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# Alterations in Polyadenylated RNA during Pollen Maturation and Germination

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**Abstract.** Developmental changes in poly(A)-bearing RNA in male tobacco gametophyte were examined by sedimentation analysis and by hybridization with $^3$H-poly(U). The results indicate that the transition of microspore undergoing postmeiotic division to mature pollen is accompanied by characteristic changes in RNA and poly(A) content and the size of poly(A)$^+$RNA.

The volume of pollen grain increases about 2times, total RNA per grain from 34 to 230 pg and poly(A) from 22 to 450 fg, which together with the estimated increase in the number average size of poly(A)$^+$RNA from 700 to 2 100 nucleotides suggests an approx. rise of RNA containing poly(A) from 0.3 to 2.7% of total RNA. Size distribution of the populations of polyadenylated RNAs shows progressive formation of species with a higher molecular mass and differentiation of the pollen-characteristic pattern with main sedimentation maxima close to 12S, 19S and 26S. This pattern remains almost unchanged during 8 h of pollen tube growth and is also found in polysomes formed at the beginning of germination. The amount of poly(A) decreases gradually after the onset of soaking at a rate of slightly more than 1% per h within 24 h of pollen cultivation.

As a whole, the results demonstrate that in the course of pollen maturation a specific population of polyadenylated mRNAs is formed which persists as stored mRNA in quiescent pollen and is used as template during pollen tube formation.

The opposite effects of RNA antimetabolites on RNA and protein synthesis (TUPÝ 1966) and the rapidity of polysome formation (MASCARENHAS and BELL 1969, LINSKENS *et al.* 1970, TUPÝ 1977) in germinating pollen have suggested that a stable mRNA is stored in mature pollen meeting the requirement for protein synthesis during pollen tube formation. The messenger activity of RNA from ungerminated pollen could be directly demonstrated by its translation in a cell free system (FRANKIS and MASCARENHAS 1980).

The biogenesis of most mRNA includes the addition of poly(A) segment which seems to select and stabilize functional molecules (see BRAWERMAN 1981). The experiments described here show that a specific population of polyadenylated mRNAs is formed during pollen maturation, exhibiting stored mRNA properties. The related alteration of sedimentation profile of

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poly(A)+RNA after microspore division suggests specific changes in structural gene transcription and/or nuclear RNA processing with the progress of pollen differentiation.

## MATERIAL AND METHODS

### Plant Material

*Nicotiana tabacum* L. cv. Samsun was grown under field conditions. Flower buds were harvested from vigorous plants from which the adult flowers were continuously removed to prevent seed formation. Staging of anthers was based on flower bud morphology (NITSCH and NITSCH 1969). The buds with corolla length of 13—15, 17—20, 26—29, 36—44, 48—51 and 52—57 mm corresponded to anther stages referred to as 1 to 6, respectively. Cytological investigation showed that in stage 1 in which the corolla had the same length as the calyx, anthers contained spores undergoing mitotic division. Stage 6 corresponded to buds one day before anthesis.

### Isolation of Immature Pollen

Anthers collected from 20 buds were gently pressed (just to squeeze out pollen) in glass mortar containing about 5 ml of cold autoclaved sugar-mineral pollen medium prepared according to BREWBAKER and KWACK (1963). The suspension was then diluted with about 10 ml of the pollen medium, vortexed for 20 s and filtered through cheese-cloth to remove anther debris. The volume was made to 20 ml and the pollen grains were separated by filtration under reduced pressure (TUPÝ *et al.* 1977a) and stored frozen in dry ice. Before filtration, two aliquots were taken under stirring for determination of the size and number of pollen grains. The mean pollen size was determined from 100 measurements and the grain number was counted in two volume samples containing about 10 000 grains. For approximate volume calculation the shape of pollen grains was held for spherical.

