Plant Physiol. (1976) 157, 632-639

Sequence of Reactivation of Ribonucleic Acid Synthesis during Early Germination of the Maize Embryo[1]

Received for publication August 11, 1975 and in revised form November 24, 1975

CLAUDE VAN DE WALLE[2] AND GEORGES BERNIER
Laboratory of Physiology, Department of Botany, University of Liège, Sart Tilman, B-4000, Liège, Belgium

ROGER DELTOUR AND RAYMOND BRONCHART
Laboratory of Morphology, Department of Botany, University of Liège, Sart Tilman, B-4000, Liège, Belgium

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ABSTRACT

The onset of RNA synthesis in primary roots of germinating *Zea mays* embryos was studied. Incubation of excised embryos in the radioactive precursor solution was performed after different germination periods.

It was observed that there are three successive stages in the reactivation of RNA synthesis. Stage one is characterized by the synthesis in the chromatin of heterodisperse nuclear RNA rich in adenine. Stage two is characterized by the synthesis of heterodisperse nuclear RNA in the chromatin and 2.3×10^6 rRNA precursor in the nucleolus. Stage three is characterized by the synthesis of heterodisperse nuclear RNA and rRNA precursor and by the processing of the latter in mature rRNA.

In higher plants, the growth of the embryo is arrested during maturation of the seed and a state of quiescence is established. During this period, the water content remains low and the synthetic activity, in particular RNA synthesis, is almost completely arrested (25).

Numerous studies were devoted in recent years to the reactivation of RNA synthesis during the germination process, but the question of a sequence in the onset of synthesis of the different RNA species was not often considered. During germination of the wheat embryo, Chen *et al.* (2) concluded that the earliest transcribed genes were the ribosomal ones. On the contrary, Walbot (26) observed in *Phaseolus* that there was no preferential synthesis of a particular species of RNA during the first 48 hr of germination. Recently, it was claimed that, in germinating wheat embryos, mRNA was synthesized first; rRNA and tRNA synthesis followed (8). In the radicle of maize embryos, we reported that the first RNA to be synthesized was hnRNA[3] (23).

Considering these contradictory results, further attempts were undertaken in order to establish the complete sequence of reactivation of high mol wt RNA synthesis. The maize embryo was considered advantageous for this purpose because it contains a large radicle which can be easily excised and used for RNA extraction. This allowed a biochemical study limited to the tissue where the resumption of growth first occurs (1).

MATERIALS AND METHODS

Germination Procedure. Maize seeds (*Zea mays*, var. InRA 258 or CiV 2) were germinated in the dark at 16 C. The seeds were first soaked in tap water for 6 hr and then transferred to Petri dishes. The choice of a suboptimal temperature for germination was explained in a previous paper (23). At that temperature, the protrusion of the radicle occurred between 48 and 72 hr of germination.

Incubation Procedure. Fifteen excised embryos were incubated 2 hr at 16 C in 125 μl of a radioactive solution ([G-^{3}H]adenosine, 11.8 Ci/mmole, 200 μCi/ml; or [5-H^3]uridine, 27 Ci/mmole; 200 μCi/ml; or [^{32}P]phosphate, 1 or 5 mCi/ml). Streptomycin (50 μg/ml) was added to the solution. At the end of the incubation period, the radicles were excised under a stereoscopic microscope, the root caps and coleorhizas were carefully discarded. For biochemical preparations, the radicles were stored in liquid N_2.

Autoradiographic Preparations. At the end of the incubation period, the excised radicles or root tips (1 mm) were processed in two different ways. When embedded in paraffin, fixation was performed in formalin-acetic acid-alcohol. Five-μm thick longitudinal sections were prepared and covered with an autoradiographic stripping film, Kodak Ar.10 (22).

In case of epoxy resin embedding, the radicles were washed for 0.5 hr in H_2O at 16 C, fixed for 3 to 5 hr in 6% glutaraldehyde, washed for 48 hr in sodium cacodylate buffer (15), and postfixed in 2% osmium tetroxide. One-μm thick longitudinal sections were cut and covered with a liquid emulsion, Ilford L4. After exposure, the sections were stained with 0.2% toluidine blue.

Counts of silver grains were performed in several radicles of each experimental batch. The conditions of incubation in the radioactive precursor and of autoradiography were the same for all batches. For each radicle, the grains were counted over 10 cells containing a distinct nucleolus and chosen at random in the cortical region of the radicle at about 0.5 mm from the tip. The background was estimated from counts of grains in areas adjacent to the section. Ten areas, with approximately the size of a cell, were chosen at random. Only one section per radicle was observed.

Biochemical Preparations. About 60 radicles or root tips (5 mm) were homogenized in a precooled mortar with 1 ml of the extraction buffer (0.4 M NaCl, 0.005 M EDTA; 1% Sarkosyl, 0.1 M tris-HCl, pH 9). Extraction was performed with 3 ml of buffer; 2 ml of phenol previously saturated with the buffer; 2 ml of chloroform-isoamyl alcohol (24:1). After recovery of the aqueous phase by centrifugation (5 min, 3860*g*), a second extraction was performed with 4 ml of chloroform-isoamyl alcohol. The nucleic acid was precipitated with ethanol for 2 hr at −25C.

