The massive reorganization of cyst cytoplasm required at individualization is expected to be a correspondingly complex function requiring exquisite coordination of multiple cytoplasmic functions, and may account for the previously noted high frequency with which Drosophila genes are mutable to male-sterile phenotypes.

Key words: Spermatogenesis, Individualization, Actin, Syncytium, Membrane, Drosophila

INTRODUCTION

Because spermatzoa are generally constructed and matured within a germline syncytium, the final stages of spermatogenesis require the resolution of the male germline cyst into separate gametes by packaging each spermatid into its own plasma membrane (Kierszenbaum, 1994), a process sometimes referred to as sperm individualization. Despite the apparent conservation of this feature of spermatogenesis, little is known of the cellular mechanisms by which sperm individualization is achieved. Spermatogenesis in Drosophila has been described in considerable detail (for recent review, see Fuller, 1993). The spermatogenic cyst of Drosophila provides an excellent system for the investigation of sperm individualization, because the cysts are readily distinguished from each other and because cysts at all stages of development are present simultaneously in the testis, where their spatial distribution reflects the stage of development. Moreover, the extremely polarized structure of the Drosophila spermatogenic cyst results in a correspondingly polarized yet synchronous
individualization process, the ultrastructure of which has been described in the classic studies of Tokuyasu et al. (1972). Each mature Drosophila cyst contains 64 haploid spermatids descended by 4 mitotic divisions and a meiotic division from the same primary spermatocyte. The mature cyst is an unusually large cell (~2 mm long), with a bundle of 64 needle-shaped nuclei at one end and flagellar tails extending throughout its length. Individualization is accomplished by the assembly of a cytoskeletal-membrane complex at the nuclear end of the cyst, which translocates the length of the cyst, investing each spermatid in its own plasma membrane and simultaneously extruding most of the syncytial cytoplasm from between sperm tails as it proceeds (Fig. 1). This complex, which we refer to as the individualization complex (IC), is a coordinated array of discrete ‘investment cones’ (Tokuyasu et al., 1972), which move synchronously along the spermatogenic cyst, each spermatid being individualized by a single investment cone. The movement of the IC along the sperm bundle causes an observable dilation of the cyst due to the accumulation of cytoplasmic material extruded out from between the sperm tails. The IC is then detached from the mature individualized cyst when it reaches the tail end, where it is referred to as a waste bag (Tokuyasu et al., 1972).

Here we present a simple fluorescence assay for the visualization of the major actin cytoskeletal component of the IC in spermatogenic cysts of Drosophila. A male-sterile clathrin mutation, Chc4, which fails to individualize sperm properly, displays a grossly altered IC distribution and morphology by the fluorescence assay. Applied to spermatogenic cysts of males homozygous for a number of male-sterile mutations that allow the morphogenesis of highly elongated cysts, the assay distinguishes several distinct classes of mutant phenotypes, differing in the assembly, structure and dynamics of the IC. Combined structural, genetic and molecular analysis of these mutations may allow new insights into cellular mechanisms operating in postmeiotic spermatogenesis, including individualization.

MATERIALS AND METHODS

Fly husbandry
Drosophila stocks were maintained at room temperature and grown on standard cornmeal, agar and molasses medium. Fly manipulations were as described by Matthews (1994) and Greenspan (1997). scat/ms(2)30B, ms(2)46C, ms(2)42A and tho186E P insertion mutations, previously described by Castrillon et al. (1993), were obtained from the Bloomington stock center.

ms(2)4210, ms(2) 5970, ms(2) 5720 and ms(2) UK are chromosome 2 mutations generated in the lab of M.T. Fuller by P-element mutagenesis using P[lacW] (Bier et al., 1989) in a white background and maintained using CyO as a balancer chromosome. ms(3)3915 is a chromosome 3 mutation generated in an identical fashion and maintained using TM3 as a balancer chromosome. ms(2) Hb328, obtained via M.T. Fuller, is a chromosome 2 mutation generated in the laboratory of J. Hackstein by chemical mutagenesis (Hackstein, 1991).

Cytology and ultrastructural analysis
Blebbing assay for individualization defects
Testes were dissected from adult males in Drosophila Ringers solution with Pipes (Castrillon et al., 1993), squashed and examined directly by phase-contrast microscopy.

