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# Poly(A)$^+$ RNA Synthesis in Germinating Pollen of *Nicotiana tabacum* L.

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**Abstract.** Poly(A)$^+$RNA is synthesized during the first hours of pollen germination and is rapidly incorporated into polysomal structures. After a 2-h pulse with uracil-$^{14}$C, 42% of the transcribed fraction of polysomal RNA is polyadenylated. Following 4 h of germination the amount of the newly-made poly(A)$^+$RNA decreases steadily at the rate of about 14% per h, whereas that of rapidly-labelled poly(A)$^-$RNA continues to grow. Beginning 1 h of cultivation the ratio of poly(A)$^-$/poly(A)$^+$RNA increases exponentially. Similarly as in non-polyadenylated mRNA the main portion of the synthesized polysomal poly(A)$^+$RNA sediments at a rate of 4 to 14 S and its mean size decreases slightly with the time of labelling. RNA isolated from nuclei and cell wall containing pollen tube fraction differed from the polysomal one in higher specific radioactivity and the polyadenylated RNA exhibited higher size distribution. The comparison of the results with earlier observations suggests the involvement of poly(A)in mRNA translation in pollen tubes.

There is evidence of the presence of stored mRNA in mature pollen grains meeting the requirement for protein synthesis at early germination (Mascarenhas and Bell 1969, Linskens *et al.* 1970, Tupý 1977). Pollen of *Nicotiana tabacum* L. has also been shown to synthesize both ribosomal and messenger RNA at the earliest times after the onset of germination (Tupý 1977). The new mRNA formed associates rapidly with monosomes increasing for a certain time the amount of polysomes. However, our knowledge of its properties and functions in the development of male gametophyte is still very poor. In general, actinomycin D does not affect early germination, but inhibits later pollen tube growth and prevents the formation of the sperm cells (for review see Mascarenhas 1975). More recent results from *Tradescantia* pollen indicated that the RNA species synthesized during the first 2—3 h of growth and their translation is required for mitotic division of the generative cell (Lafleur and Mascarenhas 1978).

In this communication we show that the early synthesized pollen tube mRNA is highly polyadenylated, whereas that transcribed later on is essentially without poly(A) segments.

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## MATERIAL AND METHODS

Pollen of *Nicotiana tabacum* L. cv. Samsun free from microbial contamination was cultivated in shaken suspension cultures at 25 °C (TUPÝ *et al.* 1977a). One cultivation flask contained 50 mg of pollen and 10 ml of 0.3 M sucrose solution with 0.01% boric acid and 10 μCi of uracil-2-$^{14}$C (s.a. 44 mCi mmol$^{-1}$). At different times of incubation the mass of pollen tubes was separated from the liquid by filtration (TUPÝ *et al.* 1977a) and rapidly frozen on the surface of solid $CO_2$.

Polysomes were isolated by a modification (TUPÝ *et al.* 1977b) of the procedure of SUN *et al.* (1975) in the presence of 2% Triton X-100. RNA was extracted (TUPÝ *et al.* 1977b) from the polysomal pellet and from pollen tube residue sedimenting during separation of the postmitochondrial supernatant (20 min at 20 000 g).

The isolation of poly(A)$^+$RNA was achieved by affinity chromatography on poly(U) Sepharose 4B according to the procedure of DWORKIN and INFANTE (1976). Fractions containing the non-retained and retained RNAs were pooled and precipitated with 2 vol. of ethanol after addition of pollen ribosomal RNA as carrier to the poly(A)$^+$RNA fraction.

The sedimentation analyses were carried out on 5—20% sucrose gradients made with 12 ml of 10 mM Tris-HCl, pH 7.6; 100 mM NaCl; 0.1% SDS. The gradients were spun in a Beckman SW 40 Ti rotor for 14 h at 25 000 rpm, 20 °C.

Radioactivity was measured in a toluene-based scintillation fluid with an efficiency of 75 to 80%.

## RESULTS

### RNA Extraction

As deproteinization of RNA extracts with phenol alone results in considerable loss of poly(A)$^+$ containing RNA (BRAWERMAN *et al.* 1972, PERRY *et al.* 1972), several methods based on phenol-chloroform extraction have been developed for the isolation of this type of RNA. We have compared the phenol-chloroform extraction at pH 6 (PERRY *et al.* 1972) and at pH 9 (DELSENY *et al.* 1977) with the chloroform procedure currently used in our laboratory for RNA extraction from pollen tubes (TUPÝ *et al.* 1977b). Although the phenol-chloroform procedures give higher yields of the total 260 nm-absorbing material, there are only very slight differences in rapidly labelled poly(A)$^-$RNA and the amount of poly(A)$^+$RNA extracted by the chloroform method appears to be the highest (Fig. 1). This was confirmed in several experiments and for this reason as well as for greater simplicity, we used this method in further work. Additional purification of RNA extracts by CsCl centrifugation (GLISIN *et al.* 1974) did not change the proportions of the two types of RNA.

