Membrane-Bound Ribosomes in Germinated Uredospores

R. C. Staples, L. Ramakrishnan, and Z. Yaniv

Boyce Thompson Institute, Yonkers, New York 10701.

Supported in part by grants from the U.S. National Science Foundation (GB-5492X) and the Herman Frasch Foundation.

Accepted for publication 25 July 1969.

ABSTRACT

Ribosomes from differentiated uredospores of the bean rust fungus (*Uromyces phaseoli*) that were germinated for 4 hr were partly bound to cellular membranes. Compared with total ribosomal RNA extracted by a phenol and sodium lauryl sulfate procedure, 37% of the ribosomes were extracted directly, while the remainder were bound to membranes and required release to be recovered. The percentage of ribosomes that were aggregated into polyribosomes was 57%. These polyribosomes were ribonuclease-sensitive. Ribosomes extracted with trypsin were not active in amino-acid incorporation. Phytopathology 60:58-62.

Germinating intact uredospores incorporate radioactive amino acids into protein (12). When spore extracts were analyzed on sucrose density gradients after incorporation, most of the radioactive protein sedimented as a very heavy fraction which was dispersed with sodium deoxycholate (DOC) (12). This suggested that the protein was bound with ribosomes to a membrane such as the endoplasmic reticulum. The fraction was found, by electron microscopy, to contain membrane elements.

During a study of polyribosomes in germinated spores, DOC was required for polyribosome extraction, even though they were demonstrated without detergents in nongerminated spores (15). This suggested again that ribosomes in germinating uredospores are bound to membranes. The present studies were made to explore the extractability, degree of binding, polymerization, and activity of polyribosomes from germinated uredospores. Since differentiated spores represent the final developmental stage of these fungi apart from the host (7), spores having germ tubes with appressoria were utilized for the work.

MATERIALS AND METHODS.—Uredospores of the bean rust fungus, *Uromyces phaseoli* (Pers.) Wint., were collected from infected leaves of bean (*Phaseolus vulgaris* L. 'Pinto') plants grown in controlled environmental chambers at 20° C and a 16-hr day as described previously (14, 15).

Differentiation.—Differentiation of germ tubes was initiated by germinating hydrated spores on collodion membranes containing 1% paraffin oil using the procedure developed by Wynn & Gajdusek (18). Twenty membranes were required to germinate 1 g of spores. One hundred mg of hydrated spores were dusted onto each 550-cm^2 membrane (half of these were lost), and the spores were incubated in the dark at 20° C. Spores were hydrated by floating on a water surface for 16 hr at 4° C.

Extraction of RNA.—Ribonucleic acid (RNA) was extracted by the phenol-SLS method (12). One g of spores was ground in a ball mill for 1 hr in 10 ml of 0.01 M Tris [tris (hydroxymethyl)amino methane] buffer, pH 7.5, 2 ml of 2% sodium lauryl sulfate (SLS), and 20 ml redistilled phenol. The suspension was then centrifuged at 13,000 g for 10 min, and the aqueous layer was removed by aspiration. This procedure was repeated twice, and the pooled fractions were added to an equal volume of phenol and recentrifuged at 13,000 g for 10 min. The aqueous layer was removed, precipitated with 0.15 M potassium acetate, and made to 70% ethanol. The precipitated RNA was centrifuged down, redissolved in 2 ml of distilled water, and passed through a 2.5 × 30-cm column of Sephadex G50. The first optical density peak (260 mμ) was collected, reprecipitated as before, and stored at −15°C.

Extraction of ribosomes.—Ribosomes were extracted from spores germinated for 4 hr by grinding 20 membranes, 50 ml of grinding buffer (14), and 30 g of coarse sand together in an ice-cold mortar for 5 min. Using rubber gloves, the mixture of sand and tissue was squeezed through four layers of cheesecloth and centrifuged twice at 20,200 g for 10 min. The supernatant solution was frozen for storage.

Estimation of ribosomal binding.—The supernatant solution was thawed and divided into two equal parts. One part was treated with 1 ml of 1% DOC for every 3 ml of solution, while the other part was diluted with water. After centrifugation at 265,000 g for 30 min, the pellets were collected in suspension buffer, the aggregates removed by centrifugation at 20,200 g for 5 min, and the absorbancy at 260 mμ was determined. The amount of bound ribosomes in the suspension was estimated, using the step gradient as described below.