Phalloidin assay for the IC
Testes were removed from freshly eclosed males in TB1 [15 mM potassium phosphate (equimolar di- and monobasic) pH 6.7, 80 mM KCl, 16 mM NaCl, 5 mM MgCl2, 1% PEG 6000] and dissected in TB1 on poly-L-lysine-treated slides to expose spermatogenic cysts. Generally, this involves rupturing the testis at one end or the other and teasing the sperm bundles out with a fine forceps. Tissue samples were flattened under a siliconized coverslip and frozen in liquid nitrogen. Coverslips were flicked off the slides using a razor blade and the samples remaining on the slides were placed in ethanol cooled to −20°C and fixed in 3.7% paraformaldehyde/PBS (130 mM NaCl, 7 mM NaH2PO4·2H2O, 3 mM Na2HPO4·2H2O). The slides were then washed in PBS, blocked in PBTB (PBS, 0.1% Tween, 3% BSA) and incubated for two hours at 37°C in a solution of 6 Units/ml rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR) in PBTB. Slides were then washed in PB, stained with DAPI (1 µg/ml), washed again with PBS and observed under a Nikon microphot SA microscope with epi-fluorescence illumination. A minimum of three independent preparations of each genotype were examined using this assay, with a range of 15 (dud) to 110 (Chc4) squashes prepared from mutant males.

Electron microscopy
Testes were dissected from adult males 2-5 days after eclosion and prepared for electron microscopy as described by Tokuyasu et al. (1972), except that tissues were infiltrated and embedded in LX112 resin (Ladd Research Industries, Burlington, VT). Thin sections were observed and photographed using a Jeol 100CX transmission electron microscope at 80 kV accelerating voltage.

In situ hybridization
Polytene chromosome preparation and hybridization reactions were carried out essentially as described by Pardue et al. (1986). Biotinylated probes specific for the Drosophila white gene were generated by the random primer method (Feinberg and Vogelstein, 1983) using linearized pCasPer4 (Pirotta, 1988) as template and a commercial kit (BrightStar DECAprime-Biotin, Ambion, Inc., Austin, TX). Hybridization signals were visualized using streptavidin-conjugated horseradish peroxidase (Gibco BRL, Gaithersburg, MD). The peroxidase detection reaction was enhanced by the addition of NiCl2 to a final concentration of 0.4 mg/ml.

RESULTS

Actin cytoskeletal dynamics of wild-type spermatogenic cysts
Previously, a dense network of ~6 nm fibrils had been localized to cone-shaped structures that form around each axoneme within the IC (Tokuyasu et al., 1972). Using rhodamine-conjugated phalloidin as a probe, we have confirmed the identity of these fibrils as actin fibers (Fig. 2). Simple preparations of elongated cysts teased out of wild-type testes reveal numerous dense, coherent foci of F-actin staining (Fig. 2A). Many of these signals colocalize with the spermatid nuclear bundles, while some appear to have progressed caudally along the length of the spermatogenic cyst (Fig. 2B). The appearance of these F-actin-rich complexes in testes squash preparations is consistent with the proposed mechanism of the individualization process (Tokuyasu et al., 1972), in which the IC assemblies around the spermatid nuclear bundle...
and subsequently travels caudally along the length of the cyst (Fig. 2C,D). As the IC translocates, it extrudes much of the cyst cytoplasm from between the sperm tails, resulting in a noticeable cystic bulge (Fig. 2E). When the IC has completed the individualization process and reaches the end of the spermatogenic cyst, it is transformed into a detachable ‘waste bag’ (Fig. 2F), the contents of which are presumably degraded within the lumen of the testis (Tokuyasu et al., 1972).

Mutations disrupting the individualization process

Our studies of the sperm individualization process were initially prompted by the finding that a semilethal allele of the Drosophila clathrin heavy chain gene, $Chc^4$, allowed the survival of hemizygous males and homozygous females at significant frequencies (Table 1). $Chc^4/Y$ males are invariably sterile, although $Chc^4/Chc^4$ females do produce some viable eggs (Bazinet et al., 1993). Spermatogenesis is therefore the only event in the Drosophila life cycle absolutely blocked by the $Chc^4$ mutation. This suggests either a specialized role for clathrin-based transport in spermatogenesis, or extreme sensitivity of some aspect of spermatogenesis to cytoplasmic disorganization caused by defective vesicle trafficking.