### Kinetics of RNA Synthesis

The mean growth rate of tobacco pollen tubes in suspension culture is about 3.1 μm min$^{-1}$ and it does not decrease during the first 8 h of cultivation (BALATKOVÁ *et al.* 1977). The uptake of nutrient solution thus remains constant and one can assume that the uptake and concentration of uracil-$^{14}$C in tube protoplasm do not change significantly. This can also be concluded

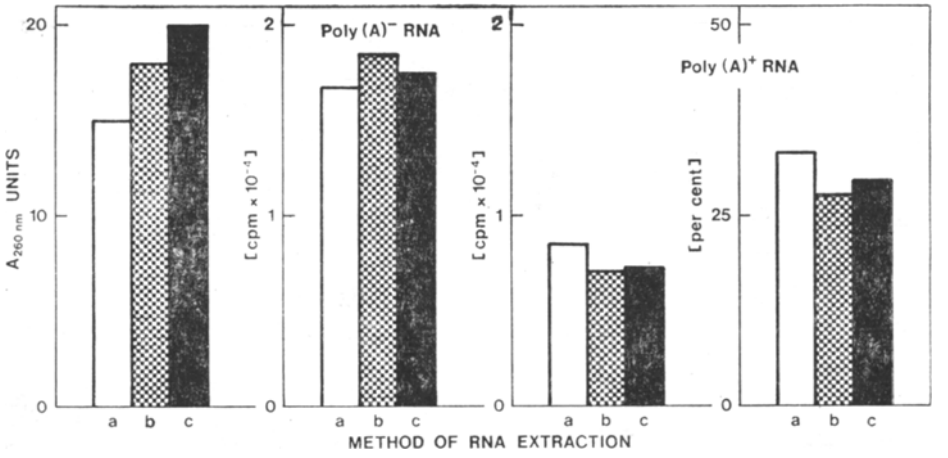


**Fig. 1.** Comparison of three methods of RNA extraction from pollen polysomes. 50 mg of pollen was cultivated during 4 h, polysomes were isolated as described in Material and Methods section and RNA was extracted: a — by chloroform method (TUPÝ *et al.* 1977b); b — by the method of PERRY *et al.* (1972) utilizing phenol-chloroform deproteinization at pH 6; and c — by the pH 9 phenol-chloroform procedure according to DELSENY *et al.* (1977).

from the earlier observation that the total incorporation of uridine-$^{14}$C into ribosomes increases at a constant rate throughout the 12 h of pollen cultivation (TUPÝ 1977).

As the total amount of RNA in pollen tubes decreases with time due to its release into the medium (TUPÝ *et al.* 1974) and to possible tube bursting, the results on RNA labelling are expressed as radioactivity per mg of RNA extracted (Fig. 2). It is shown that a great part of the RNA synthesized at the earliest time of germination is polyadenylated and that it is rapidly incorporated into the polysomal structures. The total amount of this poly(A)$^+$-RNA, rises, however, during the first 4 h only, decreasing later on. On the contrary, the specific labelling of poly(A)$^-$RNA continues to grow for the whole time of pollen cultivation. The proportion of the newly-made polysomal RNA which binds to the poly(U)-columns thus falls rapidly and that from 42% after 2 h of germination to 12% in 8-h culture. The plot of the poly(A)$^-$/poly(A)$^+$ ratios against time shows an exponential evolution. The experimental data conform to the equation $y = a^x$, where $a$ is time in hours beginning one hour of incubation and $x$ equals 1.33.

An essentially similar situation is found with RNA isolated from pollen tube residue after the separation of polysomes. This RNA represented about 42%of the total pollen RNA isolated and probably contained, in addition to nuclear and mitochondrial RNA, a portion of RNA of polysomes which may partly sediment with mitochondria during the centrifugal fractionation. The specific radioactivity of this RNA, as compared with that of RNA isolated from polysome structures is about 3 and 2 times higher in poly(A)$^-$ and poly(A)$^+$ fraction, respectively (Fig. 2). The level of the transcribed polyadenylated fraction undergoes smaller changes than in polysomes, showing a slight increase during the first 6 h followed by a slow decrease.