The nucleic acid precipitate was recovered and dissolved in the electrophoresis buffer (sodium acetate 2×10^{-2} M, EDTA 2×10^{-3} M; tris 4×10^{-2} M, pH 7.8 with acetic acid) containing, in addition, 10% sucrose and 10% sodium lauryl sulfate.

The separation was performed with 2.2% acrylamide gel ac-

[1] This work was supported by the Fonds de la Recherche Fondamentale Collective Grant 10.306.

[2] Chercheur qualifié du Fonds National de la Recherche Scientifique.

[3] Abbreviation: hnRNA: heterodisperse nuclear RNA.

Table I. *Counts of Silver Grains in Autoradiographs of Maize Radicles (CiV 2) at Different Stages of Germination*

Germination time[2]	No. of Grains[1] ± Standard Error					No. of Grains in % of Total		
	Nucleolus	Chromatin	Cytoplasm	Total	Background	Nucleolus	Chromatin	Cytoplasm
hr								
0	2.1 ± 0.3	32.9 ± 2.1	8.4 ± 0.7	43.4	0.9	5	76	19
4	1.3 ± 0.2	17.0 ± 1.1	2.5 ± 0.3	20.8	0.4	6	82	12
6	1.5 ± 0.3	18.9 ± 1.6	2.9 ± 0.4	23.3	0.6	6	81	13
8	8.9 ± 0.7	40.8 ± 3.2	6.0 ± 0.7	57.7	1.4	22	73	11
48	16.0 ± 1.5	23.2 ± 2.1	32.3 ± 4.2	71.5	3.1	16	33	45

[1] Mean of grains counted over 10 cells per radicle, background not deduced. Five radicles were observed in all cases, except in the 8-hr batch where three radicles only were used.

[2] Germination period of intact seeds before the 2-hr incubation period of embryos in [^{3}H]uridine.

cording to Loening (11). The gels were prepared in Perspex tubes (8 × 0.7 cm), and electrophoresis was carried out at room temperature under 10 mamp per tube at approximately 65 v. The scanning was performed at 265 nm with a Joyce Loebl UV scanner; the gels were then frozen to their exact length and cut in 1 or 2 mm thick slices with a homemade slicer. In the case of ^{3}H labeling, each slice was dissolved in 0.5 ml of hydrogen peroxide (Perhydrol 30%, Merck) at 70 C overnight; 10 ml of scintillation liquid were added to each vial (toluene, 1 liter; Triton X-100, 0.5 liter, Omni-Fluor, NEN Chemicals, 4 g/l). In the case of ^{32}P labeling, the gel slices were dried overnight at room temperature and put in scintillation vials containing 1 ml of toluene-Omni-Fluor scintillation liquid. Counting was performed with a Packard scintillation counter.

The base composition analysis of the ^{32}P-labeled hnRNA was performed after filtration of the nucleic acid preparation on a Sephadex G-100 column (144 × 0.6 cm) (23).

The base composition analysis of ^{32}P-labeled rRNA was performed directly after gel electrophoresis. After scanning, the gel was frozen and cut in 1-mm thick slices. The radioactivity of each slice was counted with a Geiger-Müller detector. Three to five slices were dipped in 2.5 ml of potassium hydroxide (0.3 M) and kept overnight at 37 C for hydrolysis of the RNA. The nucleotide solution recovered was neutralized with perchloric acid and chilled. The fractionation of the four nucleotides and the radioactivity determinations were performed as already described (23).

RESULTS

Stage One. We have previously reported that, in the radicle of the maize embryo, the chromatin was exclusively labeled when incubation in [^{3}H]uridine was performed during the first hours of germination (5, 22). The same result was obtained with the seeds used in the present study. When incubation was performed with nongerminated embryos or with embryos excised from seeds germinated for 4 or 6 hr, 80% of the labeling appeared in the chromatin (Fig. 1A, Table I). The majority of nucleoli had very few (0-2) grains; these grains were very often localized on the edge of the nucleolus (Fig. 1A, arrows). The majority of them could therefore be generated by radioactive RNA molecules synthesized in the chromatin. In 8 cells among the 150 observed, 5 to 7 grains were counted per nucleolus. The grains counted over the cytoplasm represented a weak labeling, considering the fact that the cytoplasm represented 70% of the cell cross-sectional area; the nucleolus and the chromatin represented only 5 and 25% of the cell cross-sectional area, respectively.

The photograph of Figure 1A was taken in the cortical region of the radicle, approximately at equal distance between the tip and the base. As seen in Figure 2, an identical pattern of labeling was obseved in cells taken in any locations within the radicle. This uniformity of the labeling pattern allowed the extraction of nucleic acid from the entire radicle.

