Distribution of Poly(A)-containing RNA during Normal Pollen Development and during Induced Pollen Embryogenesis in *Hyoscyamus niger*

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ABSTRACT The distribution of poly(A)-containing RNA [poly(A)+RNA] in pollen grains of *Hyoscyamus niger* during normal gametophytic development and embryogenic development induced by culture of anther segments was followed by *in situ* hybridization with [^{3}H]-polyuridylic acid as a probe. No binding of the isotope occurred in pollen grains during the uninucleate phase of their development. Although [^{3}H]polyuridylic acid binding sites were present in the generative and vegetative cells of maturing pollen grains, they almost completely disappeared from mature grains ready to germinate. During pollen germination, poly(A)+RNA formation was transient and was due to the activity of the generative nucleus, whereas the vegetative nucleus and the sperm cells failed to interact with the applied probe. In cultured anther segments, moderate amounts of poly(A)+RNA were detected in the uninucleate, nonvacuolate, embryogenically determined pollen grains. Poly(A)+RNA accumulation in these grains was sensitive to actinomycin D, suggesting that it represents newly transcribed mRNA. After the first haploid mitosis in the embryogenically determined pollen grains, only those grains in which the generative nucleus alone or along with the vegetative nucleus accumulated poly(A)+RNA in the surrounding cytoplasm were found to divide in the embryogenic pathway. Overall, the results suggest that, in contrast to normal gametophytic development, embryogenic development in the uninucleate pollen grains of cultured anther segments of *H. niger* is due to the transcriptional activation of an informational type of RNA. Subsequent divisions in the potentially embryogenic binucleate pollen grains appear to be mediated by the continued synthesis of mRNA either in the generative nucleus or in both the generative and vegetative nuclei.

A uninucleate microspore, born out of a reduction division of the microsporocyte, is the single-celled beginning of the male gametophytic generation of angiosperms. The microspore matures into the pollen grain which undergoes two characteristic divisions during its ontogeny. The first division of the pollen grain, designated as the first haploid mitosis, gives rise to a small generative cell and a large vegetative cell. This is followed by another mitotic division of the generative cell to yield two sperms. The second division generally takes place in the pollen tube formed from the vegetative cell of the germinating pollen grain. The pollen grain with a pollen tube and its contents, consisting of the generative cell devoid of a cell wall and the vegetative cell nucleus loose from its cytoplasm, is the mature male gametophyte.

Although pollen grains of angiosperms are thus programmed for terminal differentiation into pollen tubes and gametes, culture of anthers of certain plants at an appropriate stage of development in a simple nutrient medium has been shown to evoke repeated divisions in a small proportion of the enclosed pollen grains. The multicellular pollen grains formed afterward go through an embryogenic type of development leading to the production of embryolike structures (embryoids) and plantlets with a haploid set of chromosomes (see 29, 42, 44 for reviews). Earlier studies (28, 31) have established that in cultured whole anthers and anther segments of *Hyoscyamus niger* (henbane; Solanaceae), a large number of embryoids arise by the division of the generative cell. Here the vegetative cell does not divide or divides only sparingly to form a suspensorlike structure on the organogenetic part of the embryoid originating from the generative cell. Embryoids are also formed by the division of

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both generative and vegetative cells, but the latter alone has seldom been found to divide in the embryogenic pathway. Autoradiographic localization of DNA synthetic activity in the vegetative and generative cells and their division products during pollen embryogenesis, using [^{3}H]thymidine as a probe, has subsequently confirmed this (30).

More recent studies (32, 33) have demonstrated that certain nonvacuolate, uninucleate pollen grains of *H. niger* become embryogenically determined as early as the first hour of culture of anthers and begin to incorporate [^{3}H]uridine, a precursor of RNA synthesis, into their nuclei. After the first haploid mitosis, embryogenic divisions are initiated in pollen grains in which the generative nucleus or both the generative and vegetative nuclei synthesize RNA, whereas those in which RNA synthesis occurs exclusively in the vegetative nucleus become starch filled and nonembryogenic. Thus, because pollen embryogenesis in *H. niger* appears to be correlated with an initial level of RNA synthesis in the uninucleate pollen grain and in the generative and vegetative nuclei formed afterward, it is of interest to determine whether this involves the synthesis of nonribosomal RNA that contains information for embryogenic divisions.

It is now well established that most hnRNAs and mRNAs of plant, animal, or viral origin contain a covalently linked polyadenylic acid sequence, although it has not been conclusively shown that all polyadenylic acid-containing RNA [poly(A)+RNA] is either hnRNA or mRNA (6, 11, 22). Nonetheless, the presence of poly(A) tracts on putative mRNAs makes it feasible to monitor the distribution of informational macromolecules in cells by localizing poly(A)+RNA by appropriate cytological methods. Here I have followed the distribution of poly(A)+RNA during pollen embryogenesis in *H. niger* with an *in situ* method to hybridize [^{3}H]polyuridylic acid [poly(U)] to poly(A)+RNA in histological preparations. For comparative purposes, I have also investigated the distribution of poly(A)+RNA during the development of pollen grains and their germination. It is hoped that the results will take us a step closer to describing how developmental processes in the uninucleate pollen grains are altered so as to produce embryoids and plantlets, instead of pollen tubes and gametes. A preliminary account of this work has been published (34).

MATERIALS AND METHODS

Plant Material

Plants of an annual variety of *H. niger* were raised from seeds in a growth chamber at 20°C under a photoperiod of 18 h, provided by fluorescent and incandescent lamps, and allowed to flower under the same conditions, as previously described (31).

Preparation of Stages of Pollen Development and Germination

Anthers containing microspore tetrads and pollen grains at different stages of development were dissected from flower buds and open flowers and immediately fixed in 70% ethanol-acetic acid mixture (3:1) for 24 h. They were dehydrated through 90 and 100% ethanol, *n*-propanol and *n*-butanol (12 h each) and embedded in glycol methacrylate (15). Anthers were sectioned longitudinally at 7 μm thickness on a rotary microtome equipped with a steel knife. Sections were attached to slides with drops of water and, after draining off water, slides were dried at 25°C. One slide each from at least three anthers of a flower bud or open flower was annealed in a hybridization experiment.

Later stages of development of the male gametophyte were obtained from germinating pollen grains. For this purpose, anthers from flowers open for 4–8 h were slit vertically and pollen grains squeezed into 5 ml of Millipore-sterilized (Millipore Corp., Bedford, Mass.) 2% sucrose–50 mg/l boric acid solution contained in 5-cm diameter sterile petri dishes. The pollen cultures were maintained at 25°C in a dark incubator, and samples were fixed at intervals of 0–15 min up to 1 h after sowing, and thereafter at 1-h intervals during a germination time-course of 12 h. They were processed for microtomy and sectioned as described earlier. In certain experiments, pollen grains were allowed to germinate on drops of medium placed on slides previously dipped in 0.5% gelatin and dried. Slides with pollen grains were kept in a petri dish whose halves were lined with moist filter papers and incubated at 25°C in the dark. At 1-h intervals up to 12 h, a silicone-coated cover slip was placed over the slide and gently pressed down. The slide was placed over dry ice and, after peeling off the cover slip, it was dipped in ethanol-acetic fixative for 5 min before use in *in situ* hybridization experiments. Two slides per time interval were annealed in a hybridization run.

Preparation of Slides of Pollen Embryogenesis

Flower buds containing anthers at the uninucleate stage of pollen development were sterilized for 5 min in 12% Clorox and washed several times with sterile distilled waster. One anther from each flower bud was routinely fixed in aceto-carmine to determine the stage of pollen development, and the remaining four anthers were segmented as described previously (31). All eight anther segments from a flower bud were cultured on the surface of 10 ml of Bourgin and Nitsch's (5) solidified medium contained in 1-oz French square bottles and incubated at 26°C under a 12-h photoperiod of weak fluorescent and incandescent light. Under these conditions of culture, multicellular embryoids are visible in squash preparations or sections of anther segments 3–4 d after culture (31). Immediately after sterilization, and at intervals of 1, 2, 4, 8, and 12 h and then at 12- to 24-hr intervals up to 8 d after culture, all anther segments from one flower bud were fixed in pairs in ethanol-acetic acid and processed for microtomy. One slide from each pair of cultured anther segments per time interval was annealed in a hybridization run.

In Situ Hybridization

Conditions for *in situ* hybridization with [^{3}H]poly(U) are based on a modification of the method of Capco and Jeffery (8). In the protocol followed here, 75-μl aliquots of 2.0 μCi/ml [^{3}H]poly(U) (New England Nuclear Co., Boston, Mass.; 5.2–6.1 Ci/mmol sp act) were applied to slides that were previously rinsed in the hybridization buffer (10 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM $MgCl_2$). The slides were then sealed with an acid-washed cover slip and incubated for 4 h at 30°C in a moist chamber equilibrated with the hybridization buffer (26). After annealing, slides were successively rinsed in the hybridization buffer and RNase-digestion buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 1 mM $MgCl_2$). Uncomplexed [^{3}H]poly(U) was removed by treatment of slides with pancreatic RNase A (Sigma Chemical Co., St. Louis, Mo.; 50 μg/ml RNase-digestion buffer) for 1 h at 37°C. Finally, slides were rinsed once in RNase-digestion buffer, twice in distilled water, extracted with 5% ice-cold trichloroacetic acid for 15 min, rinsed in distilled water, and air-dried. [^{3}H]Poly(U) binding sites were detected autoradiographically by dipping slides in Kodak NTB_3 liquid emulsion which had been diluted with an equal volume of water. After exposure in the dark for 4 wk, slides were developed using standard darkroom techniques. They were stained through the processed emulsion with 0.1% eosin Y, dehydrated, and mounted in euparal. To enhance visibility of nuclei in the pollen tube, some slides of germinating pollen grains were stained through the processed emulsion with acetocarmine.

Photography and Scoring of Slides

Slides were examined in a Zeiss Photomicroscope III with × 63 oil immersion objective. To determine the extent of [^{3}H]poly(U) binding in the cells of the male gametophyte and embryogenic pollen grains, the number of silver grains appearing over the nucleus and surrounding cytoplasm were counted from random samples. Silver grains were also counted from an area adjacent to the cells binding the label to provide an estimate of background. Each figure in the tables is based on counts made from at least 25 pollen grains. Photographs were made on Kodak Panatomic-X film using bright-field or Nomarski optics; for photographing autoradiographs with Nomarski optics, the direct interference contrast slider on the objective was adjusted to show silver grains black.

In situ hybridization experiments described here were done on four different occasions over a period of 2 yr, each time using slides made from a freshly fixed batch of plant materials and a new lot of [^{3}H]poly(U). Because one lot of 25 μCi [^{3}H]poly(U) is sufficient to anneal ~160 slides, this has enabled repetition of all experiments at least once. Although there were differences in silver grain counts between repetitions of the same experiment, the patterns of incorporation observed were similar. For this reason, the quantitative data given are based on examination of slides from one run of an experiment.