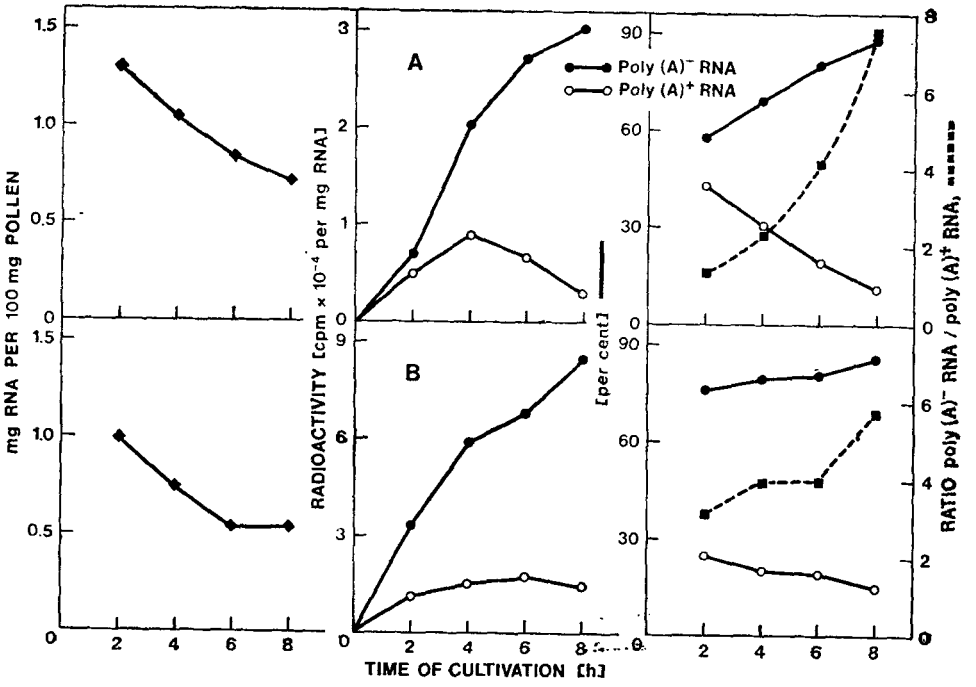


Fig. 2. Time course of the content of poly(A)$^+$ and poly(A)$^-$RNA synthesized during pollen germination. RNA was separately isolated from polysomes (A) and from the sediment obtained on isolation of the postmitochondrial supernatant (B).

**Sedimentation Properties**

Size distribution of polysomal poly(A)$^-$RNA (Fig. 3A) corresponds essentially to that found previously for total polysomal RNA (TUPÝ 1977). It is of polydisperse type, with a predominant labelling of species sedimenting between 4 S and 10 S and exhibiting a slight shift in the size of the molecules towards lower molecular weight during the first 6 h of pollen tube growth. The radioactivity of these RNAs increases steadily with the time of labelling. The radioactivity profile of poly(A)$^+$RNA shows only one major peak having the same sedimentation maximum and exhibiting the same shift with the time of pollen cultivation as the main radioactivity peak of poly(A)$^-$RNA.

The analysis of RNA from the sediment obtained on isolation of the postmitochondrial fraction reveals a relatively high content of ribosomal RNA. In comparison with the two RNA fractions of polysomes, the sedimentation profile of radioactivity of poly(A)$^-$RNA exhibits close similarity, while that corresponding to polyadenylated fraction shows somewhat higher size distribution and an additional clear cut peak sedimenting at a rate of about 9.6 S (Fig. 3B). This peak might be the nuclear poly(A)$^+$RNA which is often reported to be larger than poly(A)$^+$RNA from polysomes (ANGERMANN *et al.* 1976, RODRIGUEZ-POUSADA and HAYES 1976).