Gel electrophoresis analysis of the ^{3}H-labeled RNA synthesized during an incubation of 2 hr, after 6 hr of germination, showed an heterodisperse population of molecules, ranging approximately from 0.2×10^6 to 3×10^6 daltons and culminating in the region of rRNA. There was no peak of radioactivity corresponding either to the two rRNAs or to the rRNA precursor (Fig. 3). Base composition analysis of this RNA, labeled with [^{32}P]phosphate showed a high adenine content (Table II).

The localization of the labeling in the chromatin, the heterogeneity, and the base composition of the newly synthesized RNA establish that there is a period at the very beginning of germination characterized by the synthesis of hnRNA. This nuclear RNA is considered analogous to the hnRNA described in animal cell (19).

The very low number of silver grains counted over the nucleolus during this period establish that the rRNA precursor is synthesized at a very low level in only a few cells or is not synthesized at all. It is not possible to completely exclude the presence of a small amount of newly synthesized mRNA, represented by the weak cytoplasmic labeling during this first stage of germination. This labeling could also be attributed to the synthesis of RNA in mitochondria and plastids.

Stage Two. Autoradiographs of radicles incubated after 8 hr of germination clearly indicated a change in the labeling pattern (Fig. 1B). Numerous silver grains were present all over the nucleolus. The whole nucleus appeared uniformly labeled; the cytoplasm remained slightly labeled. Grain counts showed that the nucleolus was significantly more labeled than in the previous stage of germination (Table I).

As synthesis of the rRNA precursor is known to occur in the nucleolus (17, 21), the autoradiographic observations suggested that the germination process was reaching a second stage, different from the first one and characterized by reactivation of the synthesis of rRNA precursor.

This interpretation was substantiated by the electrophoretic pattern of the RNA synthesized during the second stage. Figure 4 shows the electrophoresis profile of a nucleic acid preparation extracted from radicles incubated for 2 hr in [^{3}H]uridine after 24 hr of germination. In addition to the population of hnRNA already recognized at the previous stage, a peak of radioactive molecules was detectable in the region of the 2.3×10^6 rRNA precursor. However, no peak of radioactivity was associated with rRNA.

The analysis of RNA molecules synthesized in the radicle after 8 hr of germination establishes the existence of a second stage in the germination process. This stage is characterized by the simultaneous synthesis of hnRNA and rRNA precursor. The processing of the latter into mature rRNA molecules is absent or so slow that it is not detectable within the 2 hr of incubation.

Stage Three. ^{3}H-labeled nucleic acid was extracted after 99 hr of germination and 2 hr of incubation in order to determine if the maturation of the rRNA precursor occurred at that time. The gel

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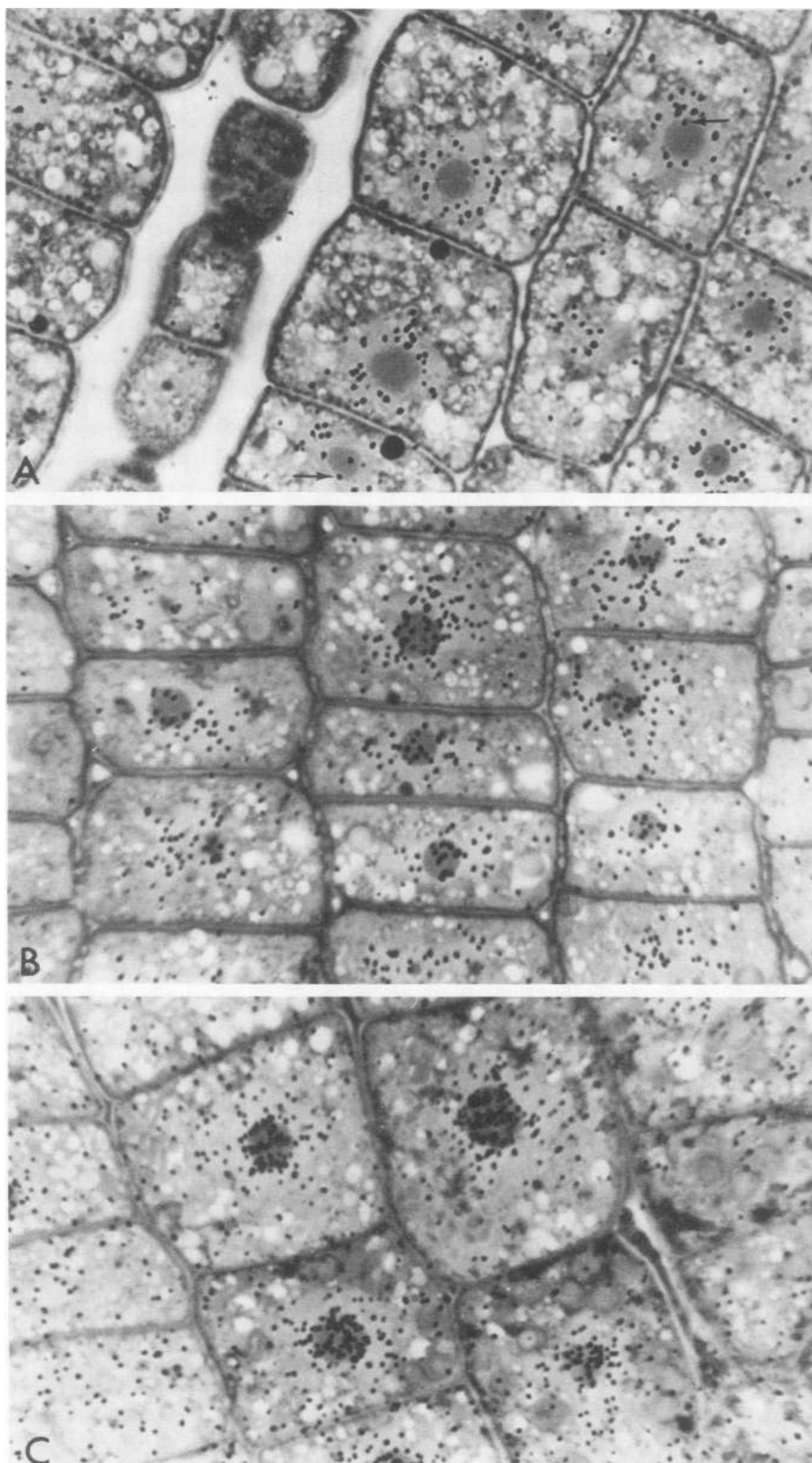