RESULTS

Specificity of [^{3}H]poly(U) Probe

The specificity of [^{3}H]poly(U) as a probe to localize the site of poly(A)+RNA accumulation by *in situ* hybridization has been established for tapetal cells of anthers of *H. niger* by pretreatments with alkali and specific RNases (35). Similar control experiments were performed in this work with sections of pollen grains allowed to germinate in the sucrose-boric acid medium for 30–45 min and of anther segments cultured for 4–8 h. Although data relating to [^{3}H]poly(U) binding in germinating pollen grains are given here, the responses of embryogenic pollen grains from cultured anther segments to the different pretreatments during *in situ* hybridization were qualitatively similar to those of germinating pollen grains. As seen in Table I, [^{3}H]poly(U) binding sites present in germinating pollen grains are selectively removed by pretreatment of sections with RNase A dissolved in a buffer containing 10 mM KCl, suggesting that they represent poly(A) sequences (1, 12). On the other hand, sections pretreated with RNase A dissolved in a high ionic strength (100 mM KCl) buffer were labeled to the same extent as untreated sections. In the experiment using RNase A dissolved in low ionic strength buffer, it was important to demonstrate that the enzyme pretreatment was effective in its interaction with pollen grains rather than by subsequent hydrolysis of [^{3}H]poly(U) by residual RNase activity. For this purpose, [^{3}H]poly(U) was recovered after hybridization of slides pretreated with RNase A. When the ability of the recovered [^{3}H]poly(U) to hybridize sections of pollen grains on untreated slides was tested, more or less the same level of radioactive binding as in the control slides was observed. This experiment thus verified that the reduction in [^{3}H]poly(U) binding sites on pollen grains pretreated with RNase A dissolved in low ionic strength buffer was not due to residual enzyme activity. In another experiment, sections were pretreated with dilute alkali which presumably hydrolyzed RNA into its constituent nucleotides, before annealing with [^{3}H]-poly(U); under these conditions, the applied probe exhibited little affinity for the alkali-resistant material of pollen grains. Similar results were obtained when sections pretreated with RNase T_2, which hydrolyzes phosphodiester linkages with adjacent adenylic acid residues (43), were subjected to *in situ* hybridization with [^{3}H]poly(U).

The most serious problem in *in situ* hybridization experiments using [^{3}H]poly(U) is the possibility that the isotope might form stable complexes with nuclear or cytoplasmic DNA of the cell. Therefore, additional control experiments were done to establish that no significant association between [^{3}H]poly(U) and DNA occurred during annealing. Data in Table I show that pretreatment of slides with DNase before annealing did not lead to a decreased binding of the isotope in the pollen grains. In fact, as noted in other investigations (8, 35), there was an appreciable increase in the number of silver grains on the pollen after DNase treatment. Although the reasons for this are not clear, the results rule out a possible interaction between [^{3}H]poly(U) and oligo-(dA:dT) sequences known to exist in nuclear (3, 4, 14, 16, 21, 38) and mitochondrial (7, 27) DNA of certain organisms; for this reason, a step involving DNase treatment of sections before annealing, used in other studies (9, 20), was not included in the protocol here. Generally, temperatures above 45°C have been shown to prevent the association of [^{3}H]poly(U) with oligo-(dA) clusters of DNA (36). However, annealing experiments described here were done at 30°C, because the plastic sections from slides annealed at higher temperatures became loose during subsequent rinsing steps. To eliminate the possibility that the applied probe was interacting with oligo-(dA) sequences of DNA at low temperatures, one set of slides was annealed at 50°C. Comparison of

TABLE I

Effect of Different Pretreatments on the Distribution of Autoradiographic Silver Grains in Germinating Pollen Grains Subjected to In Situ Hybridization with [^{3}H]poly(U)

Pretreatments	Number of silver grains per pollen grain ± SD	
	Control	Pretreated
KOH*	48.8 ± 4.6	12.6 ± 1.1
RNase A with 10 mM KCl‡	29.8 ± 2.6	4.8 ± 0.9
RNase A with 100 mM KCl‡	42.6 ± 3.9	40.3 ± 3.7
RNase T_2§	38.6 ± 4.0	8.8 ± 0.9
DNase‖	46.7 ± 4.2	59.8 ± 4.9
None¶	29.6 ± 2.8	25.3 ± 2.5
50°C**	44.2 ± 4.1	49.6 ± 4.9

* 0.3 N KOH for 24 h at 37°C.

‡ 50 μg RNase A/ml RNase-digestion buffer but with 10 mM KCl (instead of 100 mM KCl) for 16 h at 37°C. For RNase A with 100 mM KCl, the enzyme was dissolved in RNase-digestion buffer at 50 μg/ml and slides incubated for 16 h at 37°C.

§ 100 U of RNase T_2 was dissolved in 1 ml of RNase-digestion buffer, and 100-μl aliquots were applied to slides. Slides were covered with cover slips and incubated for 24 h at 37°C in a moist chamber equilibrated with RNase-digestion buffer.

‖ 100 μg DNase I/ml buffer [100 mM Tris-HCl (pH 7.6), 3 mM $MgCl_2$] for 1 h at 37°C.

¶ One set of slides (without any pretreatment) was annealed with [^{3}H]-poly(U) while another set was pretreated with RNase A in the presence of 10 mM KCl (second treatment in the Table) before annealing. After annealing, [^{3}H] poly(U) was recovered from both sets of slides and applied to new slides. Figures in "control" column indicate the number of autoradiographic silver grains on new slides annealed using [^{3}H]poly(U) recovered from slides that were not pretreated; figures in "pretreated" column refer to the number of silver grains on new slides annealed using [^{3}H]poly(U) recovered from RNase-pretreated slides.

** One set of slides (control) was annealed at 30°C, whereas the other set (pretreated) was annealed at 50°C.

TABLE II

Development of Autoradiographic Silver Grains on Pollen Grains of Different Stages of Development after In Situ Hybridization with [^{3}H]poly(U)

Stages of pollen development	Number of silver grains per pollen grain ± SD
Uninucleate, immediately after release from tetrad	1.2 ± 0.2
Uninucleate, nonvacuolate*	1.5 ± 0.2
Uninucleate, vacuolate	3.6 ± 0.5
Bicellular, starch grains not present‡	5.2 ± 0.55
Bicellular, starch grains not present	11.0 ± 1.05
Bicellular, isolated starch grains present, protoplasmic protrusion absent	38.2 ± 2.0
Bicellular, starch-filled, from a flower bud ready to open, protoplasmic protrusion present	17.0 ± 1.5
Bicellular, starch-filled, from a flower bud open for 4–8 h, protoplasmic protrusion present	4.5 ± 0.65
Bicellular, starch-filled, from a flower open for >12 h, protoplasmic protrusion present‡	3.2 ± 0.6

* Optimum embryogenic induction is obtained when anther segments containing pollen grains at this stage are cultured.

‡ Anthers collected from a different plant. Pollen grains of other stages of development shown in the table are from progressively older flower buds and open flowers of a single plant.

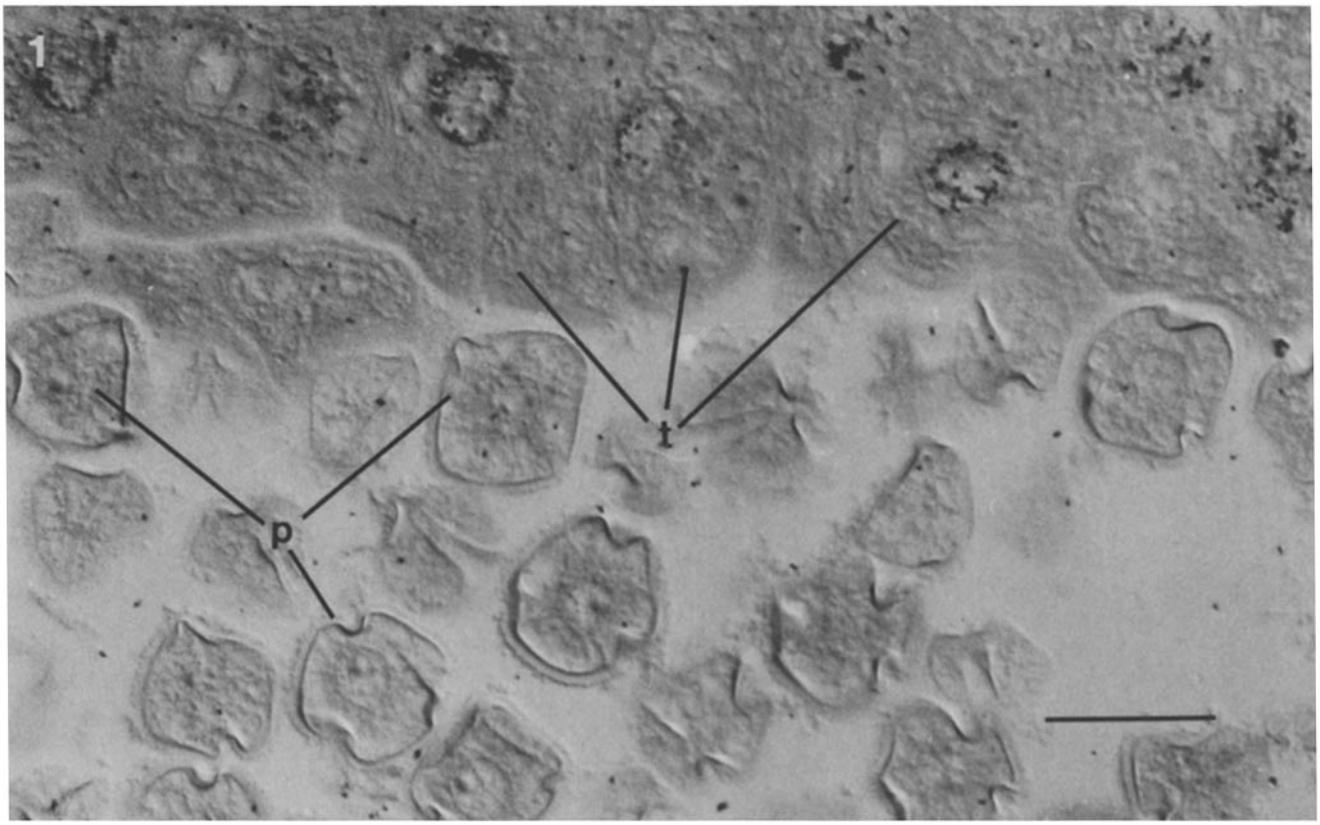


FIGURE 1 Section of an anther at the uninucleate, nonvacuolate pollen grain stage, showing [^{3}H]poly(U) binding into the tapetal cells (t). Binding of the isotope into pollen grains (p) is barely visible above background. Anthers are generally cultured at this stage of pollen development to induce embryogenic growth. Photographed with Nomarski optics. Bar, 20 μm. × 1,100.

the density of silver grains on pollen grains from sections annealed at 30° and 50°C showed no appreciable differences. Finally, another artifactual site of binding of [^{3}H]poly(U) to be taken into account is protein (10). Because ethanol-acetic acid fixative used in this work is known to remove some basic proteins from tissues (17), it is felt that such binding is probably not significant in this system, and hence no modifications were introduced in the hybridization protocol to prevent the association of the radioactive probe with cellular proteins. In summary, these experiments establish that autoradiographic localization of silver grains in pollen grains of *H. niger* following *in situ* hybridization with [^{3}H]poly(U) is due to the formation of stable complexes with poly(A) sequences.