### Cultivation and Cell Fractionation of Pollen

Mature pollen was collected and cultivated aseptically as shaken suspension (TUPÝ *et al.* 1977a) at a density of 5 mg per 10 ml in a medium containing 100 mg of boric acid, 708 mg of $Ca(NO_3)_2.4\,H_2O$, 200 mg of $MgSO_4.7\,H_2O$, 100 mg of $KNO_3$ and 1 g of casein hydrolysate per litre of 10% sucrose (TUPÝ *et al.* 1982).

Pollen culture was separated from the medium by filtration, frozen on the surface of solid $CO_2$, homogenized in a cold solution for isolation of polysomes (TUPÝ *et al.* 1977b) and centrifuged 10 min at 18 000 rev. $min^{-1}$ and 5 °C in a Beckman rotor JA-20. The supernatant was carefully separated from the pellet (referred to as preribosomal fraction) and layered on 4-ml cushion of 0.05 M Tris-HCl (pH 8.5), 1.5 M sucrose, 20 mM KCl, 5 mM mercaptoethanol, 5 μg $ml^{-1}$ cycloheximide and centrifuged 2 h at 55 000 rev. $min^{-1}$ and 5 °C in a Beckman 65 rotor. After removal of the supernatant, the tube with the ribosomal sediment was carefully rinsed with the solution used for pollen homogenization. The supernatant combined with the rinse (postribosomal fraction) was mixed with 2.5 volumes of alcohol for RNA precipitation.

**RNA Extraction and Fractionation**

RNA was extracted from pollen and from the preribosomal, ribosomal and postribosomal fractions by a procedure described previously (TUPÝ *et al.* 1977b). For further fractionation the isolated RNA was dissolved in a solution containing 0.1 M NaCl, 1 mM EDTA, 0.1% SDS, 10 mM Na-acetate, pH 5.1

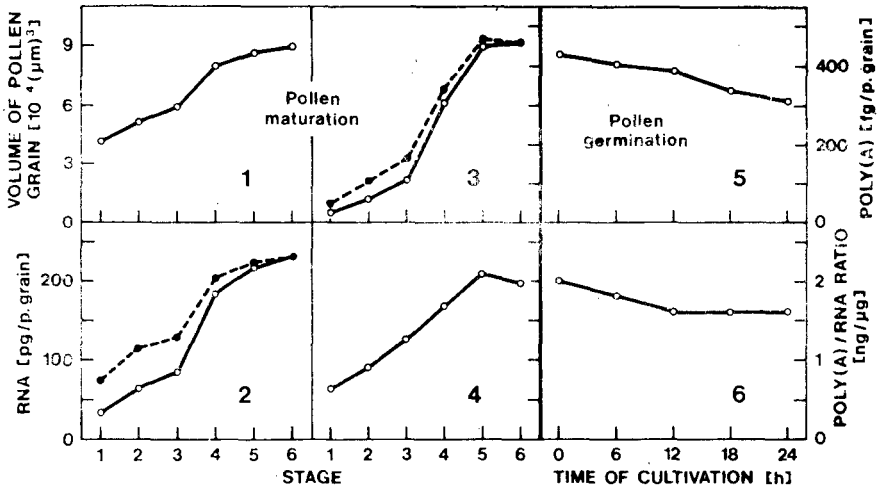


Fig. 1. Changes of pollen grain size (1) and of RNA content (2) during pollen maturation and in poly(A) content (3, 5) and poly(A)/RNA ratio (4, 6) during pollen maturation (3, 4) and *in vitro* germination (5, 6); the dashed lines express RNA and poly(A) content per volume of mature pollen.

(buffer A, SATO *et al.* 1979), and a 50-μl aliquot was layered on 5—20% sucrose gradient (3.6 ml) in the same buffer and centrifuged for 180 min at 50 000 rev. $min^{-1}$ and 20 °C in a Beckman rotor SW 56.