Squash preparations of $Chc^4/Y$ mutant testes examined by phase-contrast microscopy reveal numerous elongated cysts but a complete absence of motile sperm. When cysts are disrupted in such preparations, no mature individualized sperm

![Fig. 1. Individualization of Drosophila Spermatogenic cysts.](image)

Four mitotic divisions and a meiosis from each primary spermatocyte, with incomplete cytokinesis after each nuclear division, gives rise to a cyst containing a bundle of 64 haploid nuclei (N) (only four are shown here for simplicity). Sperm tails extend from the bundle of needle-shaped nuclei through the syncytial cytoplasm of the elongated cyst (A). Each germline cyst is sandwiched between two somatic cells known as cyst cells (c.c.). Individualization involves the assembly of a membrane-cytoskeletal complex, the individualization complex (IC), at the nuclear end of the cyst (B) that translocates the length of the cyst, encasing each spermatid in an integral plasma membrane (C) and extruding syncytial cytoplasm and other morphogenetic debris in a ‘waste bag’ (WB) subsequently eliminated from the tail end of the cyst (D). A complex membrane network (represented by fine dotted lines in the drawing) extending the length of the preindividualized cyst contains numerous cytoplasmic bridges. This membrane is either removed or remodeled during individualization. At the end of the individualization process, the waste bag is separated from the cyst and the individualized sperm bundle is tightly coiled before liberation from the two somatic cyst cells and passage out of the testis into the seminal vesicle. Dotted lines through the cyst in C represent the plane of section observed in the electron micrographs presented in this paper. A section to the left, or rostral side of the individualization complex (ind.), gives a view of individualized spermatids; a section to the right (preind.), or caudal of the IC, affords a view of a preindividualized cyst. For simplicity, the length/width ratio of the cysts is grossly underrepresented here, since the cyst grows to approximately 2 mm in length in D. melanogaster, and considerably longer in other Drosophila species. Sections through the IC itself are therefore quite rare. Adapted from Lindsley and Tokuyasu (1980), based largely on the data of Tokuyasu et al. (1972).
are distinguishable. Instead, their morphology is highly irregular, suggestive of a large variation in the width of the sperm tails, or of a failure of sperm to be separated from each other by the individualization process. In addition, numerous bulges or blebs are apparent along the length of the sperm tails (Fig. 3B,C).

As reported earlier (Bazinet et al., 1993), \(Chc^{4}\) males carrying an additional copy of the cloned wild-type \(Chc\) gene are restored to fertility. Testes squash preparations from such \(Chc^{4}\); \(P[w; Chc^{+}]\) individuals are indistinguishable from those of wild-type males, with both exhibiting numerous motile, morphologically normal sperm (Fig. 3A,D). The spermatogenic defect in \(Chc^{4}\) males is therefore attributable to the clathrin mutation and not some other mutation in the genetic background of these flies. The pronounced ‘blebbing’ defect obvious in the sperm tails of \(Chc^{4}\) testes preparations suggests a defect in the individualization process, such that pockets of cytoplasmic material normally removed during the translocation of the IC may be left behind. Alternatively, the blebs may simply result from a random redistribution of cytoplasm upon rupture of the unfixed cysts that have failed to individualize.

**Ultrastructural analysis of wild-type and \(Chc^{4}\) spermatogenic cysts**

Transmission electron microscopy of thin sections prepared from testes of \(Chc^{4}\) males confirmed a defect in the individualization process. Pre-individualized spermatogenic cysts, identifiable as such because of their rudimentary axonemal decoration, light-staining mitochondrial derivatives, enlarged minor mitochondrial derivatives and the presence of extensive cytoplasmic ground substance between the axonemes, are evident in both wild-type (Fig. 4A) and \(Chc^{4}\)
1837 Sperm individualization mutants

(Fig. 4B) testes. Although only 40 flagellar axonemes are seen in the mutant cyst (Fig. 4B) relative to 64 in the wild type (Fig. 4A), the overall cytoplasmic organization of the cysts appears quite similar in the two samples.

Highly mature spermatids in sections of wild-type cysts that have been individualized are characterized by a substantial reduction in the amount of cytoplasmic ground substance between the axonemes, more pronounced axonemal decoration (generally observable as an enhancement in radial symmetry in cross-sectional views of the axoneme), the accumulation of extensive amounts of dark-staining material in the major mitochondrial derivatives and a significant reduction in size of the minor mitochondrial derivatives (Fig. 5A). In contrast, sections through many mature Chc4 mutant cysts reveal a gross disorganization not present in immature cysts (Fig. 5B). Although these spermatids appear quite mature on the basis of axonemal decoration and the intense staining of the major mitochondrial derivatives, extensive amounts of cytoplasmic ground substance remain between the axonemes, very irregularly distributed among the spermatids. Gross distensions of the mitochondrial derivatives, usually the minor derivative, are also commonly observed in such cysts. The cytoplasm between mutant sperm tails in these cysts often appears highly disorganized, with a complex network of membranous tubules (Fig. 5B).

