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Differential segregation and modification of mRNA during spermiogenesis in *Marsilea vestita*

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Abstract

We are interested in the mechanisms that underlie cell fate determination in the endosporic male gametophytes of the fern, *Marsilea vestita*. Synchronous development is initiated by placing dry spores into water and involves the translation of stored mRNAs, with little transcription. Nine division cycles produce 32 spermatids surrounded by 7 sterile cells, and then each spermatid differentiates into a multiciliate gamete. Here, we focus on changes in the distribution of particular proteins, mRNAs, and patterns of polyadenylation as essential prerequisites for cell fate determination and gametogenesis. Earlier, we showed that α- and β-tubulin proteins become concentrated in spermatogenous initials, and that centrin mRNA is translated only in spermatogenous initials. In situ hybridizations reveal that centrin, cyclin B, and β-tubulin mRNAs are present in both sterile and spermatogenous cells, but that transcripts encoding RNA helicase and PRP-19 (a spliceosome component) become localized in spermatogenous cells. The targeted destruction of these two transcripts by RNAi treatments does not affect the numbers of division cycles, but the gametophytes exhibit anomalous patterns of cytokinesis, and a subsequent failure of spermatid differentiation. Thus, cell fate determination in the gametophyte involves localized translation, and the localization of mRNAs for proteins involved in transcript processing. We found differences in polyadenylation levels in sterile and spermatogenous cells that match the distribution of cytoplasmic poly(A) polymerase (PAP), which, in immunolocalizations, is abundant in spermatogenous cells, but undetectable in sterile cells. The activation of translation in spermatogenous initials, but not in sterile cells, may be under the control of mRNA processing enzymes, which become localized either as proteins or mRNAs in the spermatogenous subdomains before any divisions occur.

Keywords: *Marsilea vestita*; RNA polyadenylation; mRNA segregation; Spermiogenesis

Introduction

Asymmetric cell divisions produce two daughter cells that can adopt distinct fates (Scheres and Benfey, 1999). The asymmetries leading to different cell fates sometimes differ in daughter cell sizes, but always involve compositional differences between daughter cells (Kaltschmidt and Brand, 2002). Division asymmetries are usually preceded by the nonuniform distribution of cellular components, which can produce a polarized cellular axis. The generation of a polarized cell requires the asymmetric distribution of many cellular proteins. Some of these proteins can be transcription factors such as Prospero in *Drosophila* (Doe et al., 1991) or structural proteins such as actin in *Fucus* (Bouget et al., 1996). One way that proteins can be targeted to discrete subcellular locations is through the localization of the mRNAs that encode them. The cytoplasmic localization of mRNA underlies cell polarity in both somatic cells and oocytes, and provides a basis for patterning during embryonic development (Lasko, 1999; St. Johnston, 1995). Localized mRNAs may allow for site-specific translation of a protein that influences cell fate or differentiation. For example, maternal mRNAs in *Drosophila* encode some anterior and posterior determinants of polarity; bicoid and nanos mRNAs become localized to opposite poles of the oocyte, and gurken mRNA is localized to the anterior/dorsal region of the oocyte (Lasko, 1999). The localization of mRNAs that encode products required for mRNA processing can serve as the basis for different patterns in subsequent development by daughter cells that possess or lack these transcripts (Mills et al., 2002). The activation

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of maternal RNA by polyadenylation has also emerged as an important mechanism for translational regulation in embryogenesis. Studies in vertebrates have defined *cis*-acting elements in the 3′ UTRs of responding mRNAs and *trans*-acting factors that bind to these sequences. This modification of an mRNA can stimulate or activate its translation and thus serves as a mechanism for translational control (Chang et al., 1999; Richter, 1996).

In this paper, we describe changes in the distributions of particular proteins, mRNAs, and patterns of polyadenylation that underlie cell fate determination in the male gametophyte of the water fern *Marsilea vestita*. The major morphogenetic process linked with development of the endosporic gametophyte of *M. vestita* is spermiogenesis. Development of the gametophyte is initiated by placing dry microspores into water or an aqueous culture medium and reaches completion rapidly, in approximately 11 h (Hart and Wolniak, 1998, 1999; Hepler, 1976; Hyams et al., 1983; Klink and Wolniak, 2001; Mizukami and Gall, 1966; Myles and Hepler, 1977, 1982; Pennell et al., 1988; Sharp, 1914; Wolniak et al., 2000; Klink and Wolniak, 2000). The pattern of development in each male gametophyte is precise and synchronous (Hepler, 1976; Klink and Wolniak, 2001; Mizukami and Gall, 1966; Pennell et al., 1986, 1988; Sharp, 1914). Nine rapid mitotic division cycles produce a total of 39 cells. The positions, sizes, and fates of the cells produced by these divisions are constant among the gametophytes; when the division cycles are completed, there are 7 sterile cells and 32 spermatids that reside in particular positions within the spherical spore wall. Gamete maturation begins after the division cycles are completed, and occurs over a 5- to 6-h period.

The regulation of spermiogenesis in *M. vestita* occurs almost entirely at the posttranscriptional level. In a series of radiolabel uptake studies, we found that some labeled proteins accumulate during development, and that the same labeled proteins accumulate in the presence of the transcriptional inhibitor α-amanitin (Hart and Wolniak, 1998). Spermiogenesis is blocked before the first mitotic division if the spores are treated with cycloheximide, but development progresses almost to completion in spores treated with α-amanitin (Hart and Wolniak, 1998; Klink and Wolniak, 2001). In contrast, for gametophytes treated with cycloheximide, no radiolabeled proteins are synthesized (Hart and Wolniak, 1998), and development is arrested at its onset (Klink and Wolniak, 2001). Dry microspores contain large quantities of mRNA (Hart and Wolniak, 1999). In a series of in vitro translation experiments, we found that these transcripts could not be translated from newly hydrated spores, but that a large variety of proteins could be translated from mRNAs isolated from gametophytes cultured for more than 30 min (Hart and Wolniak, 1999), a result suggesting that transcripts present in the dry spore are processed during the early stages of gametophyte development before any new translation can be initiated.

Spermiogenesis requires the translation of some new proteins, such as centrin, from mRNAs already present in the dry spore (Hart and Wolniak, 1998, 1999; Klink and Wolniak, 2001). Dry microspores also contain substantial quantities of stored proteins, including α-, β-, and γ-tubulin. Immunolabeling with anticentrin and antitubulin antibodies of gametophytes fixed at different stages of spermiogenesis revealed that sterile jackets cells were completely devoid of staining with these antibodies (Klink and Wolniak, 2001, 2003). The asymmetric distributions of anticentrin and antitubulin antibody labeling suggest that protein segregation is necessary for proper progression of spermatid development. The nonrandom distribution of proteins may also result in asymmetric divisions in early spermiogenesis.