**Quantitation of Poly(A) in RNA**

The content of poly(A) sequences in the RNA preparations was assayed by hybridization with $^3$H-poly (U) using a modification ot the procedure described by SATO *et al.* (1979). Sodium salt of poly-5-$^3$H-uridylic acid (The Radiochemical Centre, Amersham, 0.7—2.6 TBq $mmol^{-1}$) was dissolved in 1.5 M NaCl, 0.15 M sodium citrate pH 7.0 and 50 μl reacted with samples in buffer A (50 to 150 μl) for 25 min at 40 °C. The hybridization was terminated by addition of 0.8 ml of pancreatic RNase A (12.5 μg in 10 mM Tris-HCl pH 7.6, 0.2 M NaCl). After incubation for 30 min at 0 °C carrier serum-albumin (100 μl, 50 μg) was added, the mixtures were precipitated with 2.5 ml of 7% trichloroacetic acid and kept overnight at 0—5 °C. To determine the acid insoluble radioactivity, the samples were filtered through 2.4-cm Synpor filters (0.40 μm) and washed by two 5-ml portions of 5% TCA. Filters were dried and counted in 0.7% PPO in toluen. A linear poly(A) standard curve was established with each run from duplicate samples of three amounts (1,2 and 3 ng) of poly(A). $^3$H-poly(U) was also incubated in the

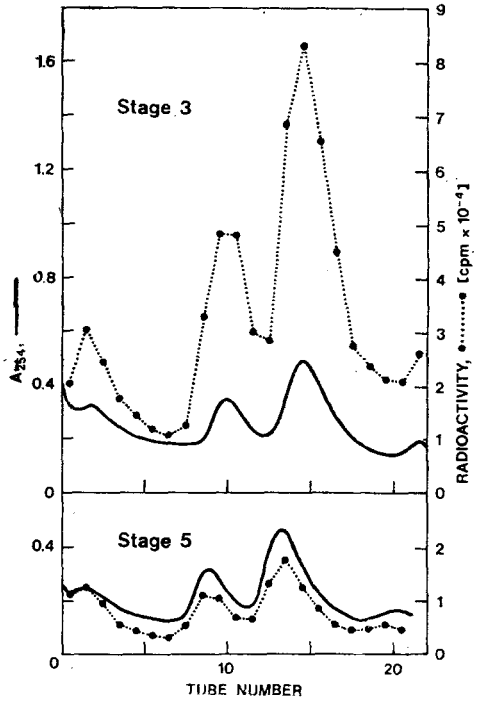


Fig. 2. RNA synthesis in immature pollen. Pollen grains isolated from anthers at stages 3 and 5 were incubated 4 h in 10 ml of sugar-mineral pollen medium in the presence of 15 kBq $ml^{-1}$ uridine-U-$^{14}C$ (75 nmol $MBq^{-1}$). RNA aliquots corresponding to pollen from 30 (stage 3) and 15 (stage 5) anthers were sedimented on sucrose density gradients.

absence of poly(A) to detect the eventual filter-retained radioactivity from unreacted molecules. The samples of RNA were incubated with $^3H$-poly(U) in duplicate and at two different concentrations, to check the linearity of the reaction.

## RESULTS

### Developmental Changes in the Amount of RNA and of Poly(A) in Pollen

During pollen development from microspore division to maturity, a pollen grain increases about 2times in volume while its content of RNA rises almost 7times and that of poly(A) sequences more than 20times (Fig. 1). The accumulation of both RNA and poly(A) proceeds most intensely between the 3rd and the 4th stage of flower bud, being very low or none during the last 2 days of pollen maturation. In the course of further development of the gametophyte into pollen tube only a slight decrease in the amount of poly(A) is registered. This decrease appears regular and reaches about 27% within 24 h.

The mature pollen grain is found to contain 230 pg of RNA and 450 fg of poly(A). Assuming that in plants the average size of the poly(A) stretch is 150 residues and that the average molecule of poly(A)+RNA is 2 100 nucleotides long (see below), then about 2.7% of mature pollen RNA is accounted for by polyadenylated RNA.