[^{3}H]Poly(U) Binding during Development of the Pollen Grain

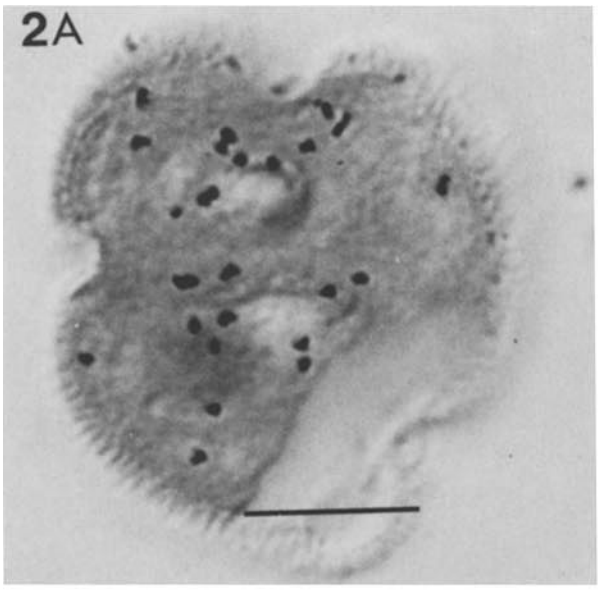
Table II presents data on [^{3}H]poly(U) binding density levels in pollen grains of different ages of *H. niger*. In agreement with a previous study (35), in sections of anthers subjected to *in situ* hybridization with [^{3}H]poly(U), little binding of the radioactive label occurred in the uni- or early bicellular pollen grains, although binding sites were detected in the tapetal cells (Fig. 1). If poly(A)+RNA accumulation reflects mRNA activity, it is clear that the early phase of development of pollen grains of *H. niger* is not accompanied by the synthesis of mRNA. These observations are consistent with the results of investigations on other plants (25, 40), which have shown that much of the total RNA synthesized in pollen grains up to the stage of the first haploid mitosis is ribosomal.

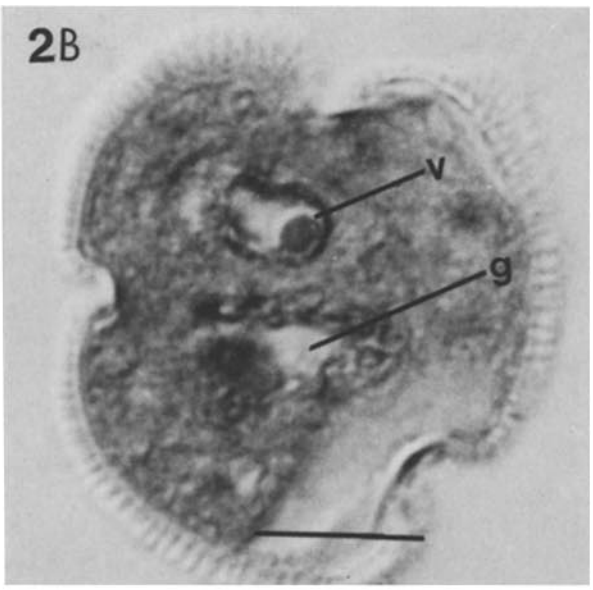
After the disintegration of the tapetum, a low level of binding of [^{3}H]poly(U) was found over the nuclei of the generative and vegetative cells of the pollen grains (Fig. 2). During further development of the pollen grains, the number of silver grains in the cytoplasm surrounding these nuclei increased until the maximum was reached shortly before the pollen grains became starch filled (Fig. 3). This presumably suggests that polyadenylated nuclear RNA is processed and exported as mRNA into the cytoplasm of the pollen cells. Although a cell wall between the generative and vegetative cells has frequently been observed in pollen grains of the type illustrated in Figs. 2 and 3, this could not be identified in the more mature pollen grains.

FIGURE 2 Section of a bicellular pollen grain showing [^{3}H]poly(U) binding into the nuclei of the generative (*g*) and vegetative (*v*) cells. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

FIGURE 3 Section of a late bicellular pollen grain showing intense [^{3}H]poly(U) binding in the generative and vegetative cells. *g*, nucleus of the generative cell; *v*, nucleus of the vegetative cell. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

FIGURE 4 Section of a bicellular pollen grain, ready to germinate, showing the absence of [^{3}H]poly(U) binding in the generative and vegetative cells. *g*, nucleus of the generative cell; *v*, nucleus of the vegetative cell. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Arrow points to the protoplasmic protrusion. Photographed with Nomarski optics. Bars, 10 μm. × 1,500.


2A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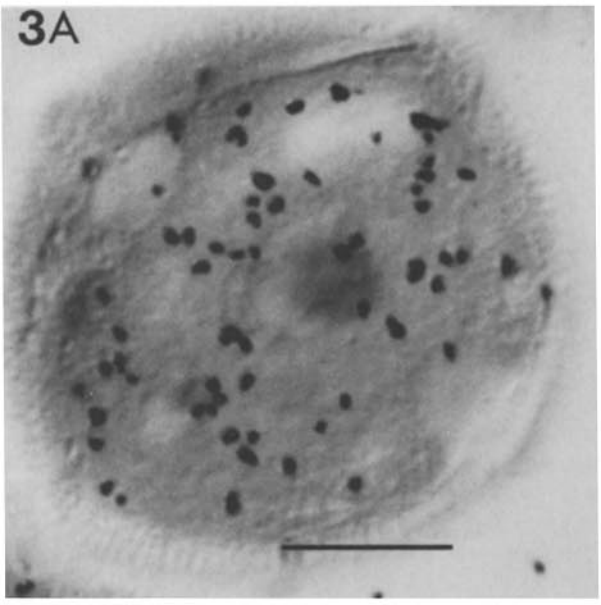
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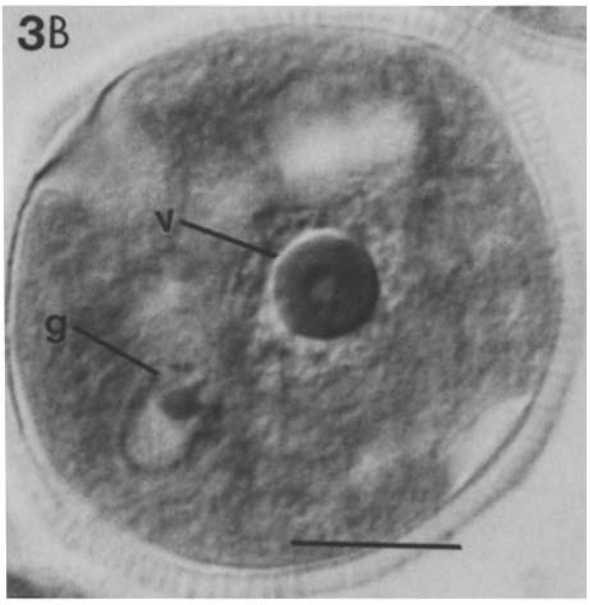
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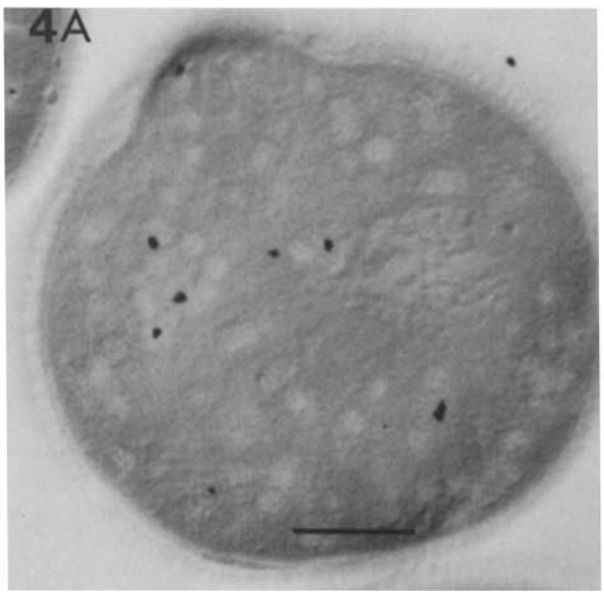
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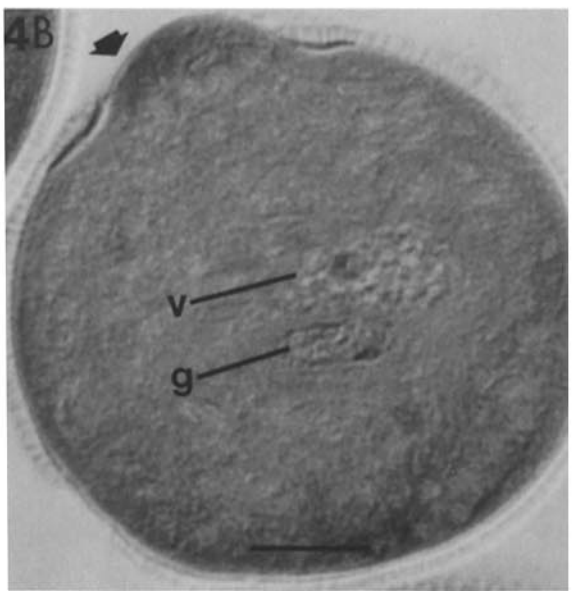
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It has not been ascertained whether this is due to the masking of the wall by starch grains or due to the ill-defined and ephemeral nature of the wall, as reported in other plants (2, 37).

After starch accumulation in pollen grains, the number of [^{3}H]poly(U) binding sites decreased steadily, and they almost completely disappeared from pollen grains of older flower buds and open flowers (Fig. 4). As seen in Figure 4, pollen grains in the advanced stages of development were characterized by a slight protrusion of the protoplast, although actual tube formation was not apparent; moreover, in such pollen grains, the generative cell was detached from the pollen wall and appeared to lie closely appressed to the nucleus of the vegetative cell.

[^{3}H]Poly(U) Binding during Pollen Germination

Pollen grains collected from flower buds and freshly open flowers failed to germinate, whereas those from flowers open for 4–8 h germinated optimally. The first sign of germination evident ~10 min after sowing was a swelling of the grain accompanied by an enlargement of the protoplasmic protrusion. Pollen tubes appeared 20–30 min after sowing and in 2–3 h the generative cell and the nucleus of the vegetative cell had migrated into the pollen tube. These inclusions could be identified by their distinctive shape and location in the pollen tube. The nucleus of the vegetative cell was large and round and generally confined to the tip of the pollen tube, whereas the generative cell was small and vermiform in shape and appeared far behind the vegetative nucleus. It should be emphasized that the generative cell in the pollen tube is constituted of a very thin layer of cytoplasm surrounding a conspicuous nucleus, and in most preparations it is difficult to make out the cytoplasm in the light microscope. Division of the generative cell to form two sperm cells is accomplished in the pollen tube 6–8 h after sowing.

In situ hybridization of germinating pollen grains with [^{3}H]poly(U) provided a means to determine the extent of poly(A)+RNA accumulation in the component cells of the male gametophyte during the terminal stages of their development. As seen from Table III, there was no appreciable binding of [^{3}H]poly(U) into pollen grains during the first 10 min after sowing. However, silver grains were found in high concentrations over the generative cell of pollen grains subjected to *in situ* hybridization 15–30 min after sowing and, during the next 30 min of germination, the labeling appeared in the cytoplasm of the vegetative cell and of the emerging pollen tubes (Figs. 5 and 6). Compared with developing pollen grains, virtually no silver grains due to [^{3}H]poly(U) binding appeared over the nucleus of the vegetative cell of the germinating pollen grain, indicating that this organelle is inactive as a source of poly(A)+RNA during germination. This result, coupled with the fact that [^{3}H]poly(U) binding first occurs in the generative cell of the germinating pollen, makes it likely that the binding sites noted in the vegetative cell and the pollen tube have their origin in the generative cell. The generative cell and the rest of the germinating pollen grain, excluding the vegetative nucleus, continued to bind [^{3}H]poly(U) until 2–3 h after sowing; but beginning at this time and coincident with the migration of the generative cell and the vegetative nucleus into the pollen tube, labeling in the pollen grain began to decrease (Figs. 7 and 8), and it nearly disappeared from the cytoplasm of pollen grains sampled 4 h after sowing and at all successive periods examined thereafter. After the migration of the generative cell and the vegetative nucleus into the pollen tube, no appreciable accumulation of silver grains was observed in the pollen tube cytoplasm. The vegetative nucleus and the generative cell in the pollen tube, and the sperm cells formed from the latter, also did not bind any [^{3}H]poly(U) (Fig. 9). These results indicate that poly(A)+RNA formation in the germinating pollen grain is due to the activity of the generative cell during a limited period in the early phase of germination.