The asymmetric divisions that occur during gametophyte development produce a prothallial cell, six sterile jacket cells, and two spermatogenous initials. The prothallial cell and jacket cells disintegrate and disappear during the progression of spermiogenesis. The mechanisms by which the male gametophyte generates asymmetric divisions remain unknown. In an effort to understand the underlying mechanism of cell fate determination in this simple system, we studied the distribution of various proteins and mRNAs during development and the pattern of polyadenylation of mRNAs in the two kinds of cells produced in the gametophyte. We show that at least two species of mRNA, one encoding an RNA helicase and the other encoding a PRP-19-like protein, both have asymmetric distribution patterns that coincide with differences in cell fates. Not surprisingly, the targeted destruction of these two transcripts by RNAi treatments results in altered development that is manifested by abnormal patterns of divisions and differentiation. Furthermore, in normal gametophytes, there are strikingly different levels of polyadenylation in sterile jacket cells and spermatogenous cells. This difference apparently results from the asymmetric distribution of cytoplasmic RNA poly(A) polymerase (PAP) protein, which is almost exclusively localized in the spermatogenous cells. Our results suggest that two distinct pathways, patterns of RNA polyadenylation and the segregation of particular transcripts during spermiogenesis, contribute to the differences in cell fates between spermatogenous and sterile cells of the rapidly developing gametophyte.

Materials and methods

Microspore culture and fixation

Dry sporocarps were collected from *M. vestita* sporophytes raised in 10 ponds at the greenhouse facilities at the University of Maryland. Microspores were isolated by grinding the dry sporocarps in a commercial coffee grinder, and sifting the microspores from the debris (Hepler, 1976; Klink and Wolniak, 2001; Tsai and Wolniak, 2001).

Gametophytes were cultured in tap water or in Laetsch's medium (Laetsch, 1967), with continuous, gentle rotational shaking in a controlled-temperature water bath (New Brunswick model RM6, New Brunswick, NJ). Gametophytes were fixed in paraformaldehyde using protocols described earlier, which involved a mechanical shock to crack the microspore walls (Hepler, 1976; Klink and Wolniak, 2000; Tsai and Wolniak, 2001).

Preparation of the probes for in situ hybridization

The pBluescript plasmids carrying cyclin B (MvU98), β-tubulin (MvU63), centrin (MvCen1-K, Centrin subclone; Klink and Wolniak, 2001), RNA helicase, and PRP-like protein (Tsai and Wolniak, 2001) were amplified through PCR reactions with primer pairs of pUC/M13 forward and reverse 17 mers. Antisense RNA probes were generated and labeled with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN) using an in vitro transcription kit from Epicentre (Epicentre Technologies; T3 AmpliScribe™ transcription kit; Madison, WI): 7.5 mM each of ATP, CTP, GTP, 2 mM UTP, 10 mM DTT, 1 μl AmpliScribe™ T3 enzyme solution, 25 mM DIG-UTP, and 1 μg of template DNA in 1× T3 reaction buffer were mixed and incubated at 37°C for 2 h. The entire reaction mixture was precipitated with an equal volume of 5 M ammonium acetate. Mixtures were incubated on ice for 15 min and centrifuged at 10000 × *g* for 15 min at 4°C. The pellet was washed in 70% ethanol and resuspended in RNase-free water. Digoxigenin-labeled probes were further quantified by electrophoresis and spectrophotometry. A 5′ end biotin-labeled, 30-nucleotide oligo(dT) probe was custom generated by Life Technology (Gaithersburg, MD). Stock solution (1 μg/μl) was made in RNase-free distilled water.

In situ hybridization

Methacrylate-embedded sections (1 to 3 μm) were placed on microscope slides that had been pretreated with 1 mg/ml of poly-D-lysine. The sections were dried at 80°C on a heat block for 1 h to increase adherence to the glass. Slides were further treated with acetone, proteinase K, paraformaldehyde, and triethanolamine according to procedures described by Steel et al. (1998). Hybridization procedures were performed as described elsewhere (Tsai and Wolniak, 2001; Warren, 1998). Sections were hybridized in 50% formamide, 5× SSC, 5× Denhardt's, 10% dextran sulfate, 2% SDS, 100 μg/ml single-stranded salmon sperm DNA, and 1 μg of an RNA probe in a moist chamber, overnight at 50°C. Slides were washed, blocked in 3% BSA, and incubated in 1:1000 dilution of anti-Digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim). The AP-conjugated antibody was detected with 4.5 μl/ml nitroblue tetrazolium (NBT) + 3.5 μl/ml 5 bromo-4 chloro-3 indolyl-phosphate (BCIP) (Boehringer Mannheim). The color was allowed to develop 30 min to overnight in a dark humid chamber at 4°C. Development of peroxidase system was carried out by mixing 1 ml of 3′,3′-diaminobenzidine (DAB) in PBS (5 mg/ml stock), 9 ml PBS/0.1 M imidazole, pH 7.6, and 10 μl 30% H_2O_2 as substrate (Hopman et al., 1997). As an alternative, 500 μl of the TrueBlue peroxidase substrate (Kirkegaard and Perry Laboratories; Gaithersburg, MD), which contains tetramethylbenzidine (TMB), was used on cell sections. The reaction was blocked within 10 min of incubation in darkness. Color development was observed with light microscopy.

Cytology and immunocytochemistry

Immunogold cytochemistry was employed to localize proteins in the gametophytes because of high autofluorescence from the spore walls. Standard immunocytochemical procedures were followed on acetone-etched methacrylate tissue sections (Baskin et al., 1992; Klink and Wolniak, 2001; Warren, 1998); 1:1000 of polyclonal antibody originally directed against *Xenopus laevis* PAP (a kind gift from Dr. J. Richter, University of Massachusetts), 1:500 of anticentrin monoclonal 20H5 directed against *Chlamydomonas reinhardtii* (gift of Dr. Jeffery Salisbury; Mayo Clinic, Rochester, MN), and 1:1000 of anti-β-tubulin (Sigma) were used as primary for in situ labeling and a 1:3000 dilution a gold-conjugated goat-anti-rabbit or mouse IgG (Research Diagnostics, Flanders, NJ) as secondary antibody. Labeled cells were observed with reflected confocal light and transmitted DIC with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, New York), using a 100× Plan Apochromatic Objective Lens. Confocal image stacks with reflected light were obtained through the 1- to 3-μm thickness of the tissue section and superimposed on a transmitted DIC image to depict antibody distribution within the etched section of the gametophyte (Tsai and Wolniak, 2001). Alternatively, we combined phase contrast and reflection interference contrast imaging with a Zeiss Antiflex Plan Neofluar 63× objective lens, and photographed the gametophytes with a Zeiss Color Axiocam microscope camera.

Electrophoresis and immunoblotting

Protein isolates were prepared from gametophytes that had developed for various intervals as previously described (Klink and Wolniak, 2001) in SDS sample buffer (62.5 mM Tris base, pH. 6.8; 2% SDS, 25% urea; 10% glycerol; 5% β mercaptoethanol; 0.02% bromophenol blue). Protein samples were boiled for 5 min and then placed on ice for 5 min. The samples were clarified at 3000 × *g* for 5 min and the supernatant was collected. Protein concentrations were determined by using assays developed by Bradford (1976). For these experiments, 30 μg of protein were loaded per lane onto 10% discontinuous SDS-polyacrylamide gels (Laemmli,

1970). SDS-PAGE was performed using a BioRad Mini-Protean II Electrophoresis Cell (BioRad, Hercules, CA). Electrophoretic transfer of proteins onto PVDF (Immobilon P; Millipore; Bedford, MA) was performed on a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) as described previously (Hart and Wolniak, 1998). In brief, PVDF membranes were wetted in methanol for 30 s and then transferred into transfer buffer (50 mM Tris base, pH. 8.2; 0.5 M glycine; 0.01% SDS; 20% methanol). The protein gel/PVDF membrane stack was fit into the Mini Trans-Blot Electrophoretic Transfer Cell apparatus and transfer was accomplished by electrophoresis for 1 h at 60 V.