The observed increase of RNA during pollen maturation seems to be related to RNA synthesis inside the pollen grain rather than to its transfer from the

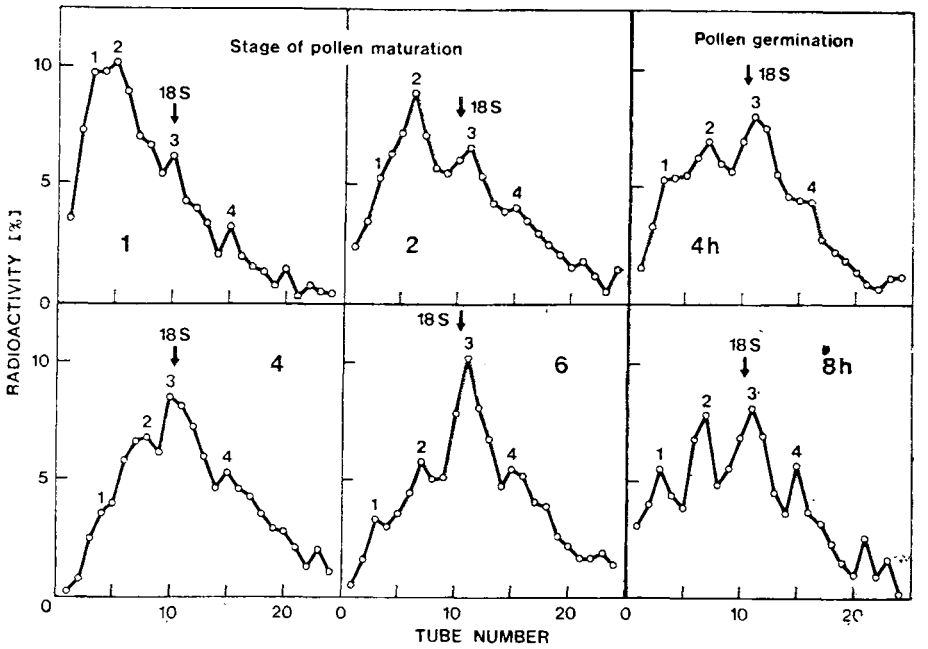


Fig. 3. Size distribution of poly(A)+RNA at different stages of pollen maturation and *in vitro* germination. Sedimentation of total RNA on sucrose density gradients.

tapetum. It is indicated by the finding of high incorporation of uridine into RNA in isolated immature pollen (Fig. 2). The newly synthesized RNA is essentially ribosomal.

**Developmental Changes in Sedimentation Pattern of Poly(A)-Containing RNA**

The size distribution of poly(A)+RNA was examined by sedimentation of total RNA through sucrose density gradient and by determination of poly(A)-containing RNA molecules in each fraction according to the formation of ribonuclease resistant hybrides with $^3$H-poly(U). At the very beginning of pollen ontogenesis, in the developmental phase of anthers in which part of microspores still complete the asymmetric mitosis, the sedimentation profile is characterized by one distinct maximum in the region of 8S (Fig. 3). With the progress of gametophytic development, the distribution of the population of polyadenylated molecules manifests a shift towards higher molecular mass and differentiation of 3 main peaks with sedimentation maxima close to 12S, 19S and 26S. Assuming that poly(A) size is the same irrespective of the size of the mRNA, the number average size of the poly(A)-containing RNA as determined from cumulative plots of the radioactive sedimentation data against molecular mass (calculated according to SPIRIN 1961), increased from 700 nucleotides at the initial stage to 2 100 nucleotides in the stage of flower buds one day prior to anthesis.

The sedimentation profile of pollen poly(A)+RNA remains essentially the same after 4 h and 8 h of germination, which provides further indication

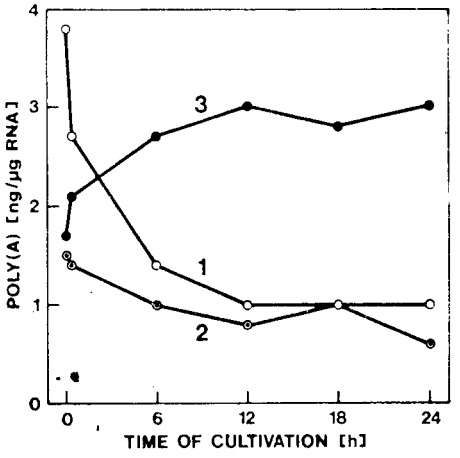


Fig. 4. Changes in cell distribution of poly(A) content during 24-h pollen cultivation. 1 — preribosomal sediment, 2 — ribosomal fraction, 3 — postribosomal supernatant.

of its relative stability. It should be noticed, however, that the pollen tubes contain a larger amount of small molecules in the 4S region than ungerminated pollen (*cf.* also with Fig. 5, curve 4). These molecules can be a free poly(A) and its increase may reflect partial liberation of poly(A) sequence from mRNA.