FIG. 1. Autoradiographs of sections of maize radicles (CiV 2) after different periods of germination. Incubation of excised embryos in [^{3}H]uridine during 2 hr. One-μm thick section embedded in epoxy resin. A: seeds germinated during 4 hr; B: seeds germinated during 8 hr; C: seeds germinated during 48 hr.

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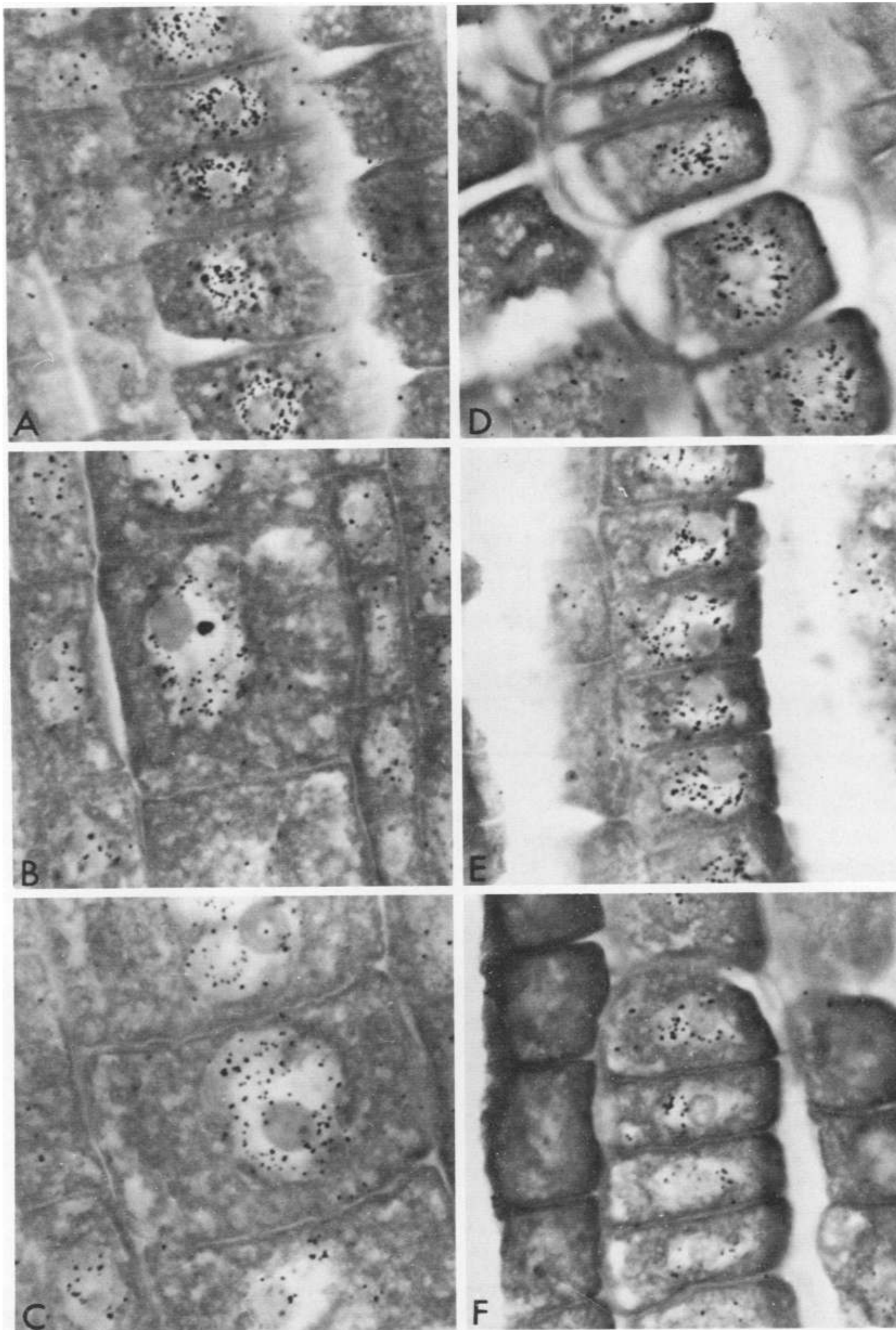