Immunoblotting was performed for the protein isolates by procedures previously described (Klink and Wolniak, 2001) using the anti-PAP (a kind gift from Dr. Joel Richter) antibody and anti-β-tubulin (NeoMarkers, Lab-Vision Corp., Fremont, CA) antibody. Secondary antibodies (antispecies horseradish peroxidase: Amersham, Buckinghamshire, UK) were diluted 1:1500 in PBST. Chemiluminescent detection was performed by immersing PVDF membranes in a 1:1 dilution of ECF (Amersham) with detection by a STORM 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The blotting assays were each repeated four times, with two different protein isolate preparations (from gametophytes grown for 4, 6, and 8 h, though only the 8-h isolate is depicted in Fig. 8) and multiple dilutions of primary antibody. We used at least two different blots for each isolate, as means to ensure that variations in loading were not significant (Klink and Wolniak, 2003).

RNAi

RNAi experiments were performed on gametophytes as described previously (Klink and Wolniak, 2001, 2003; Tsai and Wolniak, 2001). DsRNAs were made in a set of in vitro transcription reactions, using MvU11 and MvU89 cDNAs as the templates. Blast searches reveal that MvU11 (GenBank Accession # CF867680) encodes an RNA helicase, and that MvU89 (GenBank Accession # AALJ2025) encodes a PRP-11-like protein. Separate populations of microspores were treated with three different concentra-

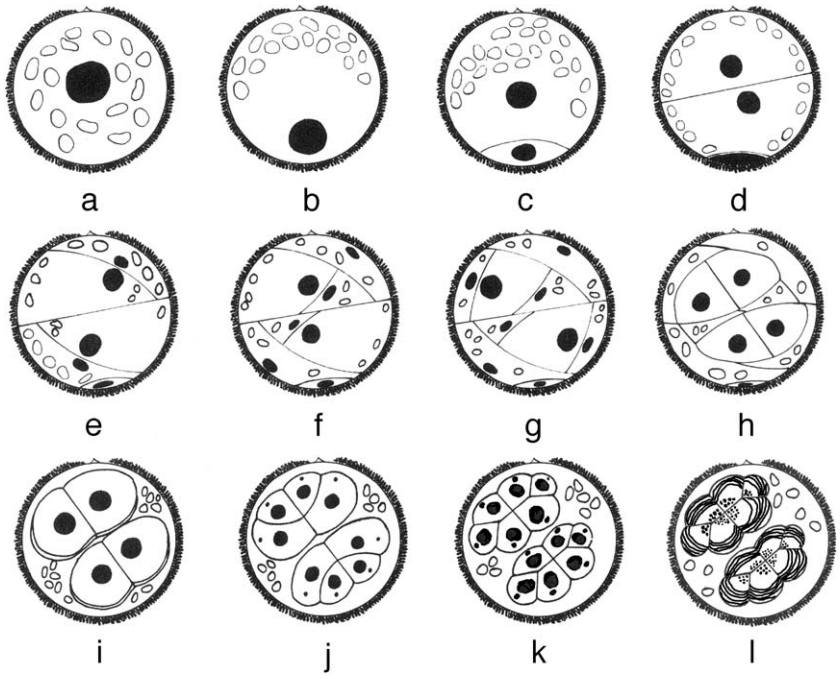

Fig. 1. Drawings of the cell division patterns during development of the male gametophyte of *M. vestita*. (a) Microspores at the time of imbibition. The nucleus is depicted as a black circle, and the plastids are depicted as open ellipses. (b) The gametophyte before the first division, when cytoplasmic reorganization occurs. (c) After the first mitotic division, a prothallial cell (bottom) is cut off from the rest of the gametophyte and no longer divides. (d) The second division gives rise to two antheridial initials, essentially of equal size. (e) Each antheridial initial undergoes an asymmetric division that produces the first of three sterile jacket cells. (f) The second sterile jacket cell is produced by another asymmetric division. (g) The antheridial initials each undergo an additional asymmetric division that produces the third sterile jacket cell for each antheridium. The other cell produced by this division is the primary spermatogenous cell. None of the jacket cells can proliferate further. (h) Each primary spermatogenous cell undergoes a symmetric division. (i) The two spermatogenous cells in each antheridium undergoes a symmetric division to produce four spermatocyte mother cells. By this stage, the jacket cells become far less conspicuous and they eventually degenerate. (j) The four spermatocyte mother cells in each antheridium undergo a division to produce eight spermatocytes. At this stage, the blepharoplast first appears, and then rapidly disappears (see Hepler, 1976). (k) Later, the blepharoplast reforms. During this last division cycle, the blepharoplast splits and functions as the centrosome, when each spermatocyte undergoes a division that produces 16 spermatids in each antheridium. (Only six of the spermatids in each cluster is depicted in this drawing.) (l) During the next 5.5 h, each of the spermatids begins to undergo maturation that culminates with the release of 32 multiciliated spermatozoids (see text).

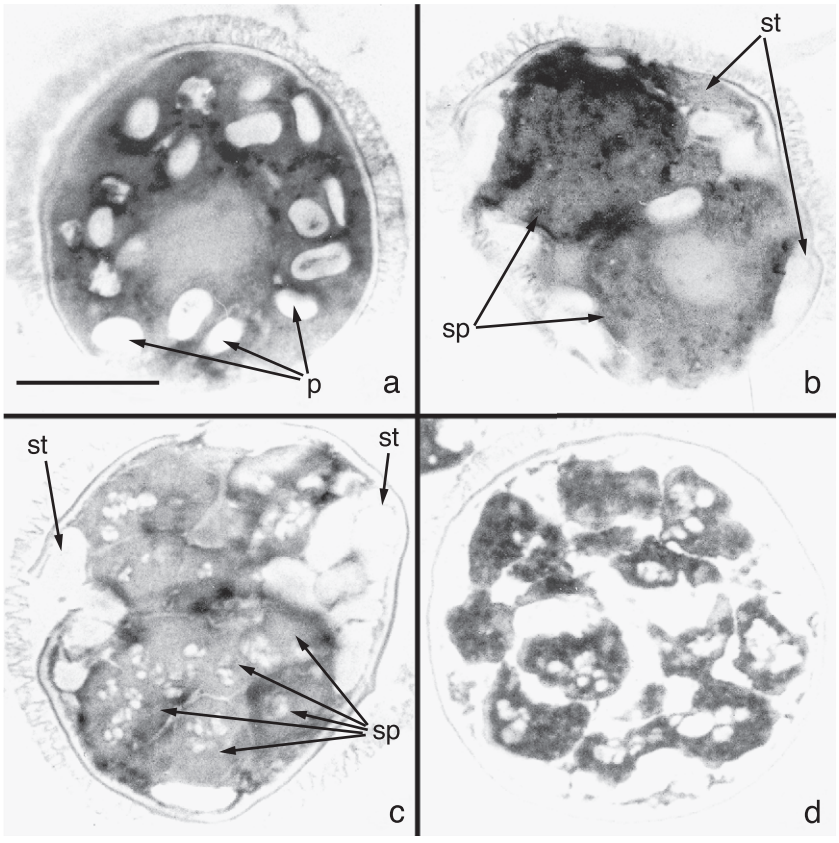

Fig. 2. Localizations of centrin mRNA distribution during spermiogenesis by in situ hybridization assays. (a) Centrin mRNA is abundant in the cytoplasm of the newly hydrated spores, and remains detectable in all cells of the gametophyte during development. (b) Two hours of development (sp = spermatogenous cells, st = sterile jacket cells). (c) Four hours of development (sp = spermatogenous cells, st = sterile jacket cells). (d) At 8 h of development, the spermatids exhibit intense labeling with the centrin RNA probe, while the peripherally distributed sterile jacket cells exhibit less staining. Scale bar = 25 μm.