**Intracellular Distribution of Poly(A)⁺RNA during Pollen Germination**

With the method of fractionation applied, 20—30% of total pollen RNA is found in preribosomal sediment, 30—40% in the ribosomal fraction and 35—45% in postribosomal supernatant. The distribution of $A_{260}$ nm absorbance on sucrose density gradients indicated that most RNA of each fraction was ribosomal (result not given). In ungerminated pollen the highest proportion of poly(A) with respect to RNA is registered in the first sediment (Fig. 4), this proportion being almost twice as high as in total pollen RNA. Although polysomes are practically absent from dry tobacco pollen (TUPÝ 1977), RNA preparation from the ribosomal fraction contained nearly 30% of pollen poly(A). This poly(A) may originate from ~ 80S and larger non-polysomal messenger ribonucleoprotein particles, which are reported to occur in eucaryotic cells (*e.g.* AJTKHOZHIN *et al.* 1973, ADAMS *et al.* 1980). Altogether, at least 60% of poly(A)⁺RNA is found to be bound to cell structures or particles sedimenting before or with ribosomes.

During the first time of germination the proportion of poly(A) decreases rapidly in the preribosomal fraction but increases in postribosomal supernatant, which contains nearly 70% of pollen tube poly(A) after 24 h of cultivation (Fig. 4). The most marked changes in redistribution of poly(A) from preribosomal to subribosomal fraction occurs during the first 20 min of pollen contact with the germination medium. After this time of incubation the size distribution patterns of poly(A)-containing RNA species from all three cell fractions exhibit the presence of the 4 main sedimentation maxima (Fig. 5) observed in maturing pollen (see Fig. 3). Their proportion in the preribosomal and in the polysomal fractions is similar to that in total RNA preparation from dry pollen showing little or no low-molecular-mass polyadenylated molecules of the 4S region. The practical absence of these mole-

cules from the polysomal fraction and the observed similarity of the sedimentation profiles demonstrate that the poly(A)-containing RNA is preserved intact during its extraction and fractionation. The small poly(A)-containing molecules are, however, the most abundant in the postribosomal supernatant. Because of the presence of rRNA, the more rapidly sedimenting

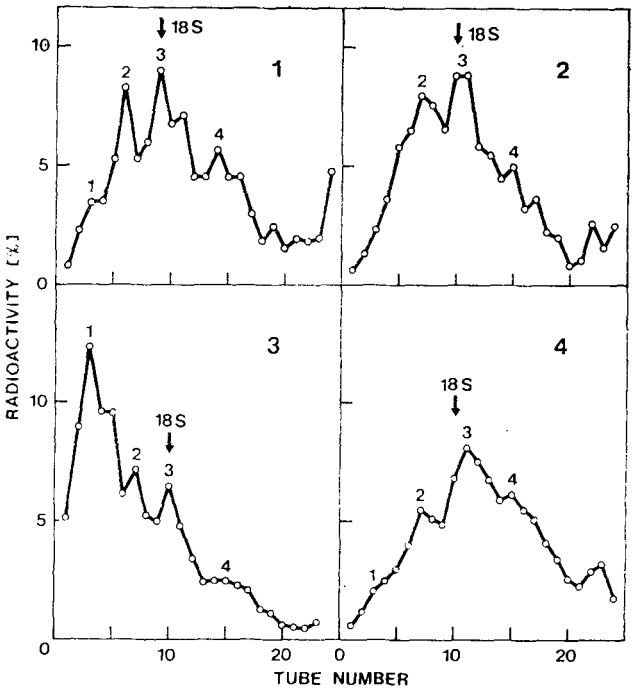


Fig. 5. Sedimentation profiles of poly(A)$^+$RNA from mature pollen on sucrose density gradients. Poly(A)$^+$RNA was isolated from preribosomal sediment (1), ribosomal fraction (2) and postribosomal supernatant (3) of pollen imbibed for 20 min and from quiescent pollen grains (4).

