The Appearance of Male Gamete-Specific Histones gH2B and gH3 during Pollen Development in *Lilium longiflorum*

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The male gametic (generative) nucleus within the nearly mature pollen of *Lilium longiflorum* contains specific variants of histone H2B and H3, which have been designated gH2B and gH3, respectively. Using specific antibodies raised against gH2B and gH3, we examined in detail the temporal and spatial aspects of the appearance of these histone variants during male gametogenesis. Neither gH2B nor gH3 was detected in microspores during meiotic division or in microspores before meiosis. However, both gH2B and gH3 were abundantly present in mid birefringent pollen and these histones continued to be detectable in germinated pollen tubes after pollination. Furthermore, immunocytochemistry revealed that these proteins were present not only in generative nuclei during the maturation process, but also in the two sperm nuclei formed by division of the generative nucleus. By contrast, these proteins were not found in the vegetative nuclei. It appears, therefore, that both gH2B and gH3 are specific to male gametic (generative and sperm) nuclei in *L. longiflorum*. The results also suggest that these male gamete-specific histones might be newly synthesized in the progenitor of sperm cells for the differentiation of male gametes, as occurs in spermatogenesis in animals. © 1995 Academic Press, Inc.

INTRODUCTION

Male gametogenesis in angiosperms is very different from that in animals, in particular after the completion of meiosis. In angiosperms, male gametes are produced in a male gametophyte (pollen or pollen tube) by two cell divisions of a haploid microspore after meiosis. In animals, by contrast, male gametes originate directly from spermatids after meiosis. The microspores liberated from pollen tetrads after meiosis undergo the first haploid mitosis within anthers. The division is a typical example of asymmetrical mitosis, giving rise to a large vegetative cell and a small generative cell. Soon the generative cell migrates into the cytoplasm of the vegetative cell. The generative cell finally divides into two sperm cells within a mature pollen grain before pollination (tricellular type) or within a germinated pollen tube after pollination (bicellular type) for double fertilization (e.g., Russell, 1991; McCormick, 1991; Tanaka, 1993). Thus, the postmeiotic development of microspores into multicellular pollen is indispensable for the differentiation of male gametes. Although the process of formation of male gametes in angiosperms is different from that in animals, the male gametes of angiosperms contain highly condensed chromatin as do the sperm of animals.

In rodents and sea urchins, specific histone variants appear in the testis and the composition of the nuclear proteins changes considerably during spermatogenesis (Poccia, 1986; Meistrich, 1989). In the rat, testis-specific variants of all histone molecules (H1, H2A, H2B, H3, and H4) are synthesized in the spermatogonia or in pachytene spermatocytes (Meistrich et al., 1985; Grimes et al., 1987). In the sea urchin, Sp H1 and H2B (variants of histones H1 and H2B) are also synthesized in spermatogonia and in premeiotic spermatocytes (Poccia et al., 1987; Poccia and Green, 1992). Thus, almost all histone variants appear before the onset of meiosis or during early meiosis and these histone variants are assumed, therefore, to be associated with specific events during meiosis, such as recombination, with the mechanics of meiosis, and with other meiotic transitions (Meistrich et al., 1985; Poccia et al., 1987). In the rat, the testis-specific histones are replaced by the testis-specific transition proteins in spermatids after meiosis, and the spermatocytes finally contain protamine in place of these basic proteins (Meistrich, 1989). In the sea urchin, somatic proteins H1 and H2B are completely replaced by phosphorylated Sp H1 and H2B, which are dephosphorylated late in spermiogenesis (Poccia et al., 1987). The protamine and dephosphorylated Sp histones are assumed to be related to the condensation of chromatin in the sperm nucleus (summarized in Kaisansky, 1989; Oliva and Dixon, 1991; Poccia and Green, 1992).

In male gametogenesis in angiosperms, meliotic histone, a variant of histone H1, has been shown to appear

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during male meiosis in lily and tulip (Sheridan and Stern, 1967). More recently, homologous proteins, designated pollen mother cell protein (PMCP) and meiotin-1, were reported in other liliaceous plants (Sasaki et al., 1990; Sasaki and Harada, 1991; Riggs and Hasenkampf, 1991; Hasenkampf et al., 1992). Moreover, the gene encoding meiotin-1 has been isolated from *Lilium longiflorum* (Riggs, 1994). Although meiotin-1 resembles somatic histone H1 in terms of its biochemical properties, its primary structure is very different from that of the somatic histone. Because meiotin-1 and PMCP appear to be distributed with strings of nucleosomes, it is assumed that meiosis-specific histone(s) is also associated with the higher-order structure of meiotic chromosomes, as is the case for many testis-specific histone variants. However, no histone variants have yet been shown to be directly involved in the differentiation of male gametes in angiosperms.