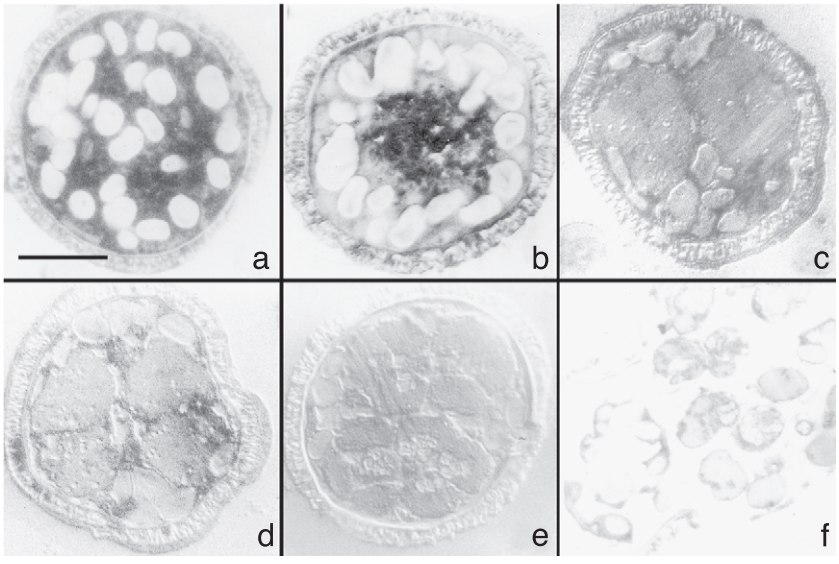

Fig. 3. Localizations of cyclin B mRNA during spermiogenesis by in situ hybridization assays. (a) Stored cyclin B mRNA was readily detectable in the dry spores. As the divisions progress (b–d), cyclin B mRNAs were present in both jacket cells and spermatogenous cells. (e, f) By 6 h after imbibition, mitotic divisions were complete and spermatid development had begun and cyclin B mRNA levels declined to barely detectable levels by 8 h (f). Scale bar = 25 μm.

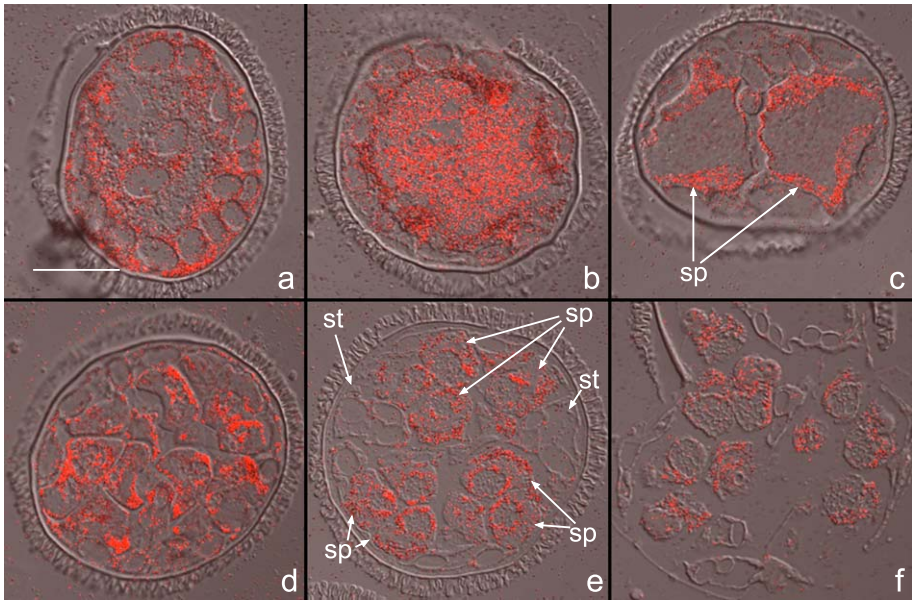

Fig. 4. Immunolocalization of β-tubulin during spermiogenesis. (a) Anti-β-tubulin antibody exhibits a uniform cytoplasmic label in the newly hydrated spores. (b) The label remains uniform at 1 h of development. (c) At 2 h of development, the anti-β-tubulin antibody localization became concentrated in the spermatogenous cells (arrows, sp) with significantly lower labels in the sterile jacket cells (arrows, st). (d) At 4 h of development, the spermatogenous cells exhibit highest concentrations of anti-β-tubulin antibody labeling. (e) Six hours of development and the spermatids exhibit pronounced staining. (f) Eight hours of development. Scale bar = 25 μm.

tions of each dsRNA, and allowed to develop for 8 h before the gametophytes were fixed and processed for light microscopic observation. Each of the treatments was performed at least twice (most were performed on three different populations of microspores), and gametophytes from all treatments were examined microscopically. At least 20 gametophytes were photographed from each sample to ensure that our assessments of developmental arrest were accurate.

Results

Protein and mRNA distributions differ in developing gametophytes

The cell division patterns in the developing male gametophyte of *M. vestita* are precise and highly predictable (Fig. 1). The nine division cycles are synchronous in populations of spores immersed into water at the same time and ulti-

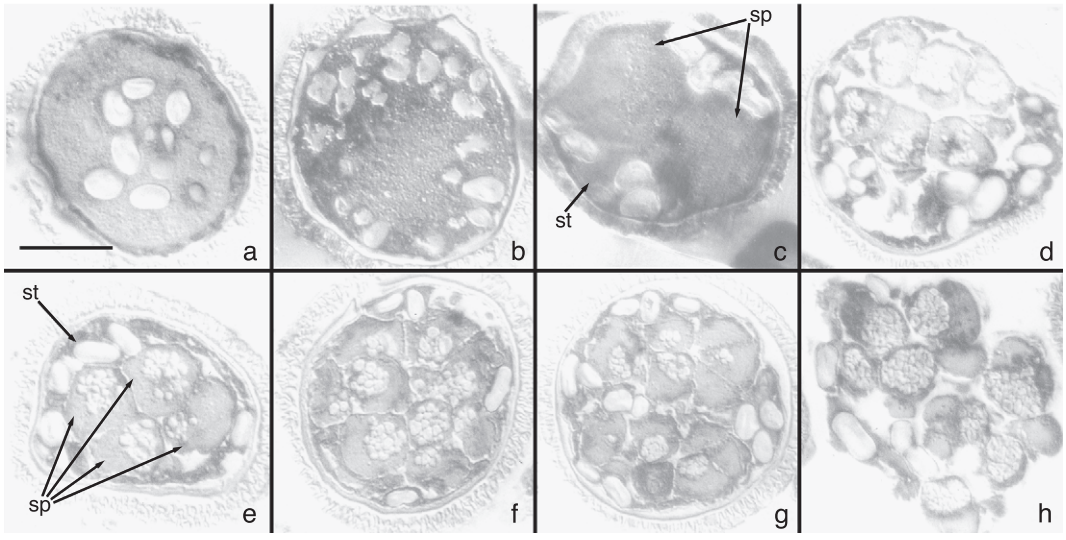

Fig. 5. Localizations of β-tubulin mRNA during spermiogenesis by in situ hybridization assays. (a) β-Tubulin mRNA is abundant in the cytoplasm of the newly hydrated spores, and remains detectable in all cells of the gametophyte during development. (b) One hour of development. (c) Two hours of development (sp = spermatogenous cells, st = sterile jacket cells). (d, e) Four hours of development (sp = spermatogenous cells, st = sterile jacket cells). (f, g) Six hours of development. (h) Eight hours of development. Scale bar = 25 μm.