TABLE III

Development of Autoradiographic Silver Grains on Germinating Pollen Grains after In Situ Hybridization with [^{3}H]poly(U)

Time after sowing	Stage of pollen germination seen in sections or in whole mounts	Number of silver grains ± SD*
min		
0	Bicellular, with protoplasmic protrusion	3.0 ± 0.2
5	Swelling of pollen grains	3.5 ± 0.22
10	Enlargement of the protoplasmic protrusion seen in section	8.5 ± 0.9
15	Protoplasmic protrusion visible outside	15.1 ± 1.2
20	As above	22.6 ± 1.5
30	Emerging pollen tubes, 20–50 μm long	41.0 ± 2.1
45	Elongating pollen tubes, 80–100 μm long	25.6 ± 1.9
60	Elongating pollen tubes, 100–120 μm long, nuclei within the pollen grain	10.2 ± 1.1

* In samples collected at a given time, pollen grains at stages earlier or later than that indicated in column 2 were also found. Figures in this column indicate the number of silver grains counted over germinating pollen (excluding pollen tube) of the stage specified in column 2.

The origin of mRNA, which codes for the first proteins of germination, is a problem of considerable interest. On the basis of actinomycin D treatment combined with [^{3}H]uridine autoradiography, Mascarenhas (23) suggested that mRNA molecules required for germination of the pollen grains and early growth of the pollen tube are preformed in the mature grain. *In situ* hybridization of developing pollen grains of *H. niger* with [^{3}H]poly(U) described earlier showed that radioactive binding sites indicative of mRNA distribution were almost totally absent from fully mature pollen grains ready to germinate. This has raised the question whether poly(A)+RNA detected during pollen germination is due to preformed mRNA or to newly synthesized mRNA. To answer this question, pollen grains were sown in the sucrose-boric acid medium supplemented with 1.0 mg/ml of actinomycin D. Samples were fixed at intervals during a 1-h time-course for *in situ* hybridization with [^{3}H]poly(U). It should be pointed out that even in this high concentration of actinomycin D, pollen germination was not inhibited but only slightly delayed, and eventually all pollen grains formed tubes. As seen from Table IV, actinomycin D was incapable of inhibiting [^{3}H]poly(U) binding capacity of germinating pollen grains and, in fact, compared with controls, treated pollen grains showed a modest increase in the number of silver grains at all points in the time-course. Although these results cannot be adequately explained at this time, the well-documented effects of actinomycin D as an inhibitor of mRNA synthesis in diverse plant and animal systems lead to the conclusion that poly(A)+RNA detected in the generative cell of the germinating pollen grain is not due to transcription of new primers. It is not determined how poly(A)+RNA formed in the developing pollen grain is conserved in an inactive form until germination. According to

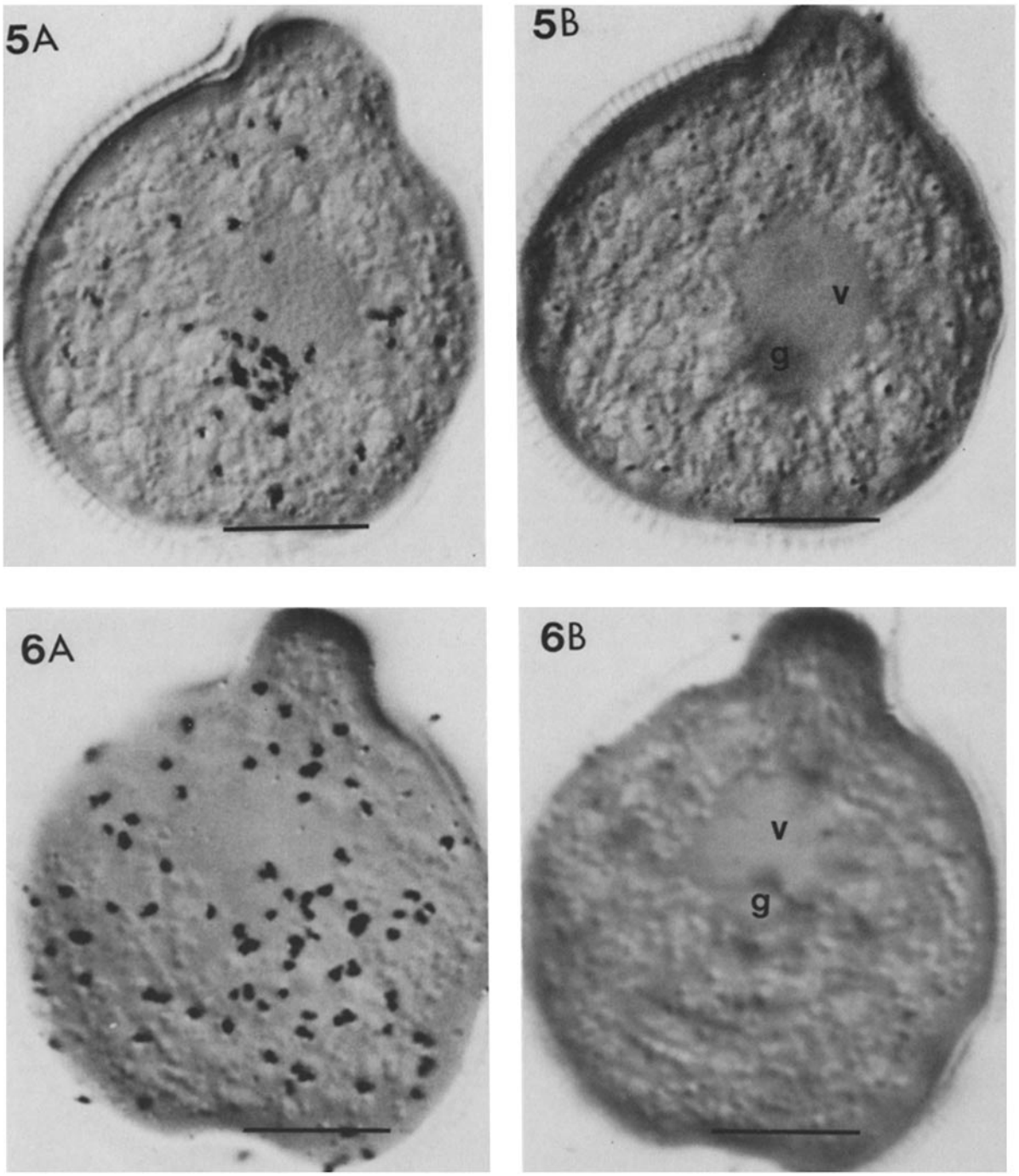
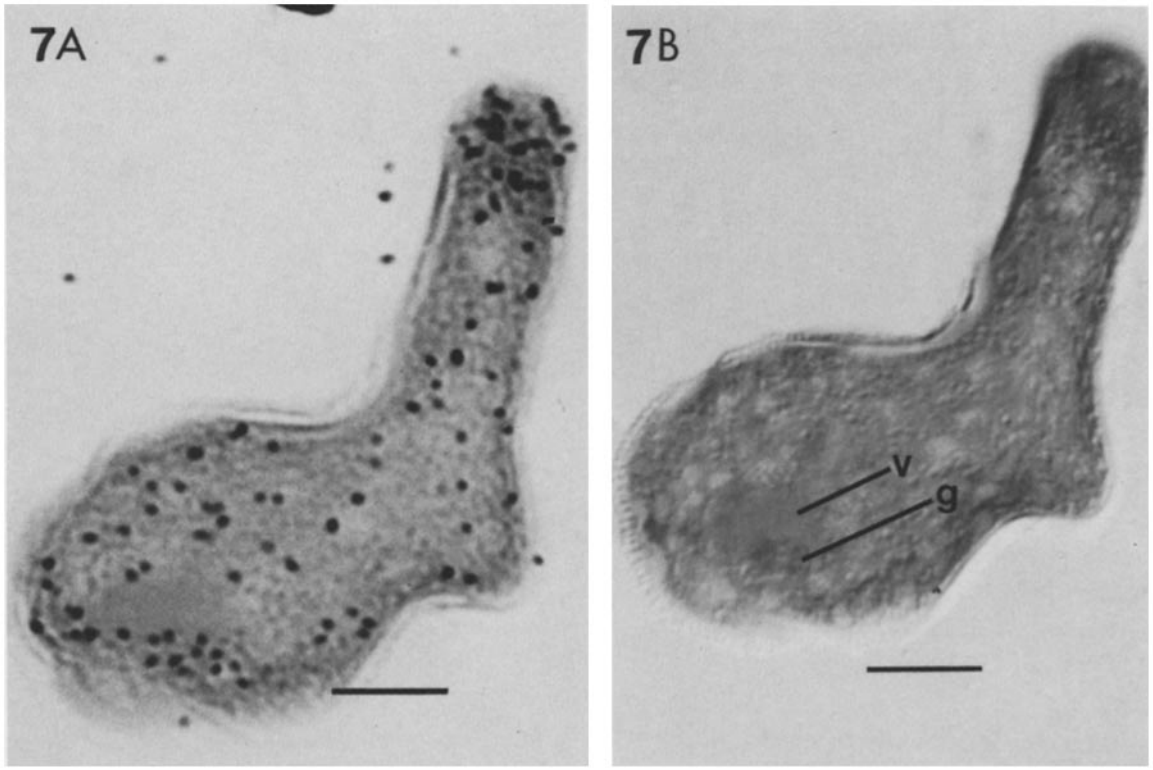


FIGURE 5 [^{3}H]Poly(U) binding into the section of a pollen grain germinated for 15 min showing label in the generative cell and the unlabeled vegetative cell. *g*, nucleus of the generative cell; *v*, nucleus of the vegetative cell. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

FIGURE 6 [^{3}H]Poly(U) binding into the section of a pollen grain germinated for 45 min, showing label in the cytoplasm of the vegetative cell. A few silver grains are found over the nucleus of the generative cell (*g*), but the vegetative cell nucleus (*v*) is unlabeled. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

some investigators (13, 39), poly(A) tails of stored mRNA in sea urchin eggs are capable of high turnover and resynthesis after fertilization. If this situation occurs in pollen grains of *H. niger*, it is conceivable that poly(A)+RNA detected in the generative and vegetative cells of developing pollen grains is turned over during maturation and is reformed in the generative cell of the germinating pollen as a new transcript, independent of simultaneous mRNA synthesis. Alternatively, it is possible that, during pollen maturation, dehydration coupled with low metabolic activity somehow masks the poly(A) sequences of mRNA or makes them unavailable for binding with [^{3}H]poly(U). In any event, this constitutes an intriguing case of molecular adaptation which is certainly deserving of further study.