We reported recently that the generative nucleus within the pollen of *L. longiflorum* contains specific basic proteins (Ueda and Tanaka, 1994) and that two of those proteins are equivalent to variants of histones H2B and H3, respectively. These proteins were designated gH2B and gH3. In this report, the appearance and longevity of gH2B and gH3 were analyzed both immunohistochemically and immunocytochemically with reference to the various stages of male gametogenesis in *L. longiflorum*.

**MATERIALS AND METHODS**

**Plant Material**

*L. longiflorum* cv. Georgia was grown in a greenhouse. The various stages of microsporogenesis and pollen development in this lily can easily be estimated from the length of buds (Erickson, 1948; Tanaka et al., 1979). Under our greenhouse conditions, meiotic division and the first microspore mitosis occur in buds that are about 13-27 and 65 mm in length, respectively, and anthesis occurs when the buds reach 170 mm in length. In this study, we obtained microsporocytes during meiotic divisions (from buds of 13-27 mm in length), uninucleate microspore (buds of 40 and 60 mm), bicellular pollen grains (buds of 90, 120, and 150 mm), and mature pollen grains (from flowers after anthesis). Pollen tubes were obtained from 5-day-old pistils after artificial pollination. Root, bulb, stem, and leaf tissues were collected from plants 3 to 4 weeks after planting. Petals, pistils, anthers, and filaments were collected from flowers 3 days after anthesis.

**Isolation of Nuclei**

For isolation of microsporocytes and young microspores, each anther was cut open at one end with sharp forceps and the cells were extruded by gentle squeezing from the cut distal end. The cells after the late microspore stage were collected from each anther with a microspatula. The developmental stage of isolated cells was determined under a light microscope after staining with aceticarmine. Before disruption of cells, tapetal nuclei and other contaminants were removed from microsporocytes and microspores by suspending the extruded contents of anthers in a solution of 0.5 M sucrose. The suspension was filtered through nylon mesh (50 μm), and the microsporocytes and microspores were collected on the nylon mesh. This treatment was repeated twice.

The isolation of nuclei from microsporocytes, microspores, and pollen grains was carried out at 0-4°C. The cells were disrupted with a Teflon–glass homogenizer in nuclear isolation buffer (NIB), which contained 10 mM 2-[(N-morpholino)ethanesulfonic acid (Mes, pH 6.0), 5 mM ethylenediaminetetraacetic acid, 10 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM 2-mercaptoethanol, and 0.5 M sucrose, and then the mixture was filtered through nylon mesh (50 μm) to remove debris. The resulting suspension was centrifuged at 600g for 10 min, and nuclei were washed three times, with centrifugation, with NIB that contained 0.1% (v/v) Triton X-100 and once with NIB. The final pellet was used as the nuclear fraction. The nuclei of stems, leaves, petals, and pistils were isolated by previously described procedures (Ueda and Tanaka, 1994). Pollen tubes, roots, bulbs, anthers (without pollen), and filaments were disrupted with a Teflon–glass homogenizer in NIB. After the pellets obtained by centrifugation had been washed three times with NIB plus 0.1% (v/v) Triton X-100, they were used for extraction of histones.

**Extraction of Histones and Two-Dimensional Gel Electrophoresis**

Histones were extracted from isolated nuclei or disrupted tissues with 0.4 N H₂SO₄, and then the protein still present in the supernatant after centrifugation at 18,000g was precipitated with 20% trichloroacetic acid (TCA). The proteins were resolved by two-dimensional gel electrophoresis. Electrophoresis in the first dimension was performed in an acetic acid/urea/Triton (AUT) gel that contained 1 N acetic acid, 8 M urea, and 0.5% (w/w) Triton X-100 and in the second dimension in a gel that contained sodium dodecyl sulfate (SDS), as previously described (Ueda and Tanaka, 1994). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (0.3% in 50% methanol and 10% acetic acid).
Preparation of Antisera and Immunoblotting

Histones were fractionated by two-dimensional gel electrophoresis (AUT-SDS PAGE), and gh2B and gh3 were separately electroeluted from gel pieces into dialysis bags. The proteins were precipitated by addition of TCA to 20% and then resuspended in 0.5 ml of phosphate-buffered saline (PBS). After emulsification with 0.5 ml of complete Freund’s adjuvant, they were injected into the footpads of rats. Blood was taken every 2 weeks after the primary injection and antibody titers were monitored by immunoblotting. Control serum was collected from a rat that had not been immunized. Antiserum raised against histone H1 of this lily was also used as a positive control.