mately cause 32 spermatids and 7 sterile cells (Hepler, 1976; Klink and Wolniak, 2001, 2003; Mizukami and Gall, 1966; Pennell et al., 1986, 1988; Sharp, 1914). We recently showed that centrin protein becomes abundant at 4 h after dry spores are immersed in water. Centrin mRNA is present as a stored transcript in the dry spore (Hart and Wolniak, 1999), and centrin translation is normal in gametophytes cultured in α-amanitin (Hart and Wolniak, 1998), a result indicating transcription is not necessary for centrin protein synthesis (Hart and Wolniak, 1998; Klink and Wolniak, 2001). Once centrin protein is made, it is detectable only in spermatogenous cells, with aggregations of the protein in the blepharoplasts (Klink and Wolniak, 2001), and centrin protein is detectable primarily at the anterior end of the spermatids in late stages of development (Klink and Wolniak, 2001). In contrast to the localized distribution of centrin protein in spermatogenous cells, we found that centrin mRNA was uniformly distributed in both sterile jacket cells and spermatogenous cells (Figs. 2a–d).

Since the spermatogenous initials continue to proliferate while the sterile cells, once formed, cease their proliferative activities, it seemed reasonable to suspect that cyclin B mRNA might become localized in the spermatogenous cells to sustain this activity. Cyclin B mRNAs were detected by in situ hybridization in newly hydrated spores and heavily concentrated inside the nucleus in gametophytes during the first 30 min of development (Fig. 3a). During the nine mitotic division cycles in normal development, cyclin B mRNAs were detected in both jacket cells and spermatogenous cells without any significant difference in abundance (Figs. 3b, c). There was no increase in the level of cyclin B mRNA in the gametophytes during development, but after the cell division phase of development was complete, the quantity of cyclin B mRNA decreased, so that by 6 h after the spores had been placed into water, cyclin B mRNA was undetectable anywhere in the gametophytes (Fig. 3d).

β-Tubulin protein is abundant in the dry spores (Hart and Wolniak, 1998; Klink and Wolniak, 2001), and it becomes segregated into the spermatogous initials early in development of the gametophyte (Figs. 4a, b). Presumably, the redistribution of tubulin protein to the spermatogenous cells involves microtubule assembly (Klink and Wolniak, 2001). After 2 h of development, the β-tubulin protein became localized within the spermatogenous cells and is almost completely absent in the jacket cells (Fig. 4c). During

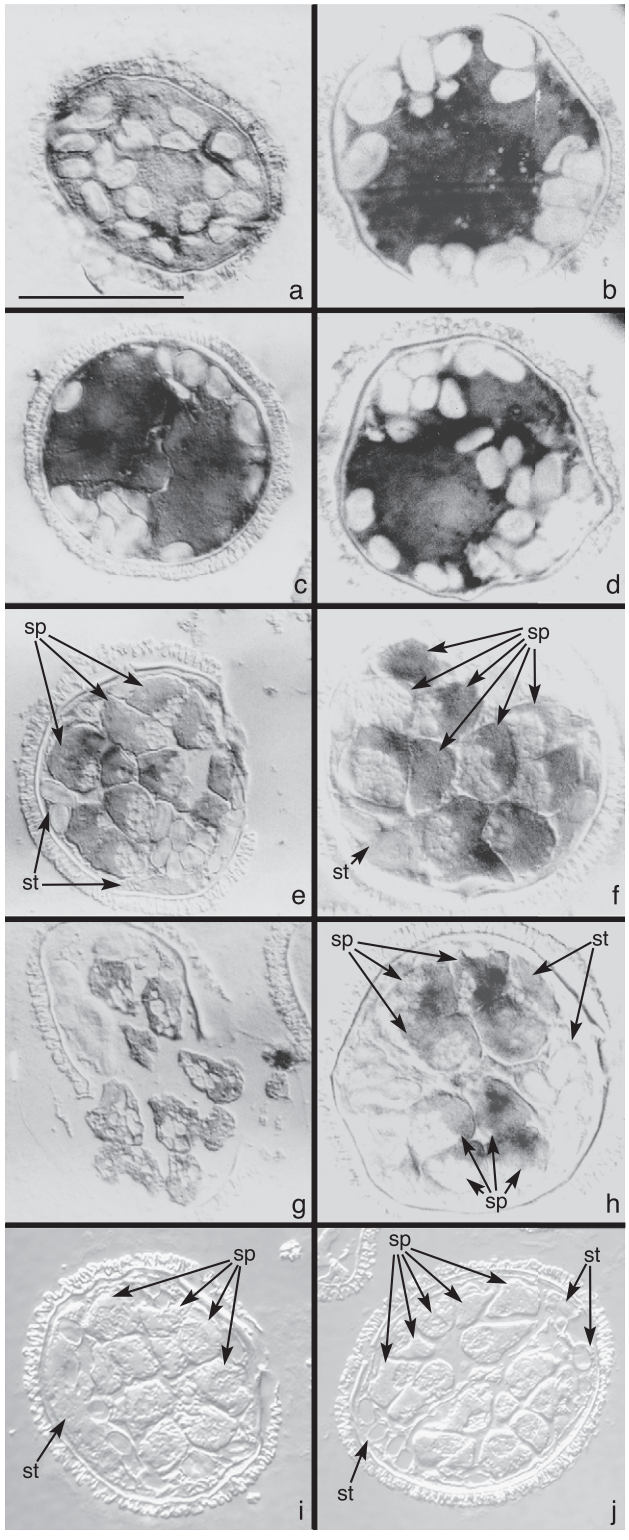

Fig. 6. Localizations of RNA helicase mRNA (a–d) and PRP-19 mRNA (e–h) during spermiogenesis by in situ hybridization assays. (a) RNA helicase mRNA is present and uniformly distributed throughout the cytosol of the newly imbibed spore. (b) At 2 h of development, RNA helicase mRNA is abundant in the central regions of the gametophyte, in cells that will later cause the spermatogenous cells. (c) At 6 h of development, RNA helicase labeling is moderate in sterile jacket cells (st) and more intense in spermatogenous cells (sp). (d) At 8 h of development, the maturing spermatids exhibit clear in situ RNA helicase labeling. (e) PRP-19 mRNA exhibits a uniform cytoplasmic distribution shortly after hydration of the spores. (f) At 1 h of development, the PRP-19 mRNA appears to be more concentrated in the central portion of the gametophyte. (g, h) At 6 h of development, the PRP-19 mRNA is almost entirely localized in the spermatogenous cells (sp) of the gametophyte, though a small amount of labeling is sometimes detectable in the sterile jacket cells (st). The concentrations of labeling seen in (h) were seen in some, but not the majority of gametophytes observed. (i, j) RNAi treatments, employing dsRNAs made from MvU11 (i) and MvU89 (j), reveal anomalies in the size of some of the spermatids, but the numbers of cell division cycles in the gametophyte appear to be normal. Scale bar = 25 μm.