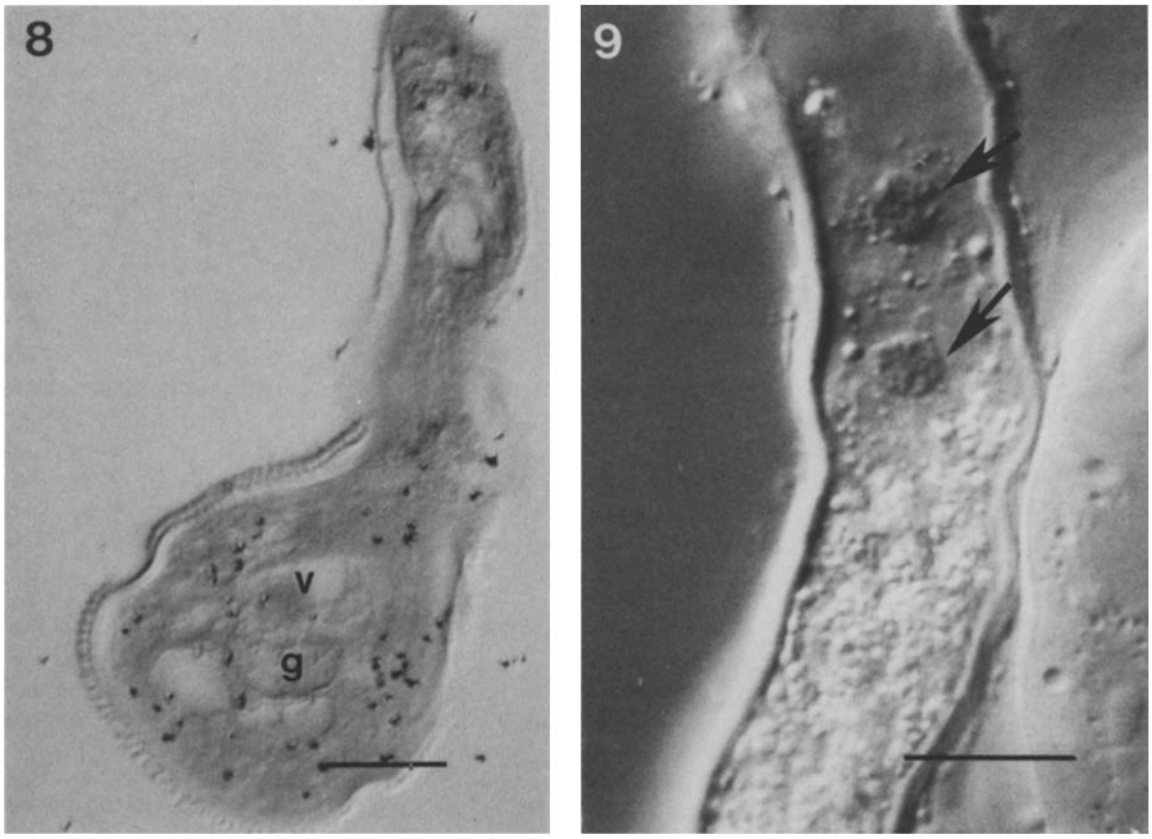
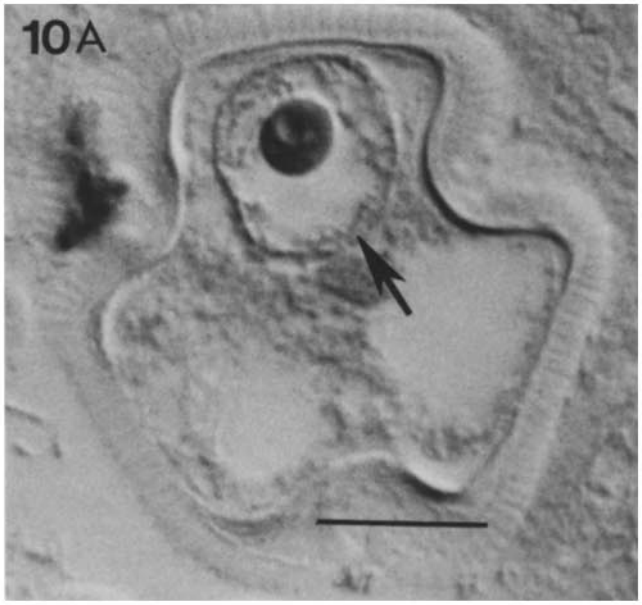
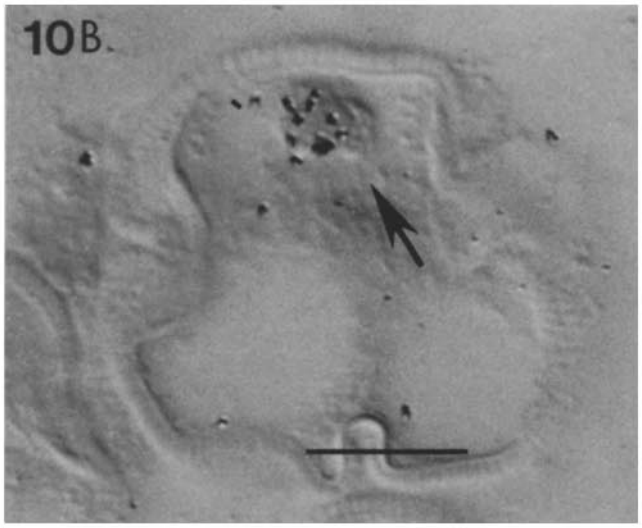


FIGURE 7 Autoradiograph of a section of a pollen grain germinated for 1 h showing the distribution of label due to [^{3}H]poly(U) binding in the component cells of the pollen grain and in the pollen tube. Some label still persists in the nucleus of the generative cell (*g*); nucleus of the vegetative cell (*v*) is unlabeled. (*A*) Focus on silver grains. (*B*) Nomarski optics photograph with focus on nuclei and cytoplasm. Bars, 10 μm. × 1,400.

FIGURE 8 Nomarski optics photograph of a section of a pollen grain germinated for 2 h showing the absence of label in the generative (*g*) and vegetative (*v*) nuclei and decreased label in the rest of the pollen grain and in the pollen tube due to [^{3}H]poly(U) binding. Bar, 10 μm. × 1,500.

FIGURE 9 Autoradiograph of a part of a pollen tube showing the absence of silver grains over the sperms (arrows) following *in situ* hybridization with [^{3}H]poly(U). For *in situ* hybridization, pollen grains were germinated directly on a slide for 6 h. Photographed with Nomarski optics. Bar, 10 μm. × 2,200.





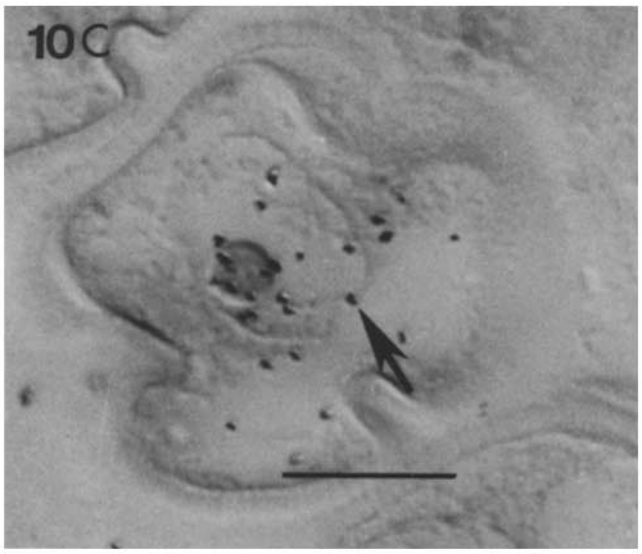


FIGURE 10 Autoradiographs of nonvacuolate, uninucleate pollen grains following *in situ* hybridization with [^{3}H]poly(U) of sections of anther segments at different times after culture. (*A*) Absence of label in the pollen grain at the time of culture. (*B*) Label in the nucleus 1 h after culture. (*C*) Label in the nucleus and cytoplasm 2 h after culture. Arrows point to the nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

TABLE IV

Development of Autoradiographic Silver Grains due to [^{3}H]-poly(U) binding on Pollen Grains Germinated for Different Times in Medium Containing Actinomycin D

Time after sowing	Stage of germination seen in sections or in whole mounts	Number of silver grains ± SD‡
min		
10	Prominent protoplasmic protrusion seen in sections (control)*	4.6 ± 0.28
	As above (actinomycin D)	6.2 ± 1.0
20	Protoplasmic protrusion visible in whole mounts (control)	18.6 ± 1.8
	As above (actinomycin D)	22.6 ± 2.0
30	Pollen tubes 20–40 μm long (control)	27.8 ± 2.0
	Pollen tubes just emerging (actinomycin D)	32.9 ± 2.6
45	Pollen tubes 50–75 μm long (control)	19.6 ± 1.6
	Pollen tubes just emerging (actinomycin D)	23.1 ± 1.9
60	Pollen tubes 75–100 μm long (control)	9.6 ± 1.0
	Pollen tubes 20–30 μm long (actinomycin D)	14.6 ± 1.2

* Control germinated in the basal medium.

‡ Figures in this column indicate the number of silver grains counted over germinating pollen (excluding pollen tube) of the stage specified in column 2.

[^{3}H]Poly(U) Binding during Pollen Embryogenesis

Now that the role of the generative and vegetative cells in poly(A)+RNA accumulation during the ontogeny of the male gametophyte of *H. niger* was established, experiments were undertaken to characterize the pattern of poly(A)+RNA formation in pollen grains whose normal gametophytic program was altered in the embryogenic pathway upon culture of anther segments. When sections of anther segments were subjected to *in situ* hybridization with [^{3}H]poly(U) immediately after sterilization before culture, either no silver grains were seen on the pollen grains or the number of silver grains seen was no greater than background (Fig. 10*A*; see also Fig. 1). However, if anther segments were cultured for at least 1 h before annealing with [^{3}H]poly(U), appreciable binding of the isotope occurred in a small number of uninucleate pollen grains (Fig. 10*B*). On the basis of their position near the tapetum, their nonvacuolate nature and their general affinity for stains, these pollen grains are judged to be embryogenically determined (31). In contrast, the majority of pollen grains confined to the center of the anther loculus were in various degrees of vacuolation and did not bind any [^{3}H]poly(U). In anther segments annealed after longer periods in culture, an increasing number of the nonvacuolate, uninucleate pollen grains were found to be labeled; moreover, an increase in grain density over the pollen grain coupled with the appearance of the label over the chromatin and cytoplasm was also noted (Fig. 10*C*). A quantitative appraisal of the changes in silver grain density in the uninucleate, embryogenically determined pollen grains during a 12-h culture period is given in Table V.

Because pollen grains did not bind any [^{3}H]poly(U) at the time of culture of anther segments (Figs. 1 and 10*A*) or at

TABLE V

Changes in the Number of Autoradiographic Silver Grains due to [^{3}H]poly(U) Binding in Uninucleate, Nonvacuolate Pollen Grains of Anther Segments Cultured for Different Time Periods

Time after culture	Number of silver grains per pollen grain ± SD
h	
0	0.8 ± 0.08
1	8.2 ± 0.70
2	18.6 ± 1.6
4	21.4 ± 1.9
8	22.3 ± 2.1
12	20.6 ± 1.8

TABLE VI

Effect of Actinomycin D on the Number of Autoradiographic Silver Grains due to [^{3}H]poly(U) Binding in Uninucleate, Nonvacuolate Pollen Grains of Anther Segments Cultured for Different Time Periods in a Medium Containing Actinomycin D

Time after culture	Number of silver grains per pollen grain ± SD	
	Control*	Actinomycin D
h		
1	9.8 ± 1.0	1.8 ± 0.45
2	16.6 ± 1.8	2.4 ± 0.8
4	18.2 ± 1.6	1.6 ± 0.4
8	15.3 ± 1.5	1.3 ± 0.4
12	21.0 ± 1.6	2.1 ± 0.5

* Cultured in the basal medium.

earlier periods in their ontogeny (see Table II), it is possible that [^{3}H]poly(U) binding sites observed in pollen grains of anther segments cultured for different periods of time are due to newly synthesized mRNA rather than to preformed mRNA. An attempt was made to resolve this issue by determining [^{3}H]poly(U) binding capacity of pollen grains of anther segments grown in a medium containing 1.0 mg/l actinomycin D. An earlier study (32) showed that this concentration of the drug blocked [^{3}H]uridine incorporation into uninucleate, nonvacuolate pollen grains, whereas division of the latter in the embryogenic pathway was also inhibited. As shown in Table VI, actinomycin D treatment of anther segments completely prevented the ability of pollen grains to bind [^{3}H]poly(U). This indicates that [^{3}H]poly(U) binding sites resident in the embryogenically determined pollen grains of cultured anther segments are due to newly synthesized mRNA. I have not eliminated here a role for polyadenylation of existing mRNA to account for [^{3}H]poly(U) binding sites observed in the embryogenically determined pollen grains.