polyadenylated RNA species detected in this fraction may come from a part of non-sedimented low ribosomal oligomers or from the presumed non-polysomal ribonucleoprotein particles.

As could be expected, most of poly(A)$^+$RNA of the polysomal fraction is found to be incorporated into polysomal structures (Fig. 6). The about 16% of poly(A) not associated with ribosomes may originate from partial degradation of polysomes which may occur during their resuspension and sucrose density gradient centrifugation.

On the whole, the results on cellular distribution of poly(A) suggest that poly(A)$^+$RNA bound in dry pollen to cell structures is progressively liberated during pollen imbibition and germination and incorporates into polysomes. During or before this incorporation, part of it seems to be degraded and to give rise to small poly(A)-molecules appearing in the cytoplasm.

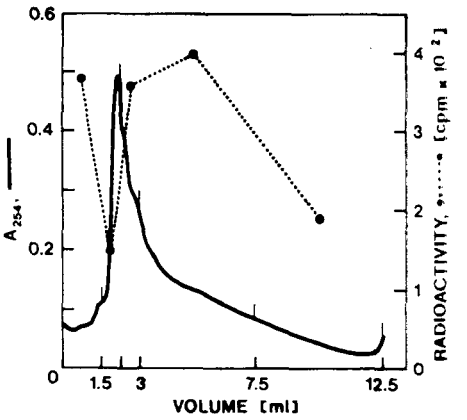


Fig. 6. Association of stored poly(A)+RNA with ribosomes after 10 min of pollen imbibition. The pellet of polysomes was resuspended in 0.4 ml of 0.05 M Tris-HCl (pH 8.5), 20 mM KCl, 5 mM mercaptoethanol, 5 μg $ml^{-1}$ cycloheximide and layered on 12 ml of 15–40% sucrose gradient in the same buffer and centrifuged for 2 h in Beckman SW 40 rotor at 32 000 rev. $min^{-1}$ 5 °C. The gradient was divided into 5 fractions and RNA extracted by the standard procedure. The radioactivity of the $^3$H-poly(U)-poly(A) complex is expressed per ml of the respective fraction.

## DISCUSSION

The pollen grain representing the male gametophytic generation in higher plants arises by mitotic division of the mature microspore, and its development can be divided into two phases, maturation and pollen tube formation. In very young pollen of tobacco and of many other species, the normal way of cell differentiation is not irreversibly predetermined and a sporophytic development can be induced by changing chemical and physical environment of the pollen. The disposition towards a change of developmental programme decreases and disappears with the progress of maturation (see SUNDERLAND and DUNWELL 1978).

The results presented here strongly suggest marked changes in gene expression during the transition of microspore into mature pollen grain. Direct evidence was obtained that the observed and already reported (see STANLEY and LINSKENS 1974) accumulation of RNA is related to the activities of rRNA genes in young pollen, and the population of polyadenylated mRNA is shown to exhibit alterations in size distribution soon after microspore division resulting in progressive formation of new species with higher sedimentation values. Poly(A)+RNA of all stages of pollen maturation differs greatly in sedimentation profile from leaf polysomal poly(A)+RNA of the same tobacco cultivar (GOLDBERG *et al.* 1978). Its approximate number average size increases from 700 to 2 100 nucleotides whereas 1 340 nucleotides are reported for poly(A)+RNA of leaf polysomes (GOLDBERG *et al.* 1978). The modifications of the spectrum of mRNA molecules during pollen maturation can be the result of differentiation of gene activities and of progressive transcription of specific gametophytic programme or might reflect pollen specific changes in posttranscriptional processing of nuclear RNA, which is demonstrated as an important mechanism of regulation of gene expression in higher plants (KAMALAY and GOLDBERG 1980). The observed accumulation of poly(A), the addition of which is considered to play an essential role in the selection of functional mRNA molecules (see BRAWERMAN 1981), supports this latter possibility. An increasing amount of poly(A)+RNA accompanied by a shift in the size of the molecules towards higher molecular mass was also found in germinating radish embryo axes (DELSENY *et al.* 1977a). The