spermatid maturation, the anti-β-tubulin antibody localization pattern became heavily concentrated in the anterior end of the spermatids, along the edges of the cells (Figs. 4d, e), where the microtubule ribbons and MLS were formed (Myles and Hepler, 1977). Anti-β-tubulin antibody labels the cytoskeleton, basal bodies, and ciliary axonemes of mature gametes (Klink and Wolniak, 2000) (Fig. 4f). β-Tubulin mRNA is detectable in newly hydrated spores (Fig. 5a), a result indicating that like centrin and cyclin B transcripts, it is present as part of a pool of mRNAs present in the gametophyte at the time the dry spore is placed into water. In contrast to β-tubulin protein, which aggregates in the spermatogenous initials (Fig. 4c), β-tubulin mRNA remained uniformly abundant in both jacket sterile cells and spermatogenous cells (Figs. 5b–e). No segregation or change in abundance of β-tubulin mRNA was observed until the sterile jacket cells degenerated, late in course of spermiogenesis (Fig. 5f).

Not all transcripts are uniformly distributed in the gametophyte

In addition to centrin, β-tubulin, and cyclin B, we analyzed the distribution patterns of an mRNA encoding RNA helicase (MvU-11, GeneBank Accession # CF867680) in developing gametophytes with in situ hybridization assays (Figs. 6a–d). We found that this transcript was readily detectable in newly hydrated spores (Fig. 6a) and that the labeling pattern became concentrated in the central portion of the gametophyte after 2 h of development (Fig. 6b). With the division cycles completed at 6 h of development, the spermatids exhibited moderately intense staining (Fig. 6b, sp) and the sterile jacket cells exhibited light, but nevertheless detectable staining for this mRNA (Fig. 6c, st). The RNA helicase transcript remained detectable in the developing spermatids in gametophytes fixed at 8 h (Fig. 6d), and was still detectable in mature gametes (data not presented).

In a fashion similar to that observed with RNA helicase mRNA, the distribution of an mRNA encoding a protein that closely resembles PRP-19 (MvU-89, GeneBank Accession # AAL92025), a component in the spliceosome complex (Cheng et al., 1993; Tarn et al., 1993), exhibited an even more distinctive segregation pattern in the gametophyte, with pronounced accumulations of the transcript in the spermatogenous cells. Our in situ hybridizations show that the PRP-19 transcript was uniformly abundant in the cytosol in newly hydrated spores (Fig. 6e), but after 2 h of development (Fig. 6f), it became localized preferentially into spermatogenous cells of the gametophytes. By 6 h of development (Fig. 6g), it was apparent that most of the mRNA had become concentrated inside the spermatogenous cells (Fig. 6g, sp: arrows); there was only light labeling in the sterile jacket cells (Figs. 6g, h, st: arrows). These mRNAs remained detectable in the spermatids (Fig. 6h, sp: arrows) through the remainder of development, and were even present in the mature spermatozoids (data not presented).

We next performed a series of RNAi experiments on gametophyte populations, targeting the destruction of MvU-11 (RNA helicase) and MvU-89 (PRP-19) mRNAs, through the addition of dsRNAs made from those two cDNAs. After 8 h of development, the presence of MvU-11 (Fig. 6i) and MvU-89 (Fig. 6j) dsRNA each alters the patterns of the cell divisions within the microspore wall. Instead of seeing two clusters of equally sized spermatids surrounded by six thin

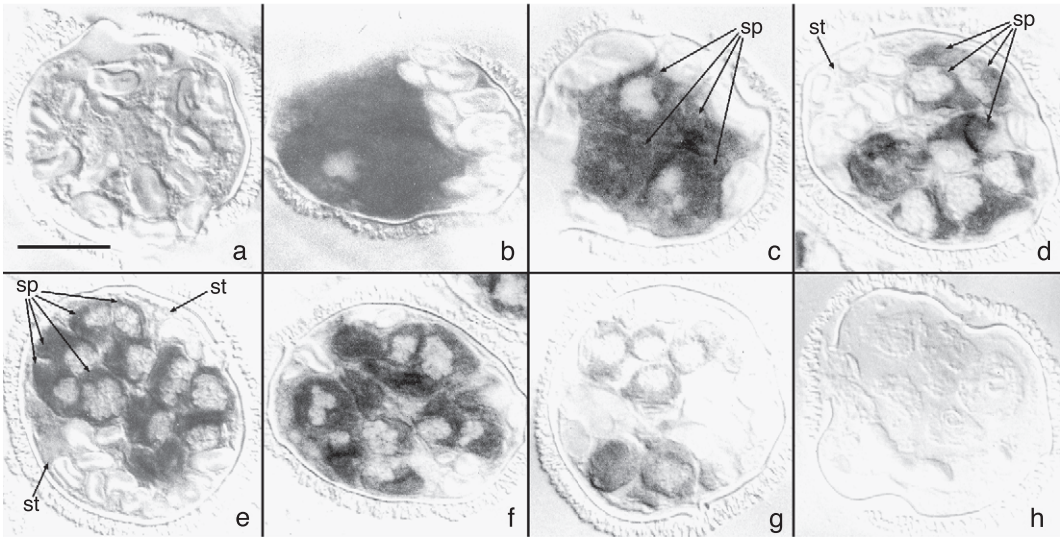

Fig. 7. Localizations of Poly(A)+ RNA distributions during spermiogenesis, by in situ hybridization assays. (a) Poly(A)+ RNA was not detectable in newly hydrated spores. (b) The label became abundant in the cytosol of all gametophytes 30 min after the spores had been placed in water. (c) Two hours of development, and only the spermatogenous cells (sp) are labeled. The unlabeled spots in (b) and (c) are nuclei. (d) Four hours of development. The spermatogenous cells of the gametophyte exhibit intense labeling with this probe. (e, f) Six hours of development. Only the spermatogenous cells of the gametophyte exhibit intense labeling with this probe. (g, h) Eight hours of development. The poly(A)+ RNA labeling pattern becomes less intense (g, h) as the spermatids mature. Scale bar = 25 μm.