Ribosomes in the pellet of aggregates were released by tryptic digestion. The pellet was resuspended in 0.5 ml of suspension buffer, 0.5 ml of trypsin (5 μg/ml) was added, and the mixture incubated on ice for 1 hr.

Ultracentrifugation.—Sucrose density-gradient centrifugation was carried out as previously described (15). For analysis of RNA, linear 5-20% gradients were used and 0.2 ml fractions were collected. For analysis of ribosomes, linear 15-30% gradients were used, and 0.4 ml fractions were collected. Of this, 0.3 ml was used for incorporation studies while 1 ml of water was added to the remainder and the absorbancy determined at 260 mμ.

Sedimentation rates and polyribosome profiles were determined at 17 C in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. All samples were centrifuged at 20,410 rpm. Plates were examined using a Nikon 6C Shadowgraph microcomparator, while areas were determined by weighing cutouts of 5-fold enlargements. Ribosomes were assumed to have a partial specific volume of 0.65 (16). The best

polyribosome profiles were obtained at a ribosomal RNA concentration of 2.0 to 2.5 mg/ml (A_{260} of 40 to 50).

When required, polyribosomes were separated from monosomes and membrane-bound ribosomes by using a step gradient (17). Usually 3.1 mg of ribosomes in 0.7 ml was layered on each gradient consisting of 3.3 ml of 0.6 M sucrose layered over 4.0 ml of 1.8 M sucrose. Polyribosomes were collected by centrifugation at 265,000 *g* for 100 min. The sucrose solutions were made in water rather than in buffers because the buffer salts caused the ribosomes partially to degrade to subunits (15). Treatment of a similar aliquot with 0.25% DOC before centrifugation to release ribosomes from membranes provided an estimation of the amount of membrane-bound ribosomes in the preparation (6).

Amino acid incorporation.—Incorporation assays were carried out as described previously (19), starting with yeast transfer RNA (tRNA) loaded with ^{14}C-phenylalanine or a mixture of 15 radioactive amino acids. The stripped yeast tRNA was loaded with amino acids using an enzyme solution prepared from rice embryos as described by App & Gerosa (1). The assays were made using 0.5 mg of ribosomes, 20 μg of polyuridylic acid, 100 μliter of supernatant (100 μg protein), and 5,000 cpm of phenylalanyl-tRNA (specific activity 25,180 cpm/mg), or 25,000 cpm of tRNA loaded with a mixture of 15 amino acids. The reaction was started by adding the ribosomes, and the reaction was stopped after 30 min by adding an equal volume (0.5 ml) of 10% trichloroacetic acid and 0.05 ml of 1% bovine serum albumin. The precipitates were washed, plated, and counted as described. The amounts of ribosomes were estimated spectrophotometrically, assuming that there was 111 μg/A_{260} unit. When required, ribosomal RNA was estimated, assuming that there was 50 μg/A_{260} unit. One μμmole of phenylalanine gave 550 cpm on the scintillation counter.

Reagents.—The sources of most reagents and their preparation were described previously (19). However, two reagents require special mention. Deoxycholate was obtained as the "Special Enzyme grade" of the sodium salt from Mann Research Laboratories, Inc., N. Y. The salt was routinely prepared as a 1% solution in water, and was used at a final concentration of 0.25%. Trypsin was obtained from Worthington Biochemical Corp., Freehold, N. J., as a twice-crystallized, lyophilized powder. It was free of detectable ribonuclease activity. A stock solution of 5 μg/ml was prepared in 0.001 N HCl.

RESULTS.—*Total RNA.*—From many 1-g lots of spores, 7.50 ± 0.7 mg of RNA was routinely extracted. According to analysis of the RNA on sucrose density gradients (Fig. 1-A), 84% of this RNA was composed of ribosomal RNA, while most of the remainder was 4.6 S tRNA. Thus, there was about 6.3 mg of extractable ribosomal RNA/g of spores.

Ultracentrifuge analysis of the fractions.—The procedure adopted for the study of ribosome binding yielded three fractions. There were a soluble fraction containing the ribosomes, an insoluble fraction of aggregates, and a suspension of polyribosomes obtained by centrifuging the soluble fraction through a step gradient. The content of each fraction was studied in the analytical ultracentrifuge.