FIG. 2. Autoradiographs of different areas in a section of a maize radicle (INRA 258). Germination of seeds during 6 hr and incubation of the excised embryo in [^{3}H]uridine during 2 hr. Five-μm thick section embedded in paraffin. A, B, C: areas of the cortical region of the radicle localized, respectively, at the base, in the middle, and near the tip. D, E, F: areas of the central cylinder chosen, respectively, at the base, in the middle, and near the tip.

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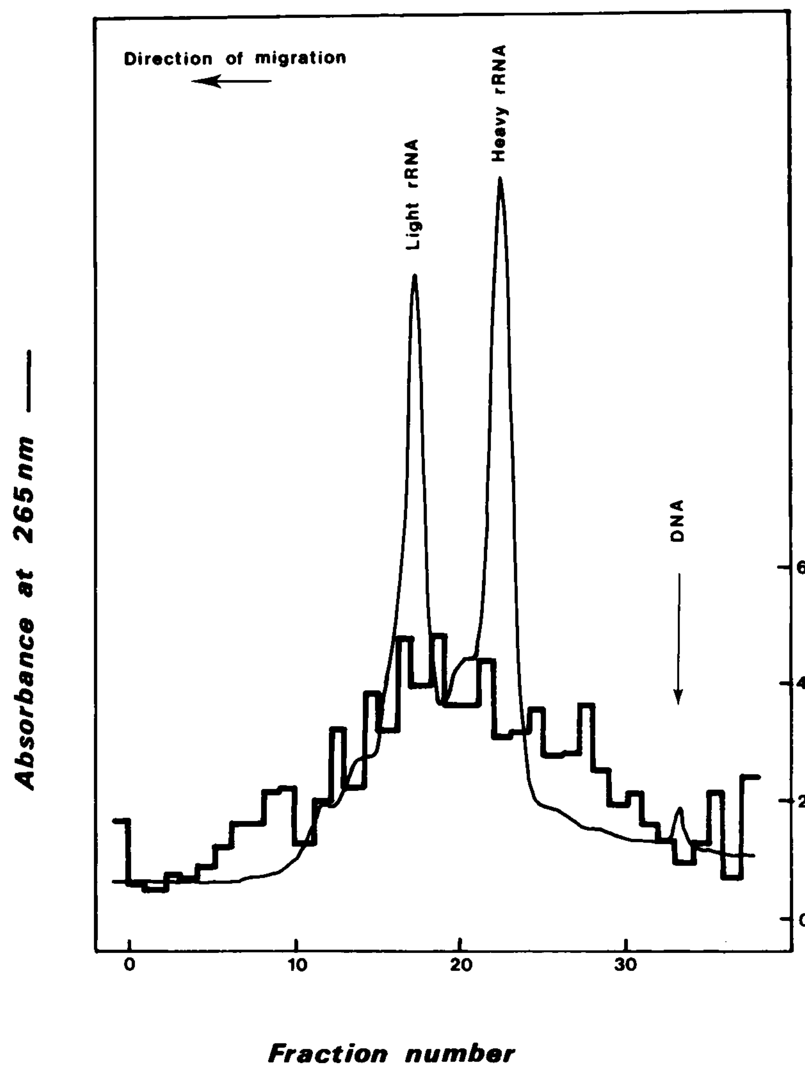

FIG. 3. Gel electrophoresis analysis of total nucleic acid extracted from maize radicles (INRA 258). Seeds were germinated 6 hr and excised embryos were incubated 2 hr in [^{3}H]uridine. Electrophoresis in 2.2% acrylamide gel, 8 × 0.7 cm, 10 mamp per gel. No absorbance unit.

Table II. *Base Composition of ^{32}P-labeled RNA from Maize Radicles (CiV 2)*

The rRNA and rRNA precursor were extracted from root tips after 99 hr of germination and 2 hr of incubation in [^{32}P]phosphate, they were separated by acrylamide gel electrophoresis. The hnRNA was extracted after 6 hr of germination and 2 hr of incubation in [^{32}P]phosphate; it was further purified by filtration on a Sephadex G-100 column.