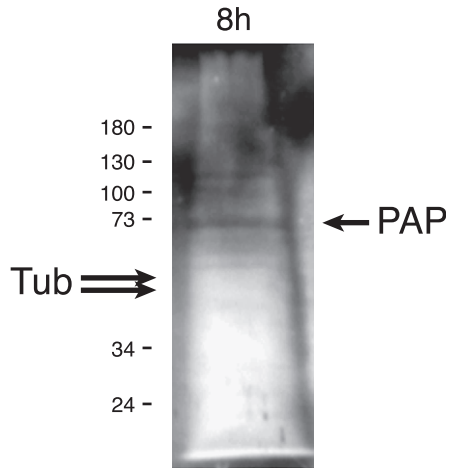

Fig. 8. Anti-PAP antibody labeling of fern gametophyte polypeptides. Polypeptides from fern gametophytes were isolated at 8 h of development, loaded onto a polyacrylamide gel, separated electrophoretically, and transferred to a blot. The isolates were probed with anti-PAP antibody that was detected by ECF fluorescence with a STORM 860 imaging scanner (Molecular Dynamics). The anti-PAP antibody binds to one major band with an apparent molecular weight of approximately 70 kDa. Several minor bands are also detected by the antibody. The blot was stripped and reprobed with anti-β-tubulin antibody, and the labeling pattern typical for the gametophyte extracts (Klink and Wolniak, 2001) is depicted at the left side of the blot with the double arrows. The band of polypeptide binding anti-PAP antibody does not overlap with that of the major tubulin bands.

sterile jacket cells, we found that the spermatids were variable in size and they exhibited anomalous shapes (Figs. 6i, j, sp: arrows). The sterile jacket cells were visible around the two groups of spermatids (Figs. 6i, j, st: arrows), but they were essentially normal in appearance. An important point about these treatments is that the numbers of cell division cycles always appeared to be normal; this result is in marked contrast to many of the RNAi treatments we have performed on gametophytes where development is arrested or greatly forestalled at a particular point during the phase of rapid cell divisions (Klink and Wolniak, 2003).

Polyadenylation of mRNA occurs only in the spermatogenous cells of the gametophyte

The distribution of Poly(A)+ RNA was monitored using a biotin-labeled oligo(dT) as a means to assess levels in RNA processing in the jacket cells and the spermatogenous cells during development. The distribution of poly(A)+ mRNA was distinctive and asymmetric during spermiogenesis (Fig. 7). The amount of mRNA polyadenylation in the dry spores was below the detection level (Fig. 7a). Within the first 30 min after imbibition (Fig. 7b), cytoplasmic polyadenylation was obvious by in situ labeling. Heavy staining was present in the cytosol of the antheridial initial and essentially undetectable inside the nucleus. After the first division took place (Fig. 7c), the distribution pattern for poly(A)+ RNA was restricted to the two cytoplasmic zones within the antheridial initial. These subdomains later become the spermatogenous cells. Thereafter, virtually all of the poly(A)+ RNA is present in the spermatogenous cells, with moderate to low labeling present in the sterile jacket

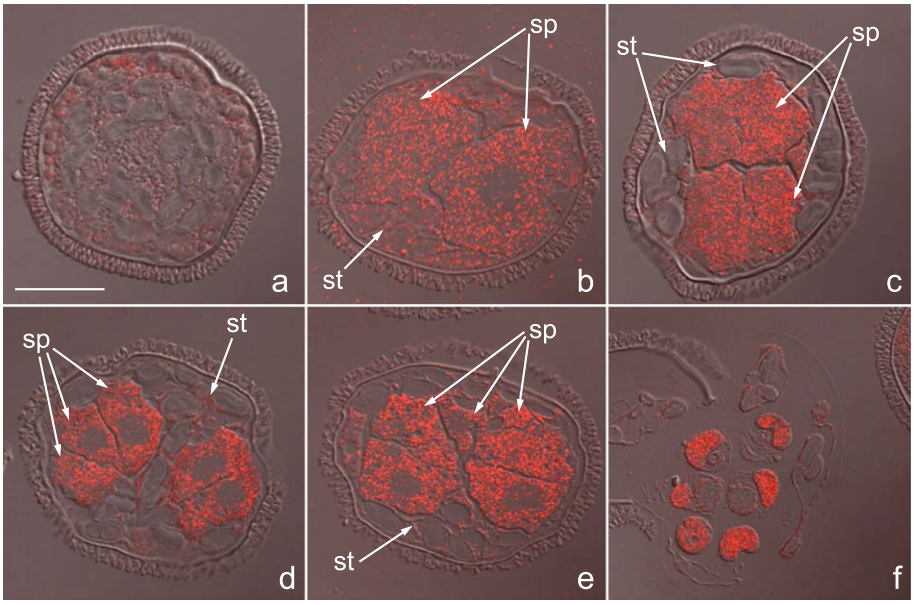

Fig. 9. Immunolocalizations of gametophytes using anticytoplasmic poly-A RNA polymerase antibody (anti-PAP) during spermiogenesis. (a) Just after spore hydration, there is a weak antibody label scattered throughout the cytoplasm of the gametophyte. (b) At 2 h of development, the anti-PAP antibody label is more abundant in the spermatogenous cells (sp) of the gametophyte, and less abundant in the sterile cells (st) of the gametophyte. The open area in the spermatogenous cell on the right is occupied by a nucleus. (c) At 4 h of development, the spermatogenous cells (sp) exhibit high levels of anti-PAP antibody labeling, while the sterile hacket cells (st) exhibit almost no detectable labeling with this antibody. (d, e) At 6 h of development, the spermatogenous cells (sp) are heavily labeled (the nuclei of these cells do not label with the antibody) and the sterile cells (st) exhibit almost no staining. (f) At 8 h of development, anti-PAP antibody labeling is particularly intense in the cytoplasmic vesicle of the maturing spermatids. Scale bar = 25 μm.

cells (Figs. 7d, e). As maturation of the spermatids neared completion at 8 h after imbibition, the amount of poly(A)+ RNA decreased significantly throughout the gametophytes, and eventually declined to low levels at the stage of spermatozoid release (Fig. 7f).

RNA PAP proteins are only present in the spermatogenous cells of the gametophyte

To study the distribution of RNA PAP proteins during spermiogenesis in *M. vestita*, we used a polyclonal antibody originally directed against *X. laevis* PAP for immunolabeling of fixed and sectioned gametophytes. The antibody binds to a few polypeptides on immunoblots that were generated from isolates from fern gametophytes, with the most prominent band exhibiting a mobility consistent with an apparent molecular weight of approximately 70 kDa (Fig. 8). On our immunoblots, we varied the amount of the antibody added to the fern protein isolate, and at dilutions of 1:500, only the 70 kDa band is recognizable (Fig. 8). This band does not overlap with the migration patterns for β-tubulin from the fern (Fig. 8); the arrows depict the positions for the tubulin bands in gametophyte isolates (Hart and Wolniak, 1998; Klink and Wolniak, 2001). For in situ immunolabeling studies, we used a primary antibody dilution of 1:1000.

Silver-enhanced immunogold localizations revealed that PAP protein was absent in the dry spores (Fig. 9a), but started to accumulate 2 h after imbibition (Fig. 9b). The anti-PAP antibody heavily labeled the cytoplasmic portion of the spermatogenous cells. Sterile jacket cells and the nucleus of spermatogenous cells showed limited anti-PAP antibody staining through successive stages of development (Figs. 9b, c). The antigen apparently becomes concentrated and appears to be abundant in the cytoplasm of the mature gametes, even near the time of their release (Fig. 9d).