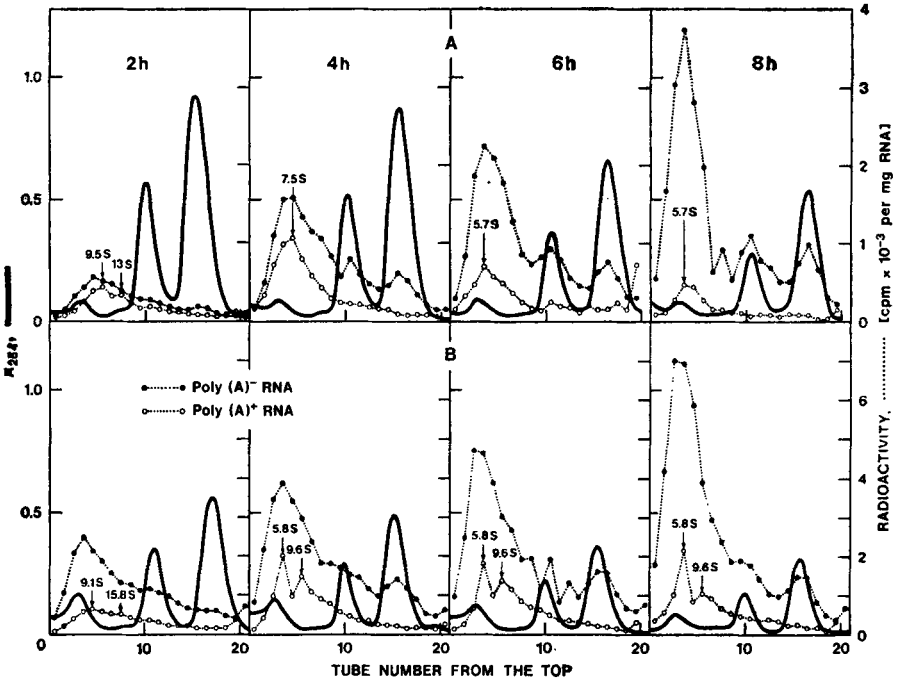


Fig. 3. Size distribution of the newly-made poly(A)$^+$ and poly(A)$^-$RNA at various stages of pollen germination. RNA isolated from polysomes (A) and from the sediment containing pollen tube debris, nuclei and mitochondria (B) was fractionated on linear sucrose density gradients.

## DISCUSSION

The results obtained present evidence that poly(A)$^+$RNA is synthesized *de novo* at the initial stages of pollen germination and that this RNA is engaged in the polyribosomal complex. The total amount of the newly-made polysomal poly(A)$^+$RNA increases, however, only during the first 4 h of incubation and decreases rapidly later on. This contrasts with the non-polyadenylated fraction, the content of which rises over the whole 8-h period of pollen cultivation. The proportion of poly(A)$^+$RNA falls from 42% at 2 h to only 12% at 8 h. The greater portion of the poly(A)$^-$RNA appears to be non-ribosomal and has very similar sedimentation properties as the poly(A)$^+$ fraction. This suggests the sequentional similarity of the polyadenylated and poly(A) lacking mRNAs. The absence of polyadenylation was already observed in a significant proportion of several specific messenger RNA species of animal (Rhoads 1975, Fromson and Verma 1976, Taylor and Tse 1976) and plant (Ragg *et al.* 1977) cells.

The reasons and significance of the initial formation and later disappearence of poly(A)$^+$RNA from polysomes are not clear. The continuing synthesis of non-polyadenylated mRNA and its sedimentation similarity with poly(A)$^+$-RNA together with the results on evolution of labelled poly(A)$^+$RNA of the nuclei containing pollen tube fraction, suggest that these changes occur due to a decrease in RNA polyadenylation rather than in consequence of a fall of transcriptional activity of the pollen genome. In higher animals the

activity of poly(A) polymerase was shown to be dependent on the physiological state of the cell (COLEMAN *et al.* 1974, JACOB *et al.* 1976). From this point of view the decline in RNA polyadenylation could be considered as a result of unfavourable conditions for long-term pollen development in artificial culture. Poly(A)$^+$RNA content is also shown to decrease rapidly soon after cell division ceases (GRIERSON and COVEY 1975) and a fall in poly(A) polymerase activity is reported to accompany the transition from the S phase to the G 1 phase of the cell cycle (ADOLF and SWETLY 1978). On the other hand, the poly(A) polymerase can also catalyse hydrolysis of poly(A) (ABRAHAM and JACOB 1978) and an increase of this activity cannot be excluded as a possible reason of the observed diminution in poly(A)$^+$RNA.

The observed decay of the newly-made polysomal poly(A)$^+$RNA demonstrates that this mRNA is translated in germinating pollen. Moreover, the changes in its level are parallelled to the earlier changes observed in proportion of ribosomes associated in polysomal structures (TUPÝ 1977) and in protein synthesizing activity (unpublished results) of tobacco pollen tubes in culture. This, together with the steadily increasing amount of the labelled polysomal poly(A)$^-$mRNA of similar sedimentation properties as the polyadenylated fraction, argues that the presence of poly(A) in at least the majority of mRNA molecules is of essential importance for their translation in germinating tobacco pollen. Such a conclusion on poly(A) function was also drawn from the results on other eucaryotic cells (*e.g.* TOBIN and KLEIN 1975, VERMA *et al.* 1975), but the existence of systems and messenger RNAs not requiring poly(A) for template activity is also reported (*e.g.* FROMSON and VERMA 1976, RAGG *et al.* 1977).

For further orientation in this research, the question appears to be of importance whether the decline in efficiency of mRNA polyadenylation is the result of changes in the physiological state of the culture due to the *in vitro* conditions or whether it has some significance for the development and normal biological functions of pollen tubes.

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