**Table 1. Summary of IC and nuclear bundle disruptions in the mutant genotypes**

<table>
<thead>
<tr>
<th>Stock</th>
<th>Viability index*</th>
<th>No. of ICs per squash (avg)</th>
<th>Intact ICs colocalizing with intact nuclear bundles</th>
<th>Disrupted ICs with associated elongated nuclei</th>
<th>Intact progressed ICs</th>
<th>Disrupted progressed ICs</th>
<th>Intact ICs with associated scattered nuclei</th>
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<td>–</td>
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<td>0%</td>
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<td>3%</td>
<td>36%</td>
<td>7%</td>
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<td>66%</td>
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<td>45%</td>
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<td>0%</td>
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<td>8%</td>
<td>80%**</td>
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</tr>
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<td>56%</td>
<td>6%</td>
<td>35%</td>
<td>3%</td>
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<th>Stock</th>
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<th>No. of ICs per squash (avg)</th>
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<th>Disrupted ICs with associated elongated nuclei</th>
<th>Intact progressed ICs</th>
<th>Disrupted progressed ICs</th>
<th>Intact ICs with associated scattered nuclei</th>
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<td>47%</td>
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<td>50%</td>
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<th>Stock</th>
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<th>Disrupted ICs with associated elongated nuclei</th>
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<td>38%</td>
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<td>8%</td>
<td>36%</td>
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<td>43%</td>
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<td>tho 86E</td>
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<td>44%</td>
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*Viability index = no. of homozygous mutant flies observed/no. of homozygous mutant flies expected, where the number of mutant flies expected = 1/2 the number of balancer heterozygotes.

**These are wild-type controls, used for mutants in rosy or white backgrounds, respectively.

*Progressed ICs are individualization complexes that do not colocalize with the nuclear bundle; i.e. ICs that have traversed caudally along the cyst.

**The IC and nuclear bundle disruptions are very mild in these squashes.

**Altered** refers to the disruption present in mlt ICs and the failure of poe ICs to enlarge.

**Actin cytoskeletal dynamics of Chc4 spermatogenic cysts**

While wild-type spermatogenic cysts often display the

*Fig. 4B* testes. Although only 40 flagellar axonemes are seen in the mutant cyst (Fig. 4B) relative to 64 in the wild type (Fig. 4A), the overall cytoplasmic organization of the cysts appears quite similar in the two samples.

Highly mature spermatids in sections of wild-type cysts that have been individualized are characterized by a substantial reduction in the amount of cytoplasmic ground substance between the axonemes, more pronounced axonemal decoration (generally observable as an enhancement in radial symmetry in cross-sectional views of the axoneme), the accumulation of extensive amounts of dark-staining material in the major mitochondrial derivatives and a significant reduction in size of the minor mitochondrial derivatives (Fig. 5A). In contrast, sections through many mature Chc4 mutant cysts reveal a gross disorganization not present in immature cysts (Fig. 5B). Although these spermatids appear quite mature on the basis of axonemal decoration and the intense staining of the major mitochondrial derivatives, extensive amounts of cytoplasmic ground substance remain between the axonemes, very irregularly distributed among the spermatids. Gross distensions of the mitochondrial derivatives, usually the minor derivative, are also commonly observed in such cysts. The cytoplasm between mutant sperm tails in these cysts often appears highly disorganized, with a complex network of membranous tubules (Fig. 5B). Qualitatively, the appearance of Chc4 cysts changes radically late in the morphogenetic process, consistent with the hypothesis that a failed attempt at individualization is the source of the pronounced loss of organization relative to wild-type cysts.
presence of a coherent bundle of actin fibers (Fig. 2), the corresponding machinery is much less frequently observed in cysts from Chc4 males prepared in parallel (Fig. 6A-C; Table 1). Infrequent ICs observed in Chc4 preparations are usually disorganized relative to wild-type ICs. Cysts from Chc4 males also reveal scattered spermatid nuclei (Fig. 6B,D) as opposed to the intact nuclear bundles present in wild-type cysts (Fig. 2B,D). When evidence of a disrupted IC is observed in Chc4 cysts, the nuclear bundle of the corresponding cyst is usually also disorganized, with numerous nuclei apparently pulled out of the bundle, or scattered caudally along the cyst (Fig. 6A-D; Table 1). The appearance of Chc4 mutant cysts indicates that the IC is sometimes assembled in this mutant, but fails to function properly. Since fertility, normal cytoskeletal appearance and intact nuclear bundle morphology are all restored by the addition of a single copy of the wild-type Chc gene on a P element (Fig. 6E,F), proper assembly and function of the IC therefore requires a fully functional Chc gene.