Discussion

We have studied the distribution of various mRNA species throughout the course of male gametophyte development in *M. vestita* to gain insight into how the gametophyte begins as a single cell and within a few hours, undergoes cytoplasmic reorganization and successive division cycles to produce two distinctly different kinds of cells. Our results indicate that at least two different translational controls commonly observed in oogenesis and embryogenesis (Richter, 1996; Wickens et al., 1996) occur during spermiogenesis in *M. vestita*: specific RNA localization and cytoplasmic polyadenylation. The localization of certain mRNAs in the developing gametophytes allows specific proteins to be synthesized in the subcellular regions where they are required and prevents their expression in regions elsewhere in the gametophyte. Specific localizations of mRNA such as cytoskeletal proteins and regulatory proteins have been described in oocytes, embryos, and differentiated somatic cells.

Among the mRNAs we surveyed, centrin (Fig. 2), cyclin B (Fig. 3), and β-tubulin (Fig. 5) exhibit a random distribution throughout the cytosol of all cells in the gametophyte. In contrast, transcripts encoding RNA helicase (Figs. 6a–d) and PRP-19 (Figs. 6e–h) exhibit localization patterns restricted largely to the spermatogenous cells of the developing gametophyte. Immunoprecipitation studies have shown that PRP-19 is associated with the spliceosome during the splicing reaction (Cheng et al., 1993), and that it functions in pre-mRNA processing. Its restricted transcript distribution pattern suggests that the spermatogenous cells may possess the capacity to process and translate mRNAs that are distributed throughout the gametophyte, while the adjacent sterile jacket cells lack the capability to perform these functions. The question about mechanisms of fate determination is whether RNA helicase and PRP-19 are transcripts that exist in the dry microspore and are moved preferentially into the spermatogenous cells, or alternatively, they represent newly transcribed products whose transcription is activated after the spermatogenous initials are formed, and localized to occur only in spermatogenous cells. RNA helicase (Fig. 6a) and PRP-19 (data not presented) mRNAs are abundant in microspores immediately after they are placed into water. Development of gametes approaches completion at normal times in the presence of the transcriptional inhibitor α-amanitin (Klink and Wolniak, 2001), but we never saw gametes released from the spores after treatment with this inhibitor; these results show that almost the entire process of spermiogenesis can occur in the absence of new transcription. Thus, we suspect that transcripts like those that encode RNA helicase and PRP-19 are already present in the gametophyte and that they are moved into cytoplasmic subdomains that later become the spermatogenous initials early in development. The mechanisms responsible for the movement of these transcripts are of obvious interest. The loss of these mRNAs, induced by RNAi treatment (Figs. 6i–j), results in changes in spermatid appearance, but not spermatid number. It is likely that the proteins encoded by these mRNAs play roles in spermatid differentiation, a facet of development that we will explore fully elsewhere.

Recent work from our lab has shown that there is an asymmetric distribution of β-tubulin and centrin proteins in the gametophyte, which is clearly observable after 4 h of development (Klink and Wolniak, 2001). This localization pattern represents the movement of β-tubulin proteins that were already present in the dry microspore, and is probably the direct consequence of microtubule assembly and disassembly patterns during the early division cycles of the gametophyte. Since centrin is translated from a pool of mRNA that was present in the dry microspore, and since centrin mRNA is present in all cells of the gametophyte (Figs. 2a–d) but centrin protein is only detectable in the spermatogenous cells (Klink and Wolniak, 2001), then it

appears likely that the spermatogenous cells retain the capacity for centrin translation while the adjacent sterile jacket cells lack (or lose) the capacity for centrin translation. It seems clear that in addition to centrin, many transcripts important for the formation of the cytoskeleton and the ciliary apparatus are translated exclusively in spermatogenous cells (Klink and Wolniak, 2003).

We analyzed cyclin B mRNA distributions during gametophyte development because the spermatogenous cells continue to divide, while the sterile jacket cells lose this proliferative capacity. It was surprising to find cyclin B transcripts distributed in all cells of the gametophyte (Figs. 3a–d), until we looked at the distribution of polyadenylated RNA. The restricted distribution polyadenylated RNA in the spermatogenous cells (Figs. 7a–f) provides a clear sense that an important facet of cell fate determination in male gametophytes of *M. vestita* is through the localization of components that are involved in the regulation of translation.

Early characterizations of maternal genes responsible for body patterning in *Drosophila* have focused on mRNA localizations but it is now clear that the localized regulation of translation is of equal importance in development (Macdonald and Smibert, 1996; Seydoux, 1996). Regulated cytoplasmic polyadenylation, a process by which dormant mRNAs are translationally activated, can involve a cytoplasmic component, PAP (Gebauer and Richter, 1995). In oocytes, untranslated maternal mRNAs are stored in messenger ribonucleoprotein particles, where a set of proteins bind to the mRNA and prevent them from interacting with the translational machinery. The activation of oocytes is often accompanied by a lengthening of the poly(A) tail. Studies in mouse, *Xenopus*, and *Drosophila* have provided clear demonstrations that cytoplasmic polyadenylation controls the translational activation of many maternal mRNAs (Salles et al., 1994). The distribution pattern of PAP enzyme in the gametophyte of *M. vestita*, assessed by immunolabeling (Figs. 9a–f), is similar to that of the poly(A)+ RNA labeling pattern (Figs. 7a–f), and it is reasonable to suspect that the enzyme is responsible for the modifications to the transcripts. An obvious question that emerges from this study centers on how PAP becomes localized in the spermatogenous cells.

Recently, work from our laboratory (Hart and Wolniak, 1999) showed that mRNA from male gametophytes is incapable of supporting protein synthesis in vitro until at least 30 min after imbibition. We suggested that some form of RNA modification takes place in spores soon after they are placed into water (Hart and Wolniak, 1999). In this study, in situ hybridizations using oligo(dT) as probes on sectioned gametophytes revealed that there was very little poly(A)+ RNA in the dry spores. After 30 min, the level of poly(A)+ RNA increases dramatically (Fig. 7b). It is likely that RNA processing early in gametophyte development is likely to include the polyadenylation of transcripts residing in the spermatogenous cells. We also observed a dramatic decrease of poly(A)+ RNA labeling in spermatids close to maturation.

This study has focused on how the male gametophyte of *M. vestita* undergoes cytoplasmic reorganization during spermiogenesis to establish a germ line of spermatogenous cells and to segregate these cells from sterile cells. The two different kinds of cells are distinct after five division cycles (Hepler, 1976; Mizukami and Gall, 1966; Myles and Hepler, 1977; Sharp, 1914), but the establishment and segregation processes that define the underlying differences between these two types of cells occur much earlier. We hypothesized that compositional differences between sterile and spermatogenous cells may result from the selective movement of mRNAs and proteins into cellular domains that later differentiate into spermatogenous cells. We followed the distribution of several mRNA species throughout the course of spermiogenesis and found that most of these transcripts are present in all cells of the gametophyte. In contrast to the majority of transcripts we surveyed, we identified two mRNA species, an RNA helicase mRNA and a PRP-19 mRNA, which are segregated largely into spermatogenous cells. If RNA processing components become restricted to the subdomains that later differentiate into spermatogenous cells, then translation could become restricted to the spermatogenous cells of the gametophyte through the segregation of relatively few components. The exclusive localization of poly(A)+ RNA and PAP protein in spermatogenous cells further support the hypothesis that only mRNAs in the spermatogenous cells are processed and then translated, although most mRNAs are randomly distributed in all cells of the gametophytes.

Acknowledgments

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