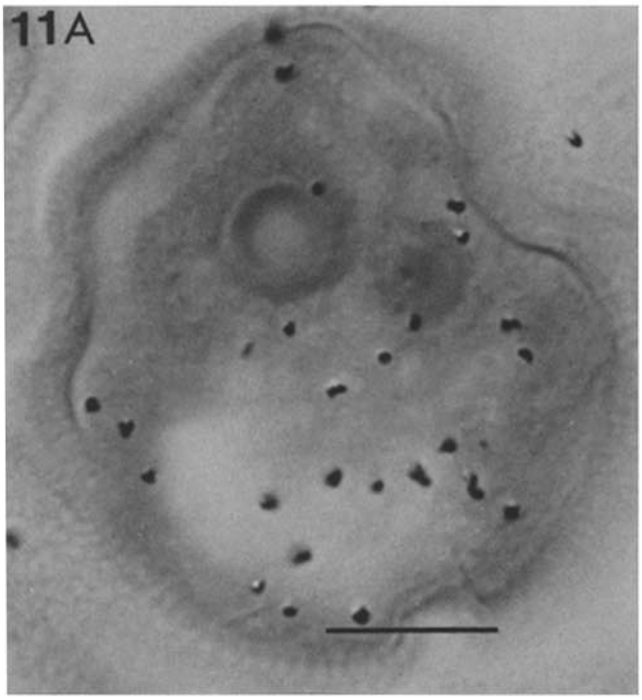
Although nuclear division was observed in occasional embryogenically determined pollen grains as early as 1 h after culture of anther segments, the majority of them gave rise to a small generative nucleus and a large vegetative nucleus 12–36 h after culture. During subsequent periods in culture, wall formation ensued to form a small generative cell and a large vegetative cell. After *in situ* hybridization of sections of anther segments cultured for 12–36 h with [^{3}H]poly(U), silver grains were generally found in the cytoplasm in the vicinity of the generative and vegetative nuclei of nonvacuolate, binucleate pollen grains. As shown in previous studies (31, 33), embryoids are formed by the subsequent division of these grains, which are thus considered to be potentially embryogenic. Depending upon the proximity of autoradiographic silver grains to the pollen nuclei, three types of labeled, potentially embryogenic binucleate pollen grains were identified in sections of anther segments subjected to *in situ* hybridization with [^{3}H]poly(U) 12–36 h after culture: (*a*) pollen grains in which the label was found in the cytoplasm surrounding the generative nucleus (Fig. 11); (*b*) pollen grains in which the label was found in the cytoplasm surrounding the vegetative nucleus (Fig. 12); and (*c*) pollen grains in which the label was found in the cytoplasm surrounding both nuclei (Fig. 13). In addition, certain pollen grains with two more or less identical nuclei were also labeled following *in situ* hybridization of sections of anther segments cultured for 12–36 h; however, in view of the small number of such pollen grains encountered, they are not considered further. Although occasional vacuolate, uninucleate pollen grains were also found to divide in anther segments sampled at 12–36 h after culture, they did not bind [^{3}H]poly(U) at these or subsequent periods in culture, but gradually disintegrated and became nonembryogenic.

For technical reasons, it was not possible to monitor in isolation the distribution of poly(A)+RNA in the different types of potentially embryogenic pollen grains during their transformation into embryoids. However, when sections of anther segments cultured for 48 h or more were subjected to *in situ* hybridization with [^{3}H]poly(U), I observed multicellular pollen grains and embryoids with varying degrees of label in the cells derived from the generative and vegetative cells, suggesting the possibility of origin of embryoids from specific types of binucleate, potentially embryogenic pollen grains referred to earlier. Fig. 14 shows an embryoid in which a modest [^{3}H]poly(U) binding occurs in the organogenetic part formed from the generative cell, and very little binding in the undivided vegetative cell; this embryoid probably had its origin in a binucleate pollen in which [^{3}H]poly(U) binding sites were found in the vicinity of the generative nucleus. In other embryoids whose organogenetic part was formed from the generative cell, the vegetative cell was found to form two or more cells which showed some affinity for [^{3}H]poly(U) (Figs. 15 and 16); such embryoids can be traced to binucleate pollen grains in which [^{3}H]poly(U) binding occurs in the vicinity of both

FIGURE 11 Autoradiograph of a nonvacuolate, binucleate pollen grain following *in situ* hybridization with [^{3}H]poly(U) of sections of an anther segment cultured for 36 h. The label is mostly in the cytoplasm surrounding the generative nucleus (*g*); *v* is the vegetative nucleus. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

FIGURE 12 Autoradiograph of a nonvacuolate, binucleate pollen grain following *in situ* hybridization with [^{3}H]poly(U) of sections of an anther segment cultured for 36 h. The label is in the vegetative nucleus (*v*) and in the surrounding cytoplasm; *g* is the generative nucleus. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.

FIGURE 13 Autoradiograph of a nonvacuolate, binucleate pollen grain following *in situ* hybridization with [^{3}H]poly(U) of sections of an anther segment cultured for 36 h. The label is in the cytoplasm surrounding the generative (*g*) and vegetative (*v*) nuclei. (*A*) Focus on silver grains. (*B*) Focus on nuclei. Photographed with Nomarski optics. Bars, 10 μm. × 2,200.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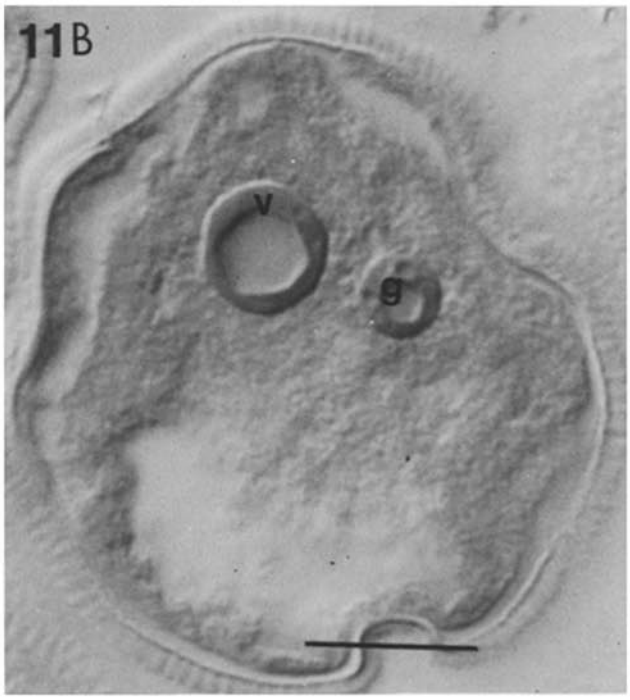
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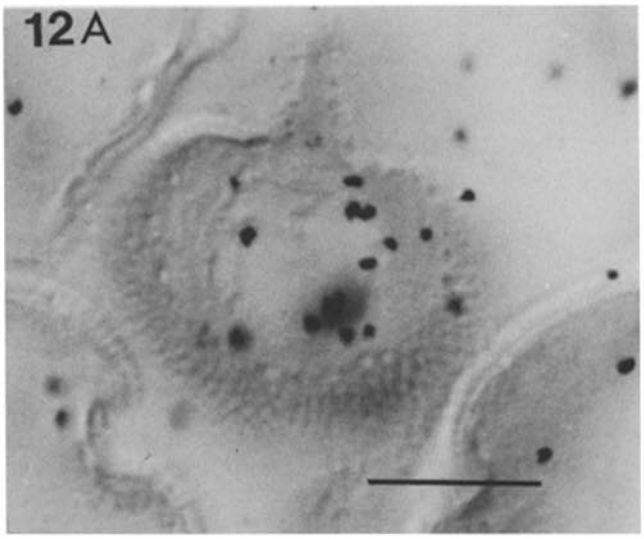
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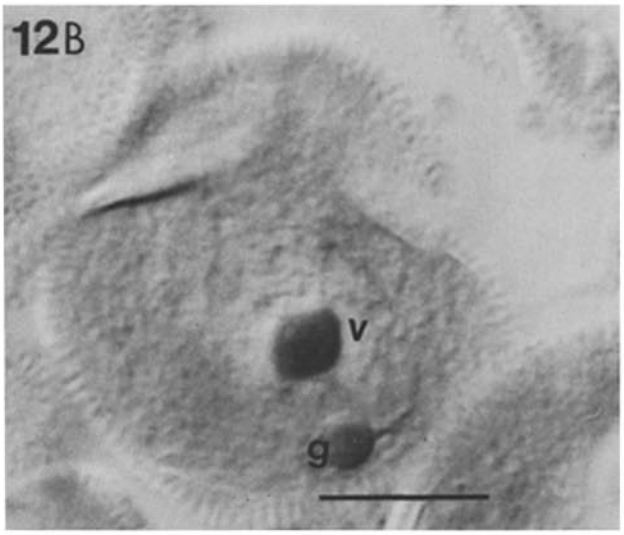
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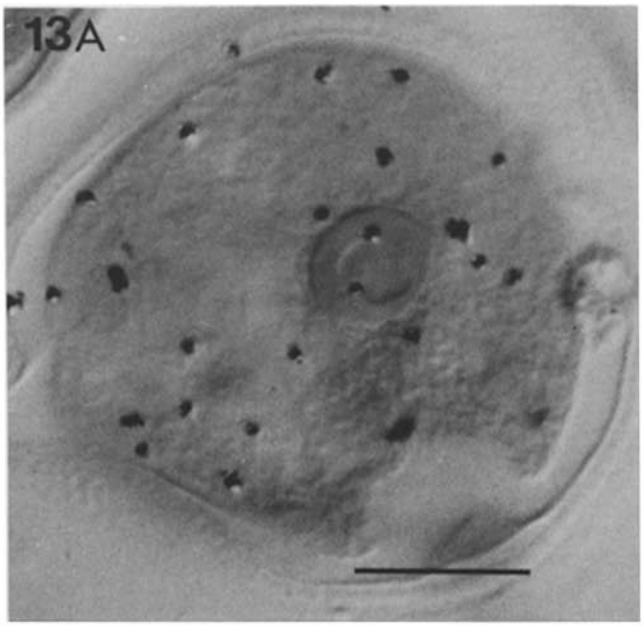
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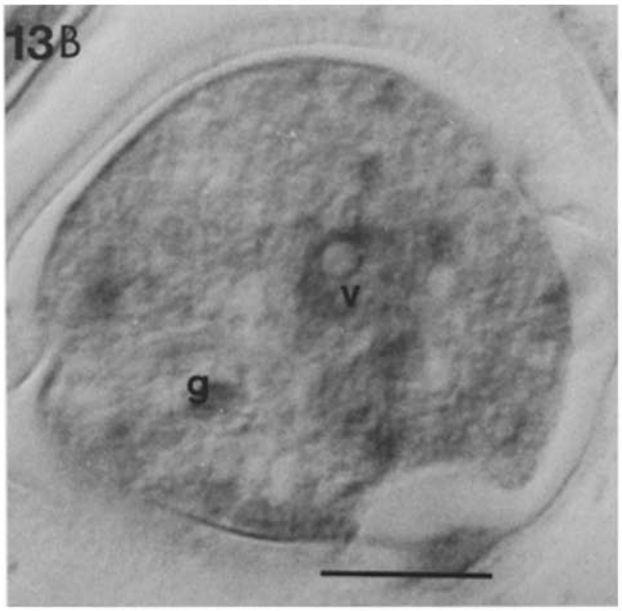
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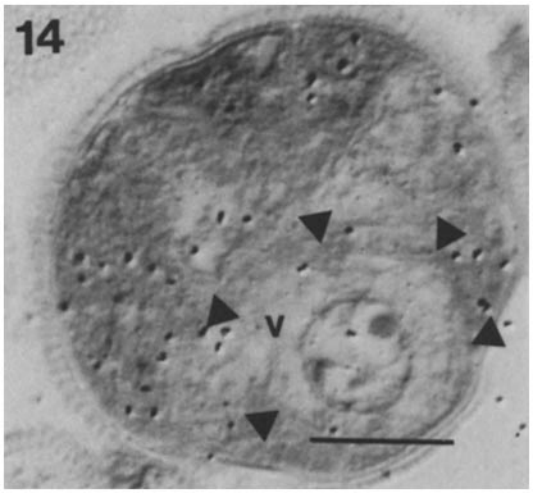