For immunoblotting, histones were separated by SDS-PAGE (Laemmli, 1970) and were then transferred electrophoretically to a nitrocellulose membrane (BioRad Laboratories, Richmond, CA). After the blotted membrane had been incubated for 1 hr in blocking solution, which consisted of 2% (w/v) bovine serum albumin (BSA) in PBS, it was incubated with primary antiserum, diluted to a final concentration of 1:500 with blocking solution, overnight at room temperature. After several washes in PBS supplemented with 0.1% (v/v) Tween 20, the membrane was incubated for 2 hr with the second antibody (biotinylated antibodies raised in sheep against rat Ig; Amersham International, Amersham, Bucks, UK) that had been diluted 1:400 with blocking solution. Finally, the membrane was incubated for 30 min with a solution of horseradish peroxidase-avidin (Vector Laboratories, Inc., Burlingame, CA) at 5 μg/ml. After several washes, the antigen was detected by incubation in a solution of 0.6 mg/ml 4-chloro-1-naphthol and 0.01% (v/v) H2O2 in PBS.

Immunofluorescence Staining

Immunocytochemistry was carried out using both isolated and cultured pollen protoplasts. Pollen protoplasts of *L. longiflorum* were isolated and cultured by the previously described procedures (Tanaka et al., 1987). Immunofluorescence staining was performed by the previously described method (Tanaka et al., 1989) with some modifications. Isolated or cultured pollen protoplasts were fixed in methanol at −20°C for 15 min and were then treated with 0.5% (v/v) Triton X-100 in PBS. The samples were affixed to coverslips that had been coated with poly-L-lysine. The coverslips were exposed to each antiserum at a final dilution of 1:200 in PBS supplemented with 0.1% (w/v) BSA and 0.1% (w/v) NaN3 overnight at room temperature in a moist chamber. After several washes, samples were treated with a 200-fold-diluted solution of biotinylated antibodies raised in sheep against rat Ig (Amersham International) for 2 hr at 37°C. The specimens were finally stained with fluorescein isothiocyanate-conjugated avidin (Vector Laboratories, Inc.) at 30 μg/ml in PBS for 1 hr at 37°C. The nuclei were stained with 4’6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, MO) at 1 μg/ml.

RESULTS

Specificity of gh2B and gh3 to Pollen

The presence of gh2B and gh3 was monitored immunochemically in several tissues of *L. longiflorum* with antisera raised against gh2B and gh3, respectively. Figure 1 shows the results of immunoblotting of histones extracted from root, bulb, stem, leaf, petal, stigma, anther (without pollen), filament, and pollen (mature pollen). Both gh2B and gh3 were detected exclusively in pollen, and they were undetectable in the other tissues (Figs. 1A and 1B). By contrast, antiserum raised against histone H1 recognized histone H1 of all tissues investigated (Fig. 1C).

The Appearance of gh2B and gh3 during Pollen Development

When histones extracted from the nuclei of microsporocytes, microspores, young bicellular pollen, and ma-
ture pollen were compared by two-dimensional gel electrophoresis, almost all proteins were found to be common to the four types of cell (Figs. 2A–2D). In particular, no significant differences were detected during meiosis and microspore development. Five classes of somatic histones, H1, H2A, H2B, H3, and H4, in this filmy were identified from comparisons with histones in somatic tissues such as leaf and petal (see Fig. 3 in Ueda and Tanaka, 1994). In addition, meiotic histone (Sheridan and Stern, 1967) was also detected in all cells examined. By contrast, gH2B and gH3 were detected only in mature pollen (Fig. 2D) and they were undetectable in microsporocytes, microsponges, and young bicellular pollen.