Assay for potential individualization defects

The analysis of the Chc4 phenotype suggests that a simple phase-contrast examination of unfixed testis squash preparations, followed by the phalloidin assay for IC formation and progress described above, might allow the identification of a number of different phenotypic classes from among male-sterile mutations that block spermatogenesis in the postmeiotic stages. Although postmeiotic arrest is the most common class of male-sterile phenotype in Drosophila (Castrillon et al., 1993), relatively little is known about cellular factors that may be required for the extensive morphogenetic changes observed in this stage.

We screened a collection of 74 male-sterile mutant fly stocks for late morphogenetic defects using this ‘blebbing’ assay and found that six mutations resulted in highly elongated, immobile, blebbed sperm tails. In addition, four other male-sterile mutations previously reported to exhibit scattered spermatid nuclei (Castrillon et al., 1993) were obtained from the Bloomington stock center. Each of these mutations also resulted in the production of elongated cysts and immotile, blebbed sperm flagella. These ten mutants exhibiting blebbing defects were further analyzed using rhodamine-phalloidin and DAPI to visualize the actin microfilament component of the IC and spermatid nuclei, respectively. Since the mutations exist in white or ry506 backgrounds, w(k) and ry506 males along with balancer heterozygotes were used as wild-type controls. Several different classes of sperm maturation phenotypes have been identified in this screen.

Mutants exhibiting defects early in the individualization process

While squash preparations of wild-type testes reveal a multitude of ICs in various stages of maturation (Fig. 2A; Table 1), testes from nanking mutant males reveal a general paucity of ICs (Table 1; Fig. 7A). However, when ICs are constructed in nanking ms (3) 3915 mutant testes, they appear to be tightly organized compared to those of most other mutants (see below) and are never seen displaced from the nuclear end of the cyst (Fig. 7C,D; Table 1). Nanking ICs often colocalize with a mildly disrupted spermatid nuclear bundle (Fig. 7D), but in general, the spermatid nuclear bundle morphology is largely unaffected (Fig. 7B). These observations suggest a primary defect in the assembly of the IC which may secondarily prevent the translocation of the IC along the spermatogenic cyst.

Robust ICs associated with intact spermatid nuclear bundles are readily observed in testes squash preparations from dud ms(2)Hb328 mutant flies (Fig. 7E,F), which construct a much greater number of ICs than nanking and occasionally exhibit more prominently scattered spermatid nuclei and IC disruptions (Table 1). These disrupted ICs often colocalize with the nuclear bundle, but fragments of the complex that have progressed away from the nuclear bundle are sometimes observed (Table 1). Intact ICs that have progressed along the spermatogenic cyst are not seen in squash preparations of testes from this mutant, suggesting that an important factor required for motility of the IC may be absent or defective. Defects characteristic of these mutations are observed much less frequently in wild-type controls.

A mutation that allows the progress of a reduced IC along the spermatogenic cyst

An IC with a significantly reduced actin signal is built around the spermatid nuclear bundle in testes preparations from ms(2)5970 males (Fig. 8A,B). In many cases, these ICs appear to progress away from the nuclear bundle and travel as a coherent unit along the spermatogenic cyst (Fig. 8B-D; Table 1). These complexes are often observed at considerable distance from the nuclear end of the cyst.
distances from the nuclear bundles. However, since we have not observed the pronounced ‘cystic bulge’ associated with highly progressed wild-type ICs (Fig. 2E) in preparations from msl(2)5970 homozygotes (Fig. 8C,D; Table 1), it appears that these mutant ICs fail to undergo an increase in volume characteristic of wild-type ICs, which are actively extruding cytoplasm from between the sperm tails.

By introducing the 2-3 transposase source (Robertson et al., 1988) into flies carrying the msl(2)5970 mutation, we found that the mutation is revertible. We mapped it by in situ hybridization, using the white gene to detect the P element, to position 28E on the second chromosome. Complementation testing revealed that the mutation is an allele of purity of essence (poe), mapped previously to the same cytological location by Castrillon et al. (1993).

**IC breakdown caused by the mulet mutation**

Spermatogenic cysts from homozygous msl(2)4210 mutant males construct an IC which by the phalloidin assay appears normal (Fig. 8E; Table 1). However, as the complex travels caudally along the cyst, it becomes severely disrupted (Fig. 8G,H; Table 1). Nuclear scattering is notably absent in these preparations, (Fig. 8F; Table 1). A gene product required to maintain the structural integrity of the IC, or to coordinate the movement of individual investment cones within the IC, may be defective or absent in this mutant. This phenotype, characteristic of homozygous msl(2) 4210 males, is rarely observed in wild-type controls (Table 1).