FIGURE 14 Autoradiograph of an embryoid with an undivided vegetative cell (*v*) showing [^{3}H]poly(U) binding following *in situ* hybridization of sections of an anther segment cultured for 96 h. Silver grains are fond mostly over cells formed from the generative cell and are sparse in the vegetative cell. Arrowheads point to the boundary between the vegetative cell and cells formed from the generative cell. Bar, 10 μm. × 1,800.

nuclei. In the embryoids illustrated in Figs. 15 and 16, the levels of [^{3}H]poly(U) binding in the cells formed from the vegetative cell are those expected of binucleate pollen grains in which the contribution of the vegetative nucleus to the final configuration of embryoids varies. Finally, binucleate pollen grains in which [^{3}H]poly(U) binding occurred in the vicinity of the vegetative nucleus were found to enlarge and accumulate starch, although some labeling persisted in such pollen grains even after they became fully starch filled (Fig. 17). I observed occasional pollen grains of this type to become embryogenic after 10–12 d in culture by the division of the generative cell, but [^{3}H]poly(U) binding pattern in them has not been followed.

DISCUSSION

In this work, an *in situ* hybridization method developed by Capco and Jeffery (8) for the detection of poly(A)+RNA in histological preparations of animal materials was used to monitor the distribution of poly(A)+RNA in pollen grains of *H. niger* during their short-lived gametophytic phase and during induced sporophytic type of growth. Under the conditions used here, it is assumed that poly(A)+RNA that interacts with [^{3}H]poly(U) includes putative informational type of RNA and that the distribution of poly(A)+RNA reflects variations in mRNA metabolism. The *in situ* hybridization method is of particular advantage in the present system because it permits the detection of local differences in the accumulation of poly(A)+RNA in the male gametophyte at crucial stages in its development or when its normal developmental program is modified in the sporophytic pathway. These results are of unique interest because they reflect the activities of individual nuclei within well-defined cell types. However, *in situ* hybridization does not yield information on the quantity and on the chemical nature of poly(A)+RNA formed by cells. Only biochemical analysis of isolated pollen grains can provide these data. Although the biochemistry of development and germination of angiosperm pollen has been studied to some extent (see 24 for review), the biochemical analysis of pollen embryogenesis has been hampered by the low percentages of pollen grains in cultured anthers that actually become embryogenic.

The results of these studies provide some of the first information on the regulatory control associated with the initiation of embryogenic divisions in pollen grains of cultured anthers. Previous autoradiographic experiments using [^{3}H]uridine have shown that RNA synthesis is initiated in a small number of nonvacuolate, uninucleate pollen grains of *H. niger* as early as the first hour of culture of whole anthers or anther segments (32, 33). Present findings suggest that at least some of the RNA synthesized is nonribosomal and possibly messenger type. The lack of [^{3}H]poly(U) binding in the uninucleate pollen grains of anther segments grown in a medium containing actinomycin D leads to the conclusion that mRNA is newly synthesized by pollen grains as they establish contact with the culture medium. This mRNA must be specifically concerned with embryogenic determination because the uninucleate pollen grains do not bind [^{3}H]poly(U) at the time of culture or at earlier stages in their ontogeny. Some investigators (18, 19) have proposed that in plants like tobacco, the potential to form embryoids is determined in a small number of morphologically distinct pollen grains during early flower formation and that culture of anthers provides only a favorable milieu for the expression of this potential. *In situ* hybridization experiments of uninucleate pollen grains of different ages with [^{3}H]poly(U) have not yielded any clear evidence supporting this thesis. A reasonable conclusion is that pollen grains become embryogenically determined as a result of the trauma of excision and culture of anther segments, and that this is accompanied by the synthesis of poly(A)+RNA. Interestingly enough, culture of anther segments also leads to the disintegration of the large majority of the enclosed pollen grains before they complete the gametophytic program.

Some speculate as to whether metabolites of tapetal origin are involved in inducing embryogenic divisions in pollen grains (41, 45). This possibility was suggested for *H. niger* on the basis of the proximity of embryogenic pollen grains to the tapetum (31). Although [^{3}H]poly(U) binding sites were present in the tapetal cells at the time of culture of anther segments, no cytological evidence was obtained to indicate that poly(A)+RNA molecules accumulated in the pollen grains were transported from the tapetum. Any idea of a tapetal origin of poly(A)+RNA seen in the uninucleate, embryogenically determined pollen grains of cultured anther segments is also incompatible with the notion that it is newly synthesized.

Comparative analysis of [^{3}H]poly(U) binding activity of the generative and vegetative nuclei during gametogenesis and during induced embryogenic growth of pollen grains provides some insight into how these nuclei respond to different developmental signals. These data have established that during the ontogeny of the male gametophyte the generative and vegetative nuclei are only transiently active in binding [^{3}H]poly(U). Moreover, although both nuclei bind [^{3}H]poly(U) during the maturation phase of the pollen grains, only the generative nucleus is active in this role during germination. Unpublished experiments have shown that both generative and vegetative nuclei of germinating pollen grains incorporate [^{3}H]uridine during the same period in the germination time-course when the latter exhibit no affinity to [^{3}H]poly(U); taken together, these results suggest that RNA synthesized by the nucleus of the vegetative cell during germination does not have any messenger properties. The striking feature of the potentially embryogenic, binucleate pollen grains is the continued transcriptional activity of the generative and vegetative nuclei and

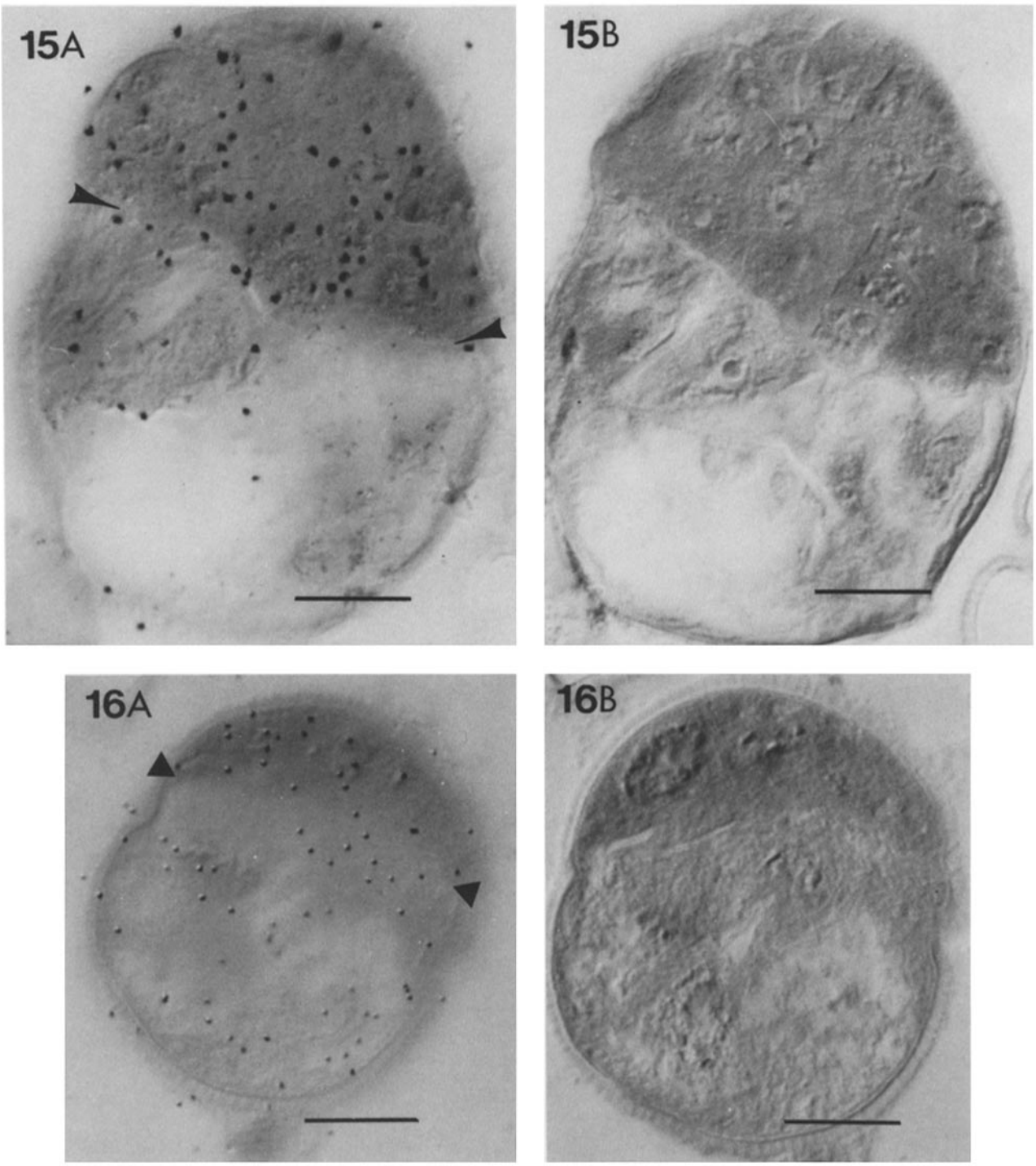

FIGURE 15 Autoradiograph of an embryoid with 5–6 cells formed from the vegetative cell and a globular mass of cells formed from the generative cell, showing [^{3}H]poly(U) binding following *in situ* hybridization of sections of an anther segment cultured for 120 h. Arrowheads indicate the boundary between cells formed from the generative cell (above arrowheads) and vegetative cell (below arrowheads). (*A*) Focus on silver grains. (*B*) Focus on cells. Photographed with Nomarski optics. Bars, 10 μm. × 1,800.