The results of immunoblotting with the antisera against gH2B and gH3 after histones had been extracted from cells at various developmental stages during the development of pollen are shown in Fig. 3. In agreement with the results of the analysis by two-dimensional gel electrophoresis, neither gH2B nor gH3 was detectable in microsporocytes or in microsponges (Fig. 3, lanes 1–4). Both proteins were, however, clearly detected in bicellular pollen from buds of 120 mm in length (Fig. 3, lanes 6–8). A weak band indicating the presence of gH2B was detected in the case of bicellular pollen from buds of 90 mm in length, whereas no bands were detected when gH3-specific antiserum was used at this stage (Fig. 3, lane 5). Moreover, although the respective bands were considerably weaker than those obtained with mature pollen, both gH2B and gH3 were also detected in pollen tubes (Fig. 3, lane 9).

Specific Distribution of gH2B and gH3 in Bicellular and Tricellular Pollen

The appearance of gH2B and gH3 during pollen development was also examined immunocytochemically. Fig.

![Image 3](image3.png)
Figure 4 shows immunofluorescence images, obtained with antisera against gH2B and gH3, of pollen protoplasts that had been isolated from bicellular pollen taken from buds of 90 and 120 mm in length. When protoplasts were stained with DAPI, two nuclei were observed within each protoplast (Figs. 4A, 4C, and 4E). It was difficult to distinguish the generative nucleus from the vegetative nucleus in pollen protoplasts from 90-mm buds, because both nuclei were very similar (Fig. 4A). By contrast, the generative nuclei in 120-mm buds could be distinguished...
from the vegetative nuclei by fluorescence staining. The generative nuclei generally fluoresced more brightly than the vegetative nuclei (Figs. 4C and 4E). The control serum, although it caused faint immunostaining of the cytoplasm, never recognized the nuclei (data not shown). The antisera against hH2B and hH3 also hardly recognized generative or vegetative nuclei in 90-mm buds. By contrast, when both specific antisera were used to immunostain pollen protoplasts from 120-mm buds, fluorescence was emitted only from the generative nuclei. No fluorescence was emitted from the vegetative nuclei (Figs. 4D and 4F). The fluorescence from the generative nuclei stained with antiserum against hH2B was considerably stronger than that from nuclei stained with the antiserum against hH3. When immunofluorescence staining of pollen protoplasts from 150-mm buds was performed, bright fluorescence was emitted from the generative nuclei that had been immunostained with each antiserum.

When the bicellular pollen protoplasts of *L. longiflorum* are cultured in White’s medium, the generative cell within each pollen protoplast divides into two sperm cells after 8–10 days of culture (Tanaka et al., 1987). Using such cultured protoplasts, we monitored the presence of hH2B and hH3 in male gametes. Figure 5 shows immunofluorescence images of pollen protoplasts after 10 days in culture. Each contained two sperm nuclei and one vegetative nucleus. The sperm nuclei were distinguishable from the vegetative nucleus by shape, size, and the intensity of fluorescence after staining with DAPI (Figs. 5A and 5C). When the cultured pollen protoplasts were stained with the antisera against hH2B and hH3, faint fluorescence was emitted from the cytoplasm of vegetative cells and bright fluorescence was emitted from the sperm nuclei but not from the vegetative nuclei (Figs. 5B and 5D).

**DISCUSSION**

In a previous report, we suggested that hH2B and hH3 might be specific to male gametic (generative) nuclei in *L. longiflorum* from an analysis by two-dimensional gel electrophoresis (Ueda and Tanaka, 1994). In the present study, such specificity was clearly demonstrated both immunocytochemically and immunofluorescence by use of specific antisera raised against hH2B and hH3. The results of immunoblotting showed that both hH2B and hH3 were present in pollen tubes, as well as in bicellular pollen that contained generative nuclei (Figs. 1 and 2). This observation suggests that sperm nuclei also contain hH2B and hH3 because pollen tubes contain many sperm nuclei as a result of the division of generative nuclei. We assume that the low intensity of bands in the case of pollen tubes was not due to a decrease in levels of hH2B and hH3 during pollen development but, rather, to contamination by acid-soluble proteins other than histones in pollen tubes. This assumption is supported by the results of immunocytochemical staining of cultured pollen protoplasts (Fig. 5). Although the sperm nuclei formed in the pollen protoplasts were artificial, the sperm nuclei contained very condensed chromatin, as do sperm nuclei in vivo (Ueda et al., 1990). Moreover, the antisera against hH2B and against hH3 stained the sperm nuclei similarly to the generative nuclei. We also confirmed that both hH2B and hH3 are absent not only from nuclei in somatic tissues, but also from nuclei of microsporocytes and microspores and from vegetative nuclei in pollen. Therefore, it appears that both hH2B and hH3 are specific to the generative and sperm nuclei in *L. longiflorum*.