The msl(2)4210 mutation is revertible by 2-3 transposase (Robertson et al., 1988) and maps to cytological position 46F. By complementation analysis, the mutation is an allele of the mulet (mlt) gene previously identified by Castrillon et al. (1993).

**Expressway mutants**

Three of the male-sterile mutants analyzed in this screen exhibit phenotypes similar to that of Chc4 (Fig. 9), in that they display disrupted ICs and elongated spermatid nuclei scattered along the cyst (Castrillon et al., 1993). IC disruptions and nuclear scattering are evident in the scat msl(2)30B (Fig. 9A-C), crossbronx (cbx) msl(2)46C (Fig. 9D) and long island expressway (lie) msl(2)42A (Fig. 9E) mutants while nearly absent from wild-type control preparations (Fig. 2A-D; Table 1). Qualitatively, lie and cbx mutant testes display the most highly disrupted ICs and the most prominent scattering of spermatid nuclei (Fig. 9D,E). Testes dissected from scat

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**Fig. 5.** High magnification views of sections through mature portions of cysts from wild-type (A) and Chc4 mutant (B) testis. Wild-type cysts assume a highly ordered, sometimes paracrystalline array after individualization, with a significant reduction in both the amount of cytoplasm between the sperm tails and the size of the minor mitochondrial derivative. Mutant cysts fail to individualize properly. In this case, more spermatids than usual appear to have been encased tightly in membrane, although grossly disorganized excess cytoplasmic material is seen within many of the spermatid membranes. ma, major mitochondrial derivative; mi, minor mitochondrial derivative; cn, complex tubular network. Bar, 1 μm.

**Fig. 6.** Squash preparations of dissected Chc4 (A-D) and Chc4 rescue (E,F) testes stained with rhodamine-phalloidin to visualize actin fibers (A,C,E), DAPI to visualize nuclei (D), or double exposed for both (B,F). Chc4 mutant cysts exhibit disrupted ICs (arrows in A-C) which could be responsible for the disruption of the spermatid nuclear bundle and the subsequent dragging of spermatid nuclei caudally along the cyst (arrowheads in B,D). Normal cytoskeletal appearance of the IC (arrows in E,F) and bundling of the spermatid nuclei (arrowheads in F) are restored by a copy of the Chc4 gene on an autosomal P-element. Bars: (A,B) 30 μm; (C,D) 20 μm; (E,F) 100 μm.
"ms(2)30B" mutant males reveal a general paucity of ICs and slightly milder disruptions of both the nuclear bundle and the IC (Fig. 9A,B; Table 1), and mild nuclear shaping defects are also evident in these testes (Fig. 9C). Because the streaming of blue fluorescent nuclei and red actin-based investment cones along the spermatogenic cysts of these mutants is reminiscent of time-lapse photographs of highways at night, we refer to this general class of mutations as expressway mutants.

**Mutations affecting nuclear morphology**

Testes from homozygous tho186E males contain cysts with complex nuclear and IC morphologies not seen in wild-type control preparations. Individual investment cones and elongated nuclei are observed scattered throughout the cyst (Fig. 9G,H; Table 1). In addition, numerous smaller DAPI signals are observed, often colocalizing with F-actin signals, suggestive of nuclear breakdown or chromosomal loss during sperm maturation (Fig. 9F; Table 1).

Some mutations allowing the assembly of highly elongated cysts appear to affect spermatid nuclear morphology earlier in development, since both ms(2) UK and ms(2) 5720 alleles of the mozzarella (moz) gene display incompletely condensed, irregularly shaped nuclei (Fig. 10B) as compared to wild-type controls. However, a significant amount of actin staining is often seen associated with the spermatid nuclear bundles of each of these mutants (Fig. 10A,B), suggesting that construction of an IC is attempted. While distinct actin staining characteristic of investment cones is often observed in preparations from both moz mutants (Fig. 10A,B; Table 1), unusual highly extended actin-based signals are also seen (Fig. 10C,D; Table 1), indicating that the IC may undergo a unique morphological change in these mutants.
Specificity of phenotypes assessed by deficiency analysis and phase-contrast observation of live preparations

Live squash preparations of the each of the new mutants have been examined by phase-contrast microscopy. In both of the relatively ‘early’ mutants, dud and nanking, irregularities are observed in earlier stages of spermatogenesis. Both mutants display defects in nebenkern formation; nebenkerns appear to be lacking in many onion-stage spermatids of dud, while multiple smaller nebenkerns seem to be present in onion-stage spermatids of nanking (data not shown). We have not yet observed any clear defect in earlier stages of mlt, poe, cbx, lie, tho, moz or scat mutant testis examined by phase-contrast microscopy.