	Cytosine	Adenine	Guanine	Uracil	Guanine + Cytosine	Guanine/Adenine
			moles % ± SD			
rRNA precursor[1]	25.3 ± 0.6	26.3 ± 0.4	29.5 ± 1.9	18.9 ± 1.4	54.8	1.12
Heavy rRNA[1]	23.8 ± 0.4	26.3 ± 0.4	32.3 ± 1.0	17.5 ± 0.5	56.1	1.22
Light rRNA[2]	22.7 ± 0.1	25.6 ± 1.0	30.6 ± 0.5	21.1 ± 0.1	53.3	1.19
hnRNA[3]	22.2 ± 0.9	30.6 ± 2.6	27.4 ± 1.4	19.8 ± 2.8	49.6	0.89

[1] Three determinations.
[2] Two determinations.
[3] Four determinations.

electrophoresis profile (Fig. 5) showed a peak of radioactive molecules in the region of the 2.3 × 10^6 rRNA precursor, corresponding to a very small absorbance peak. The two rRNA were heavily labeled and a shoulder in the radioactivity profile on the heavy side of the 1.3 × 10^6 rRNA was most probably due to the 1.4 × 10^6 heavy rRNA precursor.

Base composition analysis of the three RNA species heavily labeled at 99 hr of germination was undertaken in order to ascertain their identity (Table II). The three species were characterized by a high guanine content; the guanine/adenine ratio was above one and the guanine + cytosine content ranged from 53 to 56%. These values were similar to those obtained for rRNA and rRNA precursor in pea and artichoke tissues (18). We consider, therefore, that the peak of radioactive material which migrates in the upper portion of the gel is made of 2.3 × 10^6 rRNA precursor molecules and that a large proportion of these molecules is rapidly processed into mature rRNA during the 2 hr of incubation.

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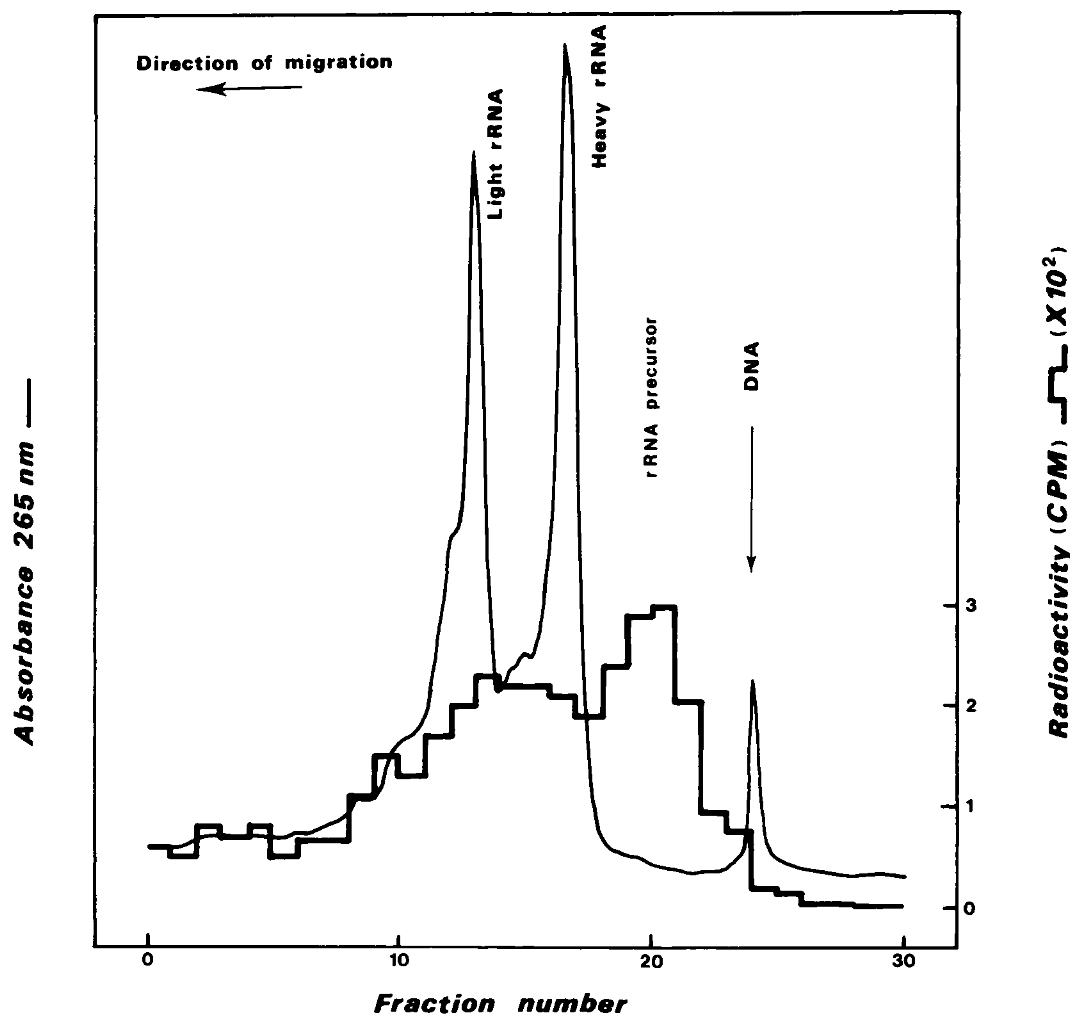

FIG. 4. Gel electrophoresis analysis of total nucleic acid extracted from maize radicles (CiV 2). Seeds were germinated 24 hr and excised embryos were incubated 2 hr in [^{3}H]uridine. The conditions of electrophoresis are the same as those in Fig. 3.

Autoradiographic observations were undertaken with root tips of seedlings incubated for 2 hr in [^{3}H]uridine after 99 hr of germination. The amount of radioactivity incorporated was so high at that time that reproducible counts of silver grains could not be obtained. The labeling pattern observed was identical to that obtained with radicles germinated for 48 hr (Fig. 1C). In these radicles, the nucleolus was more heavily labeled than the chromatin and a large number of grains was visible over the cytoplasm. Grain counts showed that 45% of the radioactivity was located in the cytoplasm (Table I). The cytoplasmic labeling at 48 hr could be correlated with the presence of radioactive rRNA detected at 99 hr (Fig. 5) and it can be suggested, that, at 48 hr, the maturation of rRNA is already reactivated.

The presence of newly synthesized hnRNA was not observed clearly in the electrophoresis gel profile during the third stage, but the observed labeling of the chromatin after 48 and 99 hr of germination suggests that hnRNA synthesis is most probably sustained.

DISCUSSION

Our data demonstrate that a sequence of events exists during the reactivation of high mol wt RNA synthesis in the germinating maize radicle. Three successive stages have been distinguished. Stage one is characterized by the reactivation of hnRNA synthesis, stage two by the reactivation of rRNA precursor synthesis, and stage three by the reactivation of a rapid processing of rRNA precursor molecules into mature rRNA.

No attempts were made to establish precisely the time of transition between these stages. It can be considered that, at 16 C, the first stage begins immediately after soaking or after a latency period shorter than 2 hr and that it lasts until the 8th hr. The second stage lasts approximately until 24 to 48 hr. The third stage begins around the 48th hr and is probably maintained permanently in the growing portion of the radicle.

There is no significant latency period before reactivation of RNA synthesis. However, such a period was observed in the radicle of another variety of maize (22) and in *Phaseolus vulgaris* axes (26).

The absence of rRNA synthesis while another RNA species is being synthesized has already been described in other plant materials. Mascarenhas and Bell (12) showed that in the growing pollen tube of *Tradescantia*, ribosomes are already present and a polydisperse RNA species, rich in adenine, is synthesized. Verma and Marcus (24) showed that in peanut cell cultures, an adenine-rich RNA species is synthesized when no rRNA synthesis occurs. This happens during the lag period between the dilution of the culture and the beginning of cell division. Cline and Rhem (3) observed the synthesis of polydisperse or messenger RNA, in the absence of rRNA synthesis, in 4- to 7-day-old *Avena* coleoptiles. The presence of several RNA polymerases with specific localization in the cell has been demonstrated in

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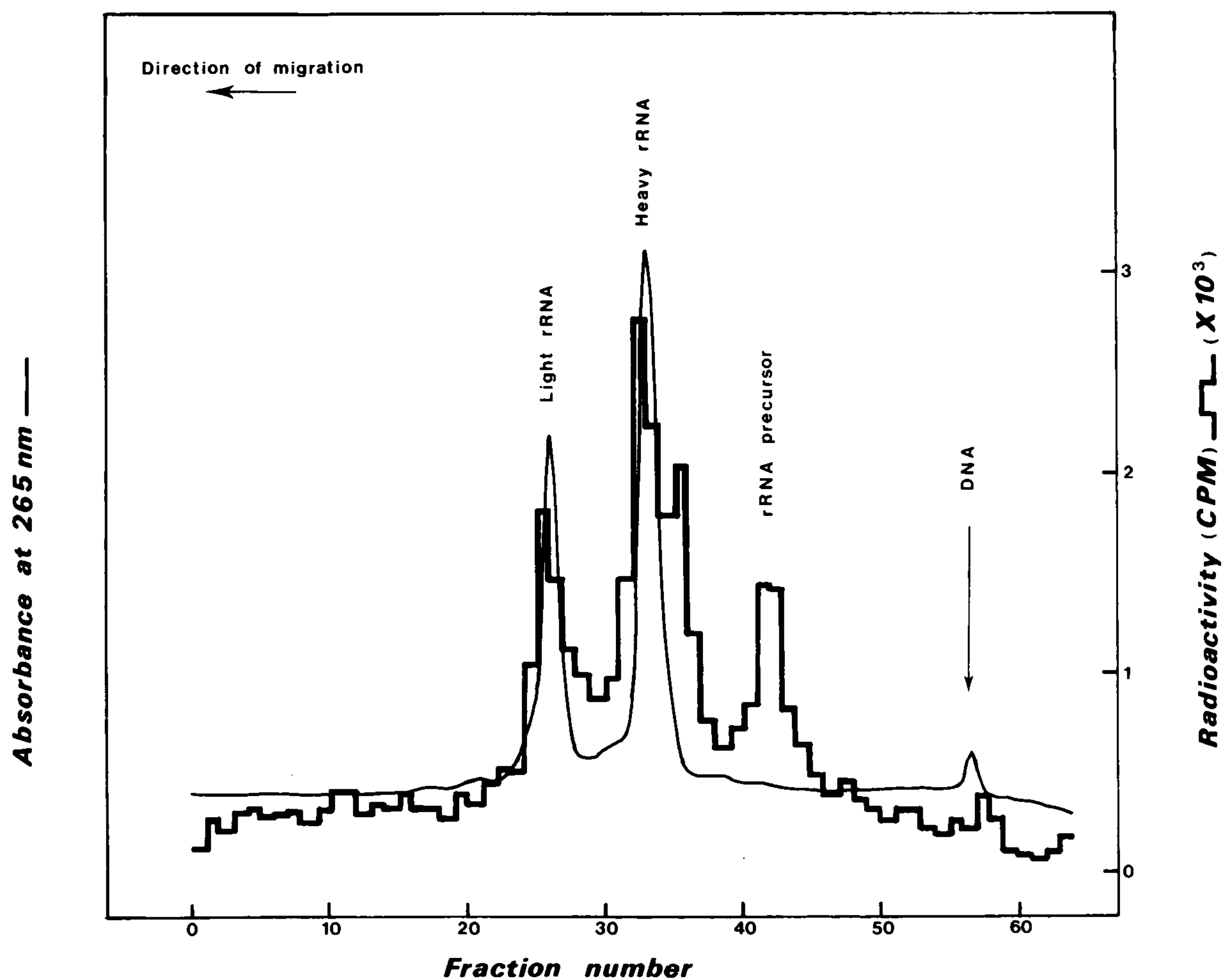

FIG. 5. Acrylamide gel electrophoresis analysis of total nucleic acid extracted from maize root tips (INRA 258). Seeds were germinated 99 hr and excised embryos were incubated 2 hr in [^{3}H]adenosine. The conditions of electrophoresis are the same as those in Fig. 3.

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plant tissues (9, 10, 13, 14, 20). The existence of a period in the germination of maize embryos characterized by the synthesis of hnRNA in the absence of rRNA synthesis could possibly be attributed to a lag in the reactivation of the nucleolar RNA polymerase while the nucleoplasmic enzyme is already activated. A similar fact was observed in onion embryos during the first 12 hr of germination (16).

The absence of rRNA synthesis during the first stage of germination could be tentatively correlated with the loss of the granular component in the nucleolus reported by de Barsy *et al.* (4) in the same material. Further work comparing ultrastructural features in the nucleolus and biochemical data on the onset of rRNA synthesis and maturation can be envisaged since the maize radicle appears to be an excellent material for such a study.

The synthesis of tRNA was not considered in this study. Preliminary results indicate that tRNA is not labeled or only very slightly labeled during the first hours of germination. A small amount of the chromatin labeling could therefore be due to tRNA. It is not established that tRNA molecules are kept into the sections with the methods of fixation and processing of the radicles used in the present study.

The sequence of reactivation of RNA synthesis in the maize embryo does not corroborate the observations previously made in other materials. The discrepancies between our results and those of Chen *et al.* (2) and Walbot (26) could be attributed to the fact that different plant species display different patterns of reactivation or to the existence of differences in the experimental procedures. The experimental systems used by these authors are characterized by conditions inducing a rapid resumption of metabolic activity. Chen *et al.* (2) used embryos of *Triticum*, excised from dry seeds, germinated and incubated in the presence of sucrose at room temperature; Walbot (26) used excised embryonic axes of *Phaseolus*, germinated and incubated in the presence of sucrose at 25 C. It was shown by Deltour (6), using maize embryos (var. INRA 258), that the period with no rRNA synthesis was shortened when germination and incubation were performed at room temperature. It cannot be excluded, therefore, that this period was already completed when Chen *et al.* (2) and Walbot (26) observed the synthesis of rRNA. The difference between our conclusions and those of Dobrzanska *et al.* (8) is perhaps less significant if it is assumed that the RNA they call mRNA is in fact nuclear pre-mRNA and that this pre-mRNA is part of hnRNA. The procedure of mRNA extraction used by these authors does not, however, establish definitely the messenger nature of the RNA and its localization within the cell.

In maize radicle, the reactivation of hnRNA and rRNA precursor synthesis occurs during a period of the germination where all cells are in the G_1 phase of the cell cycle (7).

Acknowledgments—Helpful discussions with Prof. H. Chantrenne, Dr. A. Burny, Dr. G. Marbaix, and Dr. G. Huez are gratefully acknowledged. The maize seeds var. CiV 2 were a generous gift from Mr. Legros, Centre Herbager de Promotion technique et économique, La Reid, Belgium.

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