FIGURE 16 Autoradiograph of an embryoid with several cells formed from the generative and vegetative cells, showing [^{3}H]-poly(U) binding following *in situ* hybridization of sections of an anther segment cultured for 120 h. Arrowheads indicate the boundary between cells formed from the generative cell (above arrowheads) and vegetative cell (below arrowheads). (*A*) Focus on silver grains. (*B*) Focus on cells. Photographed with Nomarski optics. Bars, 10 μm. × 1,800.

their division products as evidenced by [^{3}H]poly(U) binding in the cytoplasm in the vicinity of these nuclei. The different patterns of [^{3}H]poly(U) binding observed in the potentially embryogenic, binucleate pollen grains are consistent with the results of autoradiography of [^{3}H]uridine incorporation into these pollen grains. Data from [^{3}H]poly(U) binding imply that some of the RNA synthesized in the generative and vegetative nuclei of potentially embryogenic pollen grains as measured

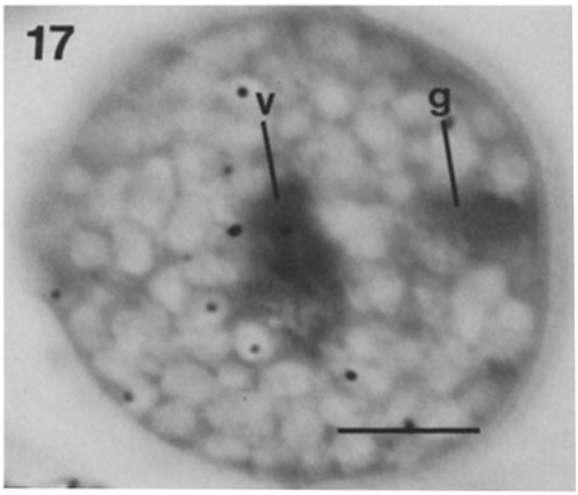

FIGURE 17 Autoradiograph of a starch-filled binucleate pollen grain showing [^{3}H]poly(U) binding following *in situ* hybridization of sections of an anther segment cultured for 120 h. *g*, generative nucleus; *v*, vegetative nucleus. Bar, 10 μm. × 1,800.

by [^{3}H]uridine incorporation is nonribosomal.

In summary, this paper's findings lead to the following interpretation of pollen embryogenesis in *H. niger*. Due to excision and culture of anther segments, a small proportion of the enclosed pollen grains begin to synthesize poly(A)-containing mRNA, which codes for the proteins necessary to induce the first haploid mitosis. Subsequent division of the binucleate pollen grains in the embryogenic pathway depends upon the synthesis of additional poly(A)+RNA by the generative nucleus or by both generative and vegetative nuclei. On the other hand, synthesis of poly(A)+RNA by the vegetative nucleus alone perpetuates a part of the gametophytic program leading to starch accumulation in the pollen grains. This probably indicates that proteins encoded by mRNA synthesized by the generative nucleus are effective in inducing continued divisions of this nucleus accompanied by cytokinesis, whereas those translated by mRNA formed by the vegetative nucleus are effective in promoting starch accumulation. Nonetheless, occasionally the vegetative nucleus divides and forms cells which contribute to the formation of the embryoid (as, for example, Figs. 15 and 16). The varying degrees of [^{3}H]poly(U) binding found in the vegetative cell and its division products might indicate that under the stimulus of a transcriptionally active nucleus of the generative cell, the nucleus of the vegetative cell might also be induced to synthesize poly(A)+RNA and divide. Because gene activation appears to be involved here, it remains to be determined whether poly(A)+RNA formed in the nucleus of the vegetative cell during its dividing and nondividing states represents distinct species of mRNA.

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REFERENCES

1. Beers, R. F. 1960. Hydrolysis of polyadenylic acid by pancreatic ribonuclease. *J. Biol. Chem.* 23:2393–2398.
2. Bhojwani, S. S., and S. P. Bhatnagar. 1974. The Embryology of Angiosperms. Vikas Publishing House Pvt. Ltd., Delhi, India.
3. Bishop, J. O., M. Rosbash, and D. Evans. 1974. Polynucleotide sequences in eukaryotic DNA and RNA that form ribonuclease-resistant complexes with polyuridylic acid. *J. Mol. Biol.* 85:75–86.
4. Blumenfeld, M., and H. S. Forrest. 1971. Is *Drosophila* dAT on the Y chromosome? *Proc. Natl. Acad. Sci. U. S. A.* 68:3145–3149.
5. Bourgin, J.-P., and J. P. Nitsch. 1967. Obtention de *Nicotiana* haploides à partir d'étamines cultivées *in vitro*. *Ann. Physiol. Vég.* 9:377–382.
6. Brawerman, G. 1974. Eukaryotic messenger RNA. *Annu. Rev. Biochem.* 43:621–642.
7. Bultmann, H., and C. D. Laird. 1973. Mitochondrial DNA from *Drosophila melanogaster*. *Biochim. Biophys. Acta.* 299:196–209.
8. Capco, D. G., and W. R. Jeffery. 1978. Differential distribution of poly(A)-containing RNA in the embryonic cells of *Oncopeltus fasciatus*. Analysis by *in situ* hybridization with a [^{3}H]poly(U) probe. *Dev. Biol.* 67:137–157.
9. Capco, D. G., and W. R. Jeffery. 1979. Origin and spatial distribution of maternal messenger RNA during oogenesis of an insect, *Oncopeltus fasciatus*. *J. Cell Sci.* 39:63–76.
10. Carmichael., G. G. 1975. Isolation of bacterial and phage proteins by homopolymer RNA-cellulose chromatography. *J. Biol. Chem.* 250:6160–6167.
11. Darnell, J. E. 1976. mRNA structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* 19: 493–511.
12. Darnell, J. E., R. Wall, and R. Tushinski. 1971. An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA. *Proc. Natl. Acad. Sci. U. S. A.* 68:1321–1325.
13. Dolecki, G., R. F. Duncan, and T. Humphreys. 1977. Complete turnover of poly(A) on maternal mRNA of sea urchin embryos. *Cell.* 11:339–344.
14. Fansler, B. S., E. L. Travaglini, L. A. Loeb, and J. Schultz. 1970. Structure of *Drosophila melanogaster* dAT replicated in an *in vitro* system. *Biochem. Biophys. Res. Commun.* 40: 1266–1272.
15. Feder, N., and T. P. O'Brien. 1968. Plant microtechnique: some principles and new methods. *Am. J. Bot.* 55:123–142.
16. Flavell, R. A., F. M. Van den Berg, and G. C. Grosveld. 1977. Isolation and characterization of the oligo (dA-dT) clusters and their flanking DNA segments in the rabbit genome. *J. Mol. Biol.* 115:715–741.
17. Gall, J. G., and M. L. Pardue. 1971. Nucleic acid hybridization in cytological preparations. *Methods Enzymol.* 21:470–480.
18. Horner, M., and H. E. Street. 1978. Pollen dimorphism: origin and significance in pollen plant formation by anther culture. *Ann. Bot. (Lond.).* 42:763–771.
19. Horner, M., and R. L. Mott. 1979. The frequency of embryogenic pollen grains is not increased by *in vitro* anther culture in *Nicotiana tabacum* L. *Planta (Berl.).* 147:156–158.
20. Jeffery, W. R., and D. G. Capco. 1978. Differential accumulation and localization of maternal poly(A)-containing RNA during early development of the ascidian, *Stylea*. *Dev. Biol.* 67:152–166.
21. Jones, K. W., J. O. Bishop, and A. Brito-da-Cunha. 1973. Complex formation between poly-r(U) and various chromosomal loci in *Rhynchosciara*. *Chromosoma (Berl.).* 43:375–390.
22. Lewin, B. 1980. Gene Expression. 2nd Edition. John Wiley & Sons, New York. 653–693.
23. Mascarenhas, J. P. 1966. Pollen tube growth and ribonucleic acid synthesis by vegetative and generative nuclei of *Tradescantia*. *Am. J. Bot.* 53:563–569.
24. Mascarenhas, J. P. 1975. The biochemistry of angiosperm pollen development. *Bot. Rev.* 41:259–314.
25. Mascarenhas, J. P., and E. Bell. 1970. RNA synthesis during development of the male gametophyte of *Tradescantia*. *Dev. Biol.* 21:475–490.
26. Pardue, M. L., and J. G. Gall. 1975. Nucleic acid hybridization to the DNA of cytologic preparations. *Methods Cell Biol.* 10:1–16.
27. Polan, M. L., S. Friedman, J. G. Gall, and W. Gehring. 1973. Isolation and characterization of mitochondrial DNA from *Drosophila melanogaster*. *J. Cell Biol.* 56:580–589.
28. Raghavan, V. 1976. Role of the generative cell in androgenesis in henbane. *Science (Wash. D. C.).* 191:388–389.
29. Raghavan, V. 1976. Experimental Embryogenesis in Vascular Plants. Academic Press, London.
30. Raghavan, V. 1977. Patterns of DNA synthesis during pollen embryogenesis in henbane. *J. Cell Biol.* 73:521–526.
31. Raghavan, V. 1978. Origin and development of pollen embryoids and pollen calluses in cultured anther segments of *Hyoscyamus niger* (henbane). *Am. J. Bot.* 65:984–1002.
32. Raghavan, V. 1979. Embryogenic determination and ribonucleic acid synthesis in pollen grains of *Hyoscyamus niger* (henbane). *Am. J. Bot.* 66:36–39.
33. Raghavan, V. 1979. An autoradiographic study of RNA synthesis during pollen embryogenesis in *Hyoscyamus niger* (henbane). *Am. J. Bot.* 66:784–795.
34. Raghavan, V. 1980. Localization of poly(A)+RNA during pollen embryogenesis in *Hyoscyamus niger*. *Bot. Soc. Am. Misc. Publ.* 158:91 (*abstr.*).
35. Raghavan, V. 1981. A transient accumulation of poly(A)-containing RNA in the tapetum of *Hyoscyamus niger* during microsporogenesis. *Dev. Biol.* 81:342–348.
36. Riley, M., B. Maling, and M. J. Chamberlain. 1966. Physical and chemical characterization of two- and three-stranded adenine-thymine and adenine-uracil homopolymer complexes. *J. Mol. Biol.* 20:359–389.
37. Sanger, J. M., and W. T. Jackson. 1971. Fine structure study of pollen development in *Haemanthus katherinae* Baker. I. Formation of vegetative and generative cells. *J. Cell Sci.* 8:289–301.
38. Shenkin, A., and R. H. Burdon. 1974. Deoxyadenylate-rich and deoxyguanylate-rich regions in mammalian DNA. *J. Mol. Biol.* 85:19–39.
39. Slater, D. W., I. Slater, and D. Gillespie. 1972. Post-fertilization synthesis of polyadenylic acid in sea urchin embryos. *Nature (Lond.).* 240:333–337.
40. Steffensen, D. M. 1966. Synthesis of ribosomal RNA during growth and division in *Lilium*. *Exp. Cell Res.* 44:1–12.
41. Sunderland, N., and M. Roberts. 1977. New approach to pollen culture. *Nature (Lond.).* 270:236–238.
42. Sunderland, N., and J. M. Dunwell. 1977. Anther and pollen culture. *In* Plant Tissue and Cell Culture. 2nd edition. H. E. Street, editor. Blackwell Scientific Publications, Oxford, 223–265.
43. Uchida, T., and F. Egami. 1971. Microbial ribonucleases with special reference to RNases T_1, T_2, N_1, and U_2. *In* The Enzymes. P. D. Boyer, editor. Academic Press Inc., New York. 4:205–250.
44. Vasil, I. K. 1980. Androgenetic haploids. *Int. Rev. Cytol. Suppl.* 11A:195–223.
45. Wernicke, W., and H. W. Kohlenbach. 1977. Versuche zur Kultur isolierter Mikrosporen von *Nicotiana* und *Hyoscyamus*. *Z. Pflanzenphysiol.* 81:330–340.