Whenever possible, the phenotypes of each of the mutations uncovering deficiencies have been examined. Poe/Df and mlt/Df phenotypes are indistinguishable from poe/poe and mlt/mlt phenotypes, indicating that the available poe and mlt mutations are null alleles.

Studies on viability reveal that Chc4, scat1 ms(2)30B, lie ms(2)42A, tho1 86E and dud ms(2)Hb328 are semilethal alleles, indicating that these genes are required for vital functions in addition to spermatogenesis. The mlt, poe, nanking, moz and cbx mutations do not significantly affect adult viability (Table 1).

**DISCUSSION**

In their classic ultrastructural study of sperm individualization in *Drosophila*, Tokuyasu et al. (1972) identified numerous parallel arrays of 6 nm filaments in the trailing half of the spindle-shaped investment cones that mediate the process. Here we have used rhodamine-conjugated phalloidin as a fluorescent probe with which to visualize this actin component of the individualization complex at the level of the light microscope. A screen of several ‘late’ male-sterile phenotypes, in which highly elongated cysts are produced, has allowed the discrimination of these ‘late failure’ phenotypes into several categories, varying in the extent to which the actin-based cytoskeletal component of the IC is assembled, its motility and the maintenance of its structural integrity as it translocates down the cyst. This assay does not, however, distinguish...
between genetic defects in cyst morphogenesis prior to individualization and defects in the individualization machinery itself. Ultrastructural analysis and subcellular localization of the affected gene products will be required to distinguish these two classes. Nonetheless, the simple fluorescence assay described here provides an entry to considerable advancements towards understanding the extensive remodeling of cyst architecture late in the spermatogenic pathway.

As suggested by Fuller (1993) individualization may provide an editing function by which defective spermatids are removed from the developmental pathway. This is probably best illustrated in males heterozygous for the segregation distorper (SD) chromosome. Within a single cyst in these males, spermatid nuclei carrying a sensitive responder element (Rsp) fail to condense properly, with the corresponding spermatids failing to become individualized, while spermatids carrying the homologous SD chromosome are successfully matured (Tokuyasu et al., 1977; Temin et al., 1991). Our finding of diverse individualization-defective phenotypic classes argues that such an editing function is not an active one, as this would be expected to result in a uniform or stereotypical individualization phenotype for most ‘late’ male-sterile mutants. Instead, each distinguishable individualization phenotype observed here might best be interpreted as symptomatic of a different class of defect, either within general cyst architecture or the individualization complex itself, each resulting in a different form of morphogenetic derailment. By this view, the failure of Rsp spermatids to be individualized is more likely a secondary or indirect consequence of incomplete nuclear condensation, resulting in a defective scaffolding or template for investment cone assembly. More recently, a *Drosophila* male-sterile nuclear lamin mutant was isolated in which elongated, defective cysts are produced, suggesting an individualization defect as a consequence of misshappen spermatid nuclei (Lenz-Bohme et al., 1997). The individualization-defective phenotype of *mozzarella*, *tho 86E* and *scat ms(2)30B* mutants, which also display nuclear morphology/condensation defects, are consistent with a similar mechanism.

Disrupted ICs and scattered spermatid nuclei are a phenotypic characteristic of *crossbronz*, *tho* and *lie* mutations. This phenotype may arise by one of several different mechanisms.

In the first, a defective IC may assemble around an initially intact spermatid nuclear bundle, with the subsequent movement of this defective complex resulting in the dragging of spermatid nuclei caudally along the spermatogenic cyst. This may be analogous to the involvement of F-actin bundles in the correct positioning of germline nuclei in *Drosophila* oogenesis, where nurse cell nuclei are anchored in place by F-actin cables during rapid cytoplasmic transport into the oocyte (Mahajan-Milkos and Cooley, 1994a). Mutations that disrupt the function of actin-bundling proteins such as *quail*, a villin homolog (Mahajan-Milkos and Cooley, 1994b), *singed*, a fascin homolog (Cant et al., 1994), and *chickadee*, a profilin homolog (Cooley et al., 1992; Verheyen and Cooley, 1994), all result in the displacement of nurse cell nuclei into the ring canals of the syncytium, obstructing transport of cytoplasm into the oocyte (Cooley and Theurkauf, 1994). In these examples, proper F-actin organization appears to be necessary in order to prevent the aberrant displacement of germline nuclei. Alternatively, in mutations such as *crossbronz*, spermatid nuclei may be splayed along the length of the cyst initially and investment cones may assemble around each nucleus independently, giving the illusion that spermatid nuclei are being dragged by defective investment cones. This may be the case in *scat ms(2)30B* mutant cysts, in which spermatid nuclei are frequently scattered, but in which the phalloidin-binding actin structures of the IC are seen relatively infrequently (Table 1).