estimated proportion of poly(A)-containing RNA of total RNA rises about 9times during pollen maturation and reaches approximately 2.7%, which corresponds to the values reported for seed-stored mRNA (DELSENY *et al.* 1977b).

No direct evidence is so far available for an eventual implication of a part of the newly formed poly(A)$^+$RNA in the direction of pollen differentiation processes. On the other hand, several indications were obtained that it represents stored mRNA and is utilized during pollen tube development. The population of its molecules exhibits a broad range of sedimentation and may thus code for a wide spectrum of polypeptides; its size distribution persists unchanged in quiescent pollen and during a number of hours of pollen tube growth and finally it readily incorporates into polysomal structures at the onset of germination. The storage of the preformed mRNA in polyadenylated form is also suggested by the observation that the amount of poly(A) does not increase at the beginning of pollen germination. Similar results are reported for seed-stored mRNA (SPIEGEL and MARCUS 1975, DELSENY *et al.* 1977b). The presence of poly(A) sequences protects mRNA molecules against nucleases (see BRAWERMAN 1981, KARPETSKY *et al.* 1981) and may thus account for the high stability of stored mRNA. The stability of these sequences may in turn require their association with a protein (ADAMS *et al.* 1980). The presence of poly(A)-protein complexes in mature pollen is suggested by the detection of non-polysomal poly(A)-containing particles. The observed relatively high stability of the level of poly(A) throughout 24 h of pollen tube growth contrasts with a rapid decay of stored poly(A)$^+$RNA in germinating seeds (DELSENY *et al.* 1977b) and suggests the persistence of the function of pollen stored mRNA in developing pollen tubes.

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## BOOK REVIEW

STRATHERN, J. N., JONES, E. W., BROACH, J. R. (ed.): THE MOLECULAR BIOLOGY OF THE YEAST *Saccharomyces*. Life Cycle and Inheritance. Vol. 11A. — Cold Spring Harbor Laboratory, 1981. 751 pp. Cloth US $ 75.—, outside US $ 90.—

The yeast *Saccharomyces* is a typical eukaryotic organism that offers the advantages of simplicity of organization and ease of handling for investigations involving classical techniques as well as techniques that are at the forefront of molecular biology. The interest in yeast stemmed at first from its role in alcohol production in the making of wine and beer. Many of the important concepts of biochemistry *e.g.* the glycolytic pathway, early research in vitamins *etc.*, owe their origins to investigations in which yeast played a key role. The 11th Monograph in the Cold Spring Harbor Monograph Series (in 2-book set) deals with the biology of yeast. In this book (Monograph 11A) the cell and life cycle and the nuclear and non-Mendelian inheritance are discussed. The second book (Monograph 11B) will present reviews on the metabolism and gene expression in yeast.

This Volume covers the following topics: a genetic mapping, genome structure and replication, cytology of life cycle, cell cycle, pheromonal regulation of development, control of cell type, mating type and mating-type interconversion, meiosis and ascospore development, mechanisms of meiotic gene conversion, mechanisms of mitotic recombination, DNA repair and mutagenesis, killer systems, mitochondrial structure and mitochondrial genetic and function. The volume provides thus a comprehensive review of all main aspects of yeast genetics and biology. Useful appendixes contain data on the genetic nomenclature of yeast, a detailed genetic map, a list of genes listed alphabetically and further an alphabetical listing of gene products and a list of genes grouped by common function.

This monograph provides a timely and authoritative reference for workers of many disciplines, involved in the surging research activity of yeast biology and genetics.

T. GICHNER (*Praha*)