The results of the present study with specific antisera also reveal temporal and spatial aspects of the appearance of hH2B and hH3 during the development of generative cells within bicellular pollen. For the immunocytochemical and immunofluorescence analysis of bicellular pollen, we chose to examine four stages; namely, early (90-mm bud), middle (120-mm bud), late (150-mm bud), and mature (after anthesis) stages. Immunoblotting with antiserum against hH2B revealed barely any antigen in the early bicellular pollen (Fig. 3A, lane 5), and no hH3 at all was detected at this stage (Fig. 3B, lane 5). However, both hH2B and hH3 were abundantly present in the mid bicellular pollen (Figs. 3A and 3B, lane 6). On the other hand, in immunocytochemical observations, no significant fluorescence was detectable at the early bicellular stage. But, at the middle stage, strong fluorescence was emitted from generative nuclei that had been stained with antiserum against hH2B, though the fluorescence after staining with antiserum against hH3 was still weak (Fig. 4). At the late and mature stages, strong fluorescence was similarly observed after staining with antiserum against hH3, as well as against hH2B (data not shown). Thus, although the timing of first appearance of hH2B and of hH3 seemed to differ, both accumulated to a remarkable extent during the mid bicellular stage.

In animals, the only known histone variant that is synthesized after meiosis is the spermatid-specific histone H2B (ssH2B) of the mouse (Moss et al., 1989), although several histone variants are known also to be synthesized in spermagonia or spermatocytes (Meistrich et al., 1985; Poccia et al., 1987). The ssH2B is present at only low levels in mature sperm (Moss and Orth, 1993). Meiotic histone in *Lilium* is also known to appear in microsporocytes before the onset of meiosis (Hasenkampf et al., 1992). By contrast, we found that both hH2B and hH3 were first synthesized in male gametic (generative) cells and were abundant in male gametes (sperm
Fig. 5. Immunofluorescence staining of cultured bicellular pollen from *L. longiflorum*. Each protoplast after 10 days in culture was double-stained with DAPI (left panels; A and C) and with primary antibodies plus FITC-labeled second antibodies (right panels; B and D). Primary antibodies were the antiserum against gH2B (B) and the antiserum against gH3 (D). s, sperm nucleus; v, vegetative nucleus; Bar, 20 μm.

cells. They also seemed to be more abundant than meiotic histone. Therefore, gH2B and gH3 appear to be new types of histone variant.

Some aspects of postmeiotic gene expression have been well documented in plants (Mascarenhas, 1990; McCormick, 1991) and animals (Erickson, 1990). Among the genes expressed in animals some are specific genes that encode sperm- or testis-specific proteins, such as the transition proteins or protamines (Erickson, 1990). The genes that are expressed postmeiotically in higher plants are all specific to the male gametophytic (vegetative) cells within pollen (McCormick, 1991; Twell, 1992), and genes expressed specifically in generative and sperm cells have not been reported. Recent studies suggest that pools of translatable mRNA exist in the generative and sperm cells of several plant species (Dumas and Mogensen, 1993; Knox et al., 1993; Zhang et al., 1993). The contents of the pools of mRNAs in these cells might have
been distributed unequally at the first microspore mitosis. However, the results of the present study suggest the possibility of gene expression that is specific to male gametic cells.

The generative cell in which both gH2B and gH3 appeared and accumulated is the immediate progenitor of the male gametes. Therefore, it can be anticipated that these proteins will be found to play an important role in the differentiation of male gametes. In general, the nuclei of male gametes of higher plants have elongated and highly condensed chromatin, as do animal sperms (McCormick, 1993; Tanaka, 1993). Our preliminary observations indicate that the generative cells change in shape from nearly spherical to ellipsoidal during growth of buds from 120 to 150 mm in length and that nuclei become elongated and highly condensed with the development of generative cells within bicellular pollen. Therefore, the accumulation of gH2B and gH3 seems to be associated with the morphological changes in the chromatin of the generative nucleus. To clarify the relationship between male gamete-specific histones and the condensation of chromatin, further detailed immunocytochemical studies and examinations by immunoelectron microscopy are required.

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