The complexity of individualization provides ample opportunity for the direct participation of a large number of gene products and predicts a correspondingly large number of genetic loci mutable to such a late phenotype. However, many indirect effects are also likely, as small effects on the structure and function of the individualization complex from minor local cytoplasmic disturbances could be expected to increment to catastrophic levels over the millimeter-scale distances traversed by the machine. For example, a subtle defect in cytoplasm organization introduced before individualization in *mlt* mutant males may interrupt the continuous travel of the IC as it moves processively along the cyst, resulting in loss of coordination or synchrony of individual investment cones. The consequent sensitivity of spermatogenesis to otherwise subtle cytoplasmic defects of the cyst could also explain the effects of the semilethal *Che* allele on individualization, the high frequency with which male-sterile alleles of lethally mutable *Drosophila* genes are observed (Lindsley and Tokuyasu, 1980; Fuller, 1993) and the large proportion of late phenotypes (i.e., resulting in highly elongated cysts) among male-sterile mutations (Castrillon et al., 1993). Distinguishing between mutations that directly affect the individualization process and those that indirectly interfere with the process through a change in general cyst organization or physiology will not be trivial; large numbers of mutants can reasonably be expected to fall into each class.

Tokuyasu et al. (1972) suggested that the microfilaments of the investment cones somehow provided the motility of the individualization complex. While the actin-based IC does progress caudally along the spermatogenic cyst, potential motor protein(s) involved in its translocation remain unknown. Recently, a novel minor myosin has been shown to be preferentially localized to the *Drosophila* testes (Miedema et al., 1995), thus implicating itself as a candidate motor component of the IC. Cytoplasmic myosin II has also been localized to the cellularization front in the syncytial blastoderm of *Drosophila* embryos (Schejter and Wieschaus, 1993a). While many of the gene products involved in the cellularization of the syncytial blastoderm such as nullo, serendipity-α (Postner and Wieschaus, 1994) and bottleneck (Schejter and Wieschaus, 1993b) appear to beblastoderm specific, the conservation of function between the two cellularization systems suggests the participation of a myosin motor in individualization. An alternative hypothesis, motility driven by assembly and disassembly of actin filaments without the active participation of motor proteins, is suggested by the similarity in appearance of the phalloidin signals in many of our images to the actin-based ‘comet tail’ structures that provide motility for intracellular parasites such as *Listeria* and *Shigella* by this simpler, and perhaps more primitive, mechanism (Theriot, 1994).
The resolution of syncytia into individual cells is a feature common to numerous morphogenetic pathways, including the cellularization of the syncytial blastoderm in *Drosophila* (Schejter and Wieschaus, 1993b) and the nuclear endosperm of some flowering plants (Lopes and Larkins, 1993). Similarly, in the mammalian bone marrow, polyploid (up to 64n) megakaryocytes partition their cytoplasm into individual platelets using reservoirs of membrane to create platelet demarcation channels (Bloom and Fawcett, 1975). Further investigation of *Drosophila* sperm individualization may provide insight into the mechanisms involved in resolving individual cells from syncytia in these and other morphogenetic systems yet to be discovered.

We are especially indebted to Diana Bartelt for generous sharing of her fluorescence microscope. We thank Margaret Fuller and Anthony Mahowald for encouragement and vital criticism, and Joseph Polak for expert assistance with electron microscopy. This work was supported by NIH Grant R15 HD 31693-01 (C. B.), NSF Grant MCB-9305287 (S. K. L.), American Heart Association, Northeast Ohio Affiliate Grant 4838 (S. K. L.), and the American Cancer Society, Cuyahoga County Unit (S. K. L). G. H. was supported by C. J. Martin Fellowship No. 937316 from the National Medical Research Council Cuyahoga County Unit (S. K. L). G. H. was supported by C. J. Martin Affiliate Grant 4838 (S. K. L.), and the American Cancer Society, 9305287 (S. K. L.), American Heart Association, Northeast Ohio Council, C. J. Martin Affiliate Grant 4838 (S. K. L.), and the American Cancer Society, 9305287 (S. K. L.).

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