Ribonucleic Acids in Spore Germination of Ustilago maydis

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ABSTRACT

Nongerminated teliospores of *Ustilago maydis,* as well as spores germinated at various periods from 0 to 16 hr, contained the following species of ribonucleic acid (RNA): cytoplasmic heavy, 25 S; cytoplasmic light, 18 S; mitochondrial heavy, 23 S; and mitochondrial light, 16 S. There was also a RNA which probably was a mixture of 5 S and 4 S, indicating the presence of transfer RNA (tRNA). Incubating the spores for 2 hr or longer resulted in the formation of a RNA that was heavier than 25 S. An extremely light RNA could be separated from other species of RNA in total RNA extracts by using a 7.5% polyacrylamide gel. The presence of functional tRNA in nongerminated spores was also demonstrated by the ability of isolated tRNA to accept amino acids. The addition of 160 μg/ml streptomycin to the germination media was needed to stop bacterial growth and thus prevent the inclusion of its RNA in the fungal extract. Phytopathology 61: 645-648.

Germination of spores is usually the sine qua non for the infection of aboveground plant parts. We now understand or have the technique for investigating many aspects of this process, the role of environmental and nutritional factors, the nature of the ultramorphological changes, and many of the biochemical functions involved in the energy-producing and synthetic mechanims of the germinating spore. In contrast, we are without information concerning the internal controls that govern the various facets of spore germination. The control of germination and infection could reside in the ribonucleic acids (RNA) of the spore, and in the presence and functioning of the various species of RNA during germination.

According to data presented previously (5), tRNA was either entirely absent in nongerminated teliospores of *Ustilago maydis* (DC.) Cda. or was present in amounts too small to be detected. Only after germination commenced was tRNA found. From then on its concentration increased with time of germination (5). In further studies, however, different and more recent methods have been used for the extraction and determination of RNA. The results indicate that small amounts of tRNA can be isolated from nongerminated teliospores and can carry on the charging reaction to form the aminoacyl-tRNA.

Materials and Methods.—Teliospores were obtained from galls on sweetcorn in 1967 and stored at 4 C. Spores were germinated in Erlenmeyer flasks containing 500 ml medium (sucrose, 1.0%; casamino acids, 0.2%) for various times on a reciprocal shaker (95 strokes/min) at 26 C. The synthesis of RNA was determined by measuring the radioactivity of RNA extracted from spores that had been incubated in media containing 100 μc uracil-2-^{14}C. To prevent bacterial growth, streptomycin sulfate was added to such media at a final concentration of 160 μg/ml. The spores were harvested by filtration through Whatman No. 1 filter paper in a Büchner funnel and washed with distilled water, 4 C. The spores were used immediately after washing or were frozen in a dry ice-acetone bath and stored at −20 C until used.

Harvested spores were suspended in 10^{-2} M cacodylate buffer, pH 7.0, containing 5.1×10^{-3} M magnesium acetate, 10^{-3} M 2-mercaptoethanol, and 10^{-5} M zinc sulphate (4). The spore suspension was ground with glass beads (200 mesh) in a Gifford Wood Mini-Mill at a setting of 20 for 20 min. The presence of zinc sulphate in buffer was to inhibit the activity of ribonuclease of the spore; all equipment with which the spore or extracts came into contact was autoclaved to inactivate any contaminating ribonuclease. In preliminary experiments, the following combination of materials gave good spore breakage: spores, 4.0 g (moist wt); glass beads, 12.0 g; cacodylate buffer, 5.0 ml; water-saturated phenol, 5.0 ml. The milling and extraction was conducted at 4 C unless otherwise stated.

After 5 ml water-saturated phenol was added to the broken cell suspension, the suspension was magnetically stirred for 15 min, then centrifuged at 30,000 *g* for 20 min. The upper aqueous phase containing RNA was carefully separated. An equal volume of water-saturated phenol was added to it and the suspension was stirred, centrifuged, and separated. The phenol extraction of the aq phase was repeated at least 3 times until no appreciable amount of protein-DNA interphase remained. Total RNA was precipitated from the final aq extract by adding 2 volumes of absolute ethanol, −20 C, and letting the suspension stand for 2 hr. The precipitate was compacted by centrifugation; the ethanol was removed; and the residue was shaken in ether to remove traces of phenol. The ether was decanted and the RNA resuspended in the E buffer (4×10^{-2} M Tris [tris(hydroxymethyl)amino methane] 2×10^{-2} M sodium acetate, 1×10^{-3} M sodium EDTA, adjusted to pH 7.2 with glacial acetic acid) (2). Traces of residual ether were removed by bubbling nitrogen through the extract. This RNA solution was used either immediately for electrophoresis or stored in liquid nitrogen.

Spore extracts were assayed by spectrophotometrically determining the E_{260}/E_{280} of suitable dilutions and referring these absorbance measurements to a nomogram from which the concentrations of total RNA were ascertained (1).

The species of RNA were separated on 2.4% pre-

swollen polyacrylamide gels (2). Ten to 30 μg RNA solution containing 10% sucrose were applied to gels and a current of 5 ma/gel (Canalco Model 300 B constant current power source) was applied for 105 min at room temperature for total RNA patterns and for 3 hr when quantitatively determining high mol wt RNA.

After electrophoresis, gels were transferred to quartz cuvettes (0.5 × 1.0 × 10.0 cm) and scanned by ultraviolet light at 260 nm using a Gilford linear transport attachment coupled to a Beckman D.U. spectrophotometer. When uracil-2-^{14}C had been included in the germination medium, the gels were transferred to a stainless steel trough with the same dimensions as the cuvette. After removal of excess water, the gel was frozen in the trough on powdered dry ice. Frozen gels were transferred to the platform of a gel-slicer (The Mickle Laboratory Engineering Co., Goomshall, Surrey, U.K.) and cut into 0.7-mm slices. These sections were placed on filter papers and allowed to dry at 70 C in scintillation vials.

To each scintillation vial was added 0.03 g 2,5-bis-[2-(5-tert-butylbenzoxazolyl)]-thiophene in 10 ml toluene. Radioactivity was counted using a Packard liquid scintillation spectrometer with counting efficiency of 65% for the tRNA charging reaction and 87% for the gel slices.

Spore tRNA was separated from total RNA according to the method of Shearn & Horowitz (10). Total RNA was dissolved in 0.5 M Tris-HCl buffer, pH 8.8, containing 0.1 M magnesium acetate, and incubated for 45 min at 37 C. Next, 20% potassium acetate, pH 5.1, was added in an amount equaling one-tenth the volume of the buffer, and the RNA was precipitated with 2 volumes of ethanol at −20 C for 2 hr. To separate tRNA from higher molecular wt RNA, the precipitate was dissolved in a buffer of 1 M NaCl; 0.2 M Tris-HCl, pH 7.3; 0.1 M magnesium acetate; then centrifuged to remove higher mol wt RNA. Potassium acetate was again added and RNA precipitated with ethanol as before. The tRNA was then dissolved in 0.1 M Tris buffer, pH 7.8, and used in the charging reaction.

The aminoacyl-tRNA synthetases were prepared according to the procedure of Muench & Berg (7) from freshly harvested 5-day-old mycelium of *Rhizoctonia solani* grown on glucose asparagine medium (6) in which thiamine hydrochloride and biotin were omitted. These cells were ground with neutral alumina (M. Woelm. Eschwege, Germany) in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.01 M $MgCl_2$ and 10% glycerol. The suspension was then centrifuged at 27,000 *g* for 30 min twice. The supernatant was centrifuged at 100,000 *g* for 2 hr, and the soluble portion passed through a diethyl aminoethyl cellulose column (24 × 2 cm) that had been previously equilibrated with buffer, 0.02 M potassium phosphate, pH 7.5; 0.02 M 2-mercaptoethanol; 0.001 M $MgCl_2$, and 10% glycerol. After the extract was applied to the diethyl aminoethyl cellulose column, 300 ml of this buffer was used to wash the column at a flow rate of 100 ml/hr. The total soluble enzyme preparation was then eluted with buffer containing 0.25 M potassium phosphate, pH 6.5; 0.02 M 2-mercaptoethanol; 0.001 M $MgCl_2$; and 10% glycerol. The fractions with greatest absorption at 280 nm were collected and concentrated by dialysis against 1 liter of buffer consisting of 0.001 M potassium phosphate, pH 6.8; 0.02 M 2-mercaptoethanol; 10% glycerol; and 15% polyethylene glycol, mol wt 6,000.

The presence of tRNA in extracts from *U. maydis* spores was determined by the ability of the tRNA to accept ^{14}C amino acids from a reaction mixture similar to that of Shearn & Horowitz (10), in which dithiothreitol was used as a reducing agent. The pH of the Tris-HCl buffer was 7.8 and an incubation time of 15 min. Five-tenths ml of the reaction mixture was used. It contained 84 μg of tRNA and 288 μg of enzyme preparation (in 50% glycerol). The formation of aminoacyl-tRNA was measured by the radioactivity in the trichloroacetic acid insoluble fraction using the filter paper disc procedure (3).

All the radioactive compounds were obtained from New England Nuclear Corp. The specific activities of the following -U-^{14}C amino acids were the same (10 mc/mmoles): L-arginine, L-aspartic acid, glycine, L-histidine, L-isoleucine, L-lysine, L-proline, L-serine, L-tyrosine, and L-valine. Other specific activities were: L-leucine-U-^{14}C (180 mc/mmole), L-phenylalanine-U-^{14}C (375 mc/mmole), and uracil-2-^{14}C (27 mc/mmole). Dithiothreitol was obtained from Sigma Chemical Co., ATP from Nutritional Biochemicals Corp., and streptomycin sulfate from Abbott Laboratories.

Results and Discussion.—Nongerminated and germinated spores of *U. maydis* contained the same species of RNA that are usually present in higher organisms. The OD profiles of RNA from spores germinated for periods of 0, 1, 2, 3, 4, 8, 12, and 16 hr all had peaks corresponding to the following species of RNA: cytoplasmic heavy ribosomal RNA (rRNA), 25 S; cytoplasmic light rRNA, 18 S; mitochondrial heavy rRNA, 23 S; and mitochondrial light rRNA, 16 S. Also present was a peak corresponding to a mixture of 5 S rRNA and 4 S tRNA (Fig. 1-A). Moreover, the total RNA extract contained 260-nm absorbing material that gave a small shoulder below the peak of 5 S and 4 S RNA. Other small 260-nm absorbing peaks heavier than 25 S appeared in extracts made from spores germinated for more than 2 hr. All peaks were found in at least three experiments.

Similar peaks were found in total RNA extracts when uracil-^{14}C was used to follow the synthesis (Fig. 1-B, C, D). The extracted RNA was radioactive after a 2-hr

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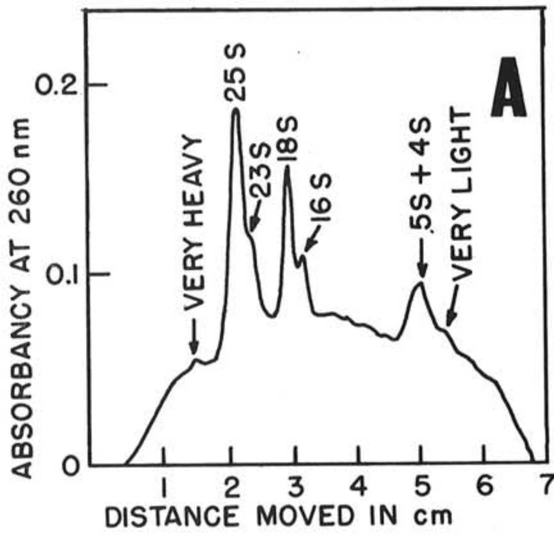
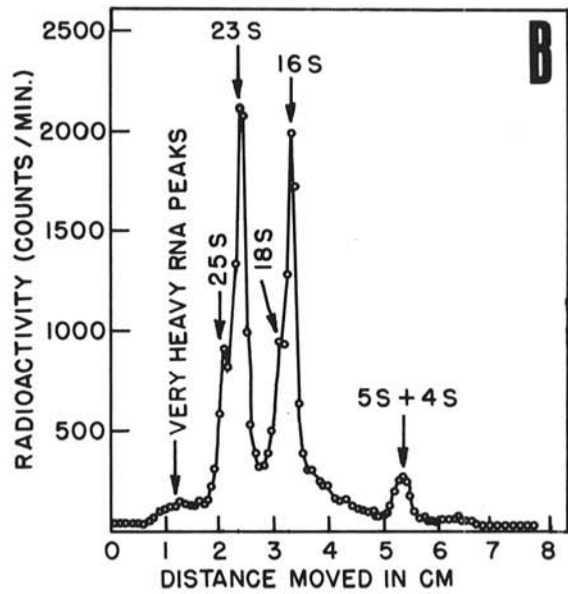
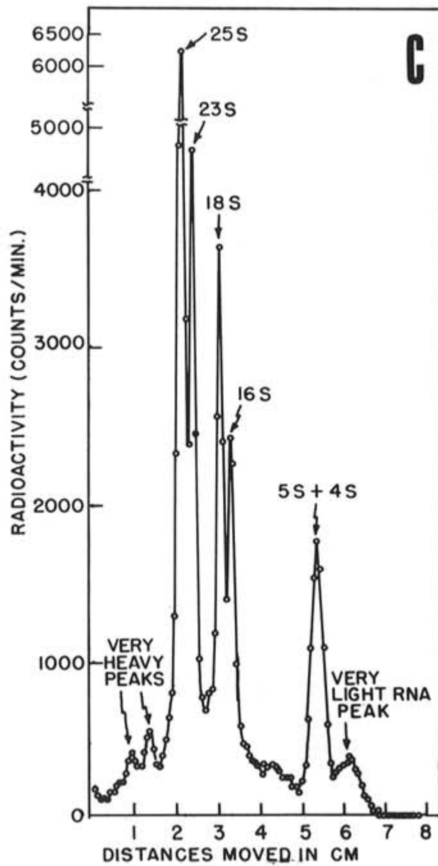
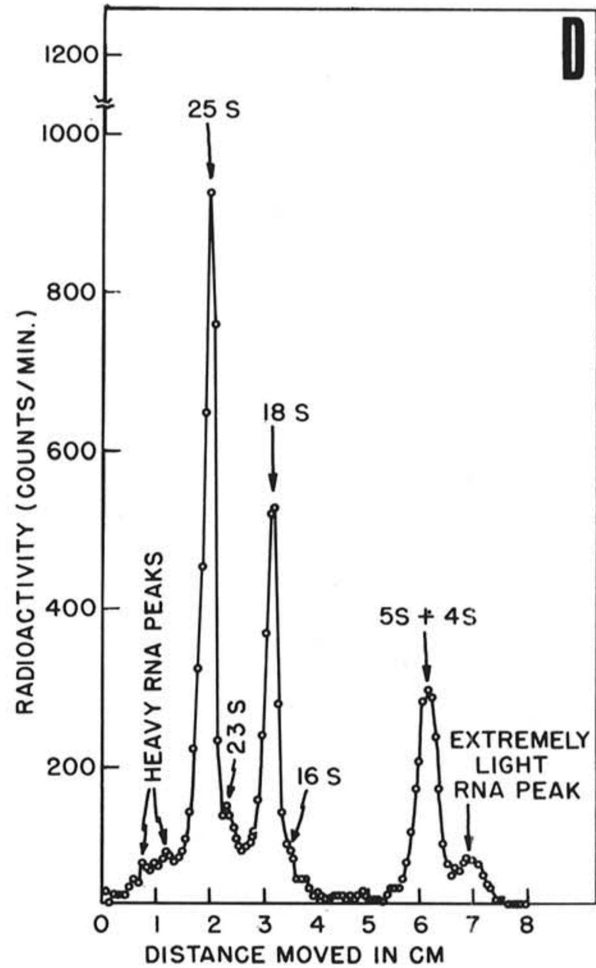
Fig. 1. Profiles from the electrophoresis of ribonucleic acid (RNA) extracted from spores of *Ustilago maydis*. The RNA was applied to preswollen 2.4% polyacrylamide gel. Absorbancy (————); radioactivity (-○-○-○-). **A)** Peaks of different species of RNA from spores germinated for 2 hr. Similar peaks were obtained with extracted RNA from spores germinated from 0 to 16 hr. **B)** Profiles of RNA labeled with uracil-2-^{14}C for 2 hr in absence of streptomycin. **C)** Profile of RNA from spores germinated with uracil-2-^{14}C for 4 hr in the absence of streptomycin. **D)** Similar to C but germinated in the presence of streptomycin.


A
ABSORBANCY AT 260 nm
DISTANCE MOVED IN cm
VERY HEAVY
25 S
23 S
18 S
16 S
5S + 4S
VERY LIGHT


B
RADIOACTIVITY (COUNTS / MIN.)
DISTANCE MOVED IN CM
VERY HEAVY RNA PEAKS
25 S
23 S
18 S
16 S
5S + 4S


C
RADIOACTIVITY (COUNTS/MIN.)
DISTANCES MOVED IN CM
VERY HEAVY PEAKS
25 S
23 S
18 S
16 S
5S + 4S
VERY LIGHT RNA PEAK


D
RADIOACTIVITY (COUNTS/ MIN.)
DISTANCE MOVED IN CM
HEAVY RNA PEAKS
25 S
23 S
18 S
16 S
5S + 4S
EXTREMELY LIGHT RNA PEAK

or longer incubation period, indicating ready synthesis of the usual RNA species even before the formation of any germ tube. The profile of radioactivity contained a marked peak for the extremely light and unknown RNA, which was only a shoulder in the OD profile (Fig. 1-C, D). The light RNA peak was more readily separated from the 4 S-5 S peak by using a 7.5% polyacrylamide gel. The peak that was heavier than 25 S RNA also occurred as one, or sometimes two, peaks in the profile of radioactivity, after incubation for 2 hr or longer (Fig. 1-B, C).

Quantitation of the total rRNA isolated from 4-hr germinated spores treated with streptomycin was made with a polar planimeter. For total RNA, the ratio between 25 S and 18 S RNA was 2.50:1, between 25 S plus 23 S and 18 S plus 16 S the ratio was 2.56:1. These ratios are close to that reported for bean cytoplasm rRNA, 2.6:1 (8). However, for newly synthesized RNA the analogous ratios were 1.72:1 and 1.74:1, respectively.

It was important to add streptomycin to the germination medium because contaminating bacteria reproduced rapidly. Without streptomycin, the ^{14}C profile had large amounts of 23 S, and 16 S peaks (Fig. 1-B, C), probably produced by bacteria, whereas in the presence of the antibiotic these peaks were greatly reduced (Fig. 1-D). Bacterial counts were exceedingly high in the absence of an antibiotic and negligible in its presence. The high peaks of radioactivity in the absence of an antibiotic could thus be attributed to the bacterial rRNA.

The presence of tRNA in ungerminated spores was confirmed by the ability of isolated spore tRNA to accept the ^{14}C amino acids. Of 12 amino acids tested, eight of them acylated spore tRNA: serine, glycine, histidine, phenylalanine, leucine, arginine, lysine, and isoleucine (Table 1). The inability of valine, proline, tyrosine, and aspartic acid to charge the tRNA may reflect the need for different conditions for the appropriate synthetases (9, 10, 11). Other causes could be the instabilities of some of these enzymes under present experimental conditions, or the inability of some synthetases from *R. solani* to promote the acylation of spore tRNA. It is also possible that the ungerminated spores lack the specific tRNA for these amino acids.

The difficulty of disrupting and extracting spores of *U. maydis* probably explains our inability to previously demonstrate the presence of tRNA and the other usual RNA species in such spores (5). Large quantities of spores and long grinding were needed to get sufficient tRNA for these studies. A total of 100 g of spores was ground, 10 g at a time, before we could obtain enough tRNA to carry out the charging reaction using the synthetase preparation from *R. solani*. The demonstration of charging with eight amino acids is evidence for the presence of tRNA in ungerminated spores. Therefore, our data are in accord with those of Van Etten et al. (12), who demonstrated tRNA in ungerminated *Botryodiplodia theobromae* and *Rhizopus stolonifer* spores by the charging reaction.

TABLE 1. Specific acceptor activity of transfer ribonucleic acid (tRNA) from ungerminated spores of *Ustilago maydis*

Amino acid	Acceptor activity as picomoles of aminoacyl-tRNA formed/mg RNA
L-Phenylalanine	44.1
L-Leucine	106.0
L-Arginine	253.6
L-Lysine	183.2
L-Isoleucine	152.8
L-Serine	168.0
L-Histidine	15.2
Glycine	122.0
L-Tyrosine	0
L-Valine	0
L-Proline	0
L-Aspartic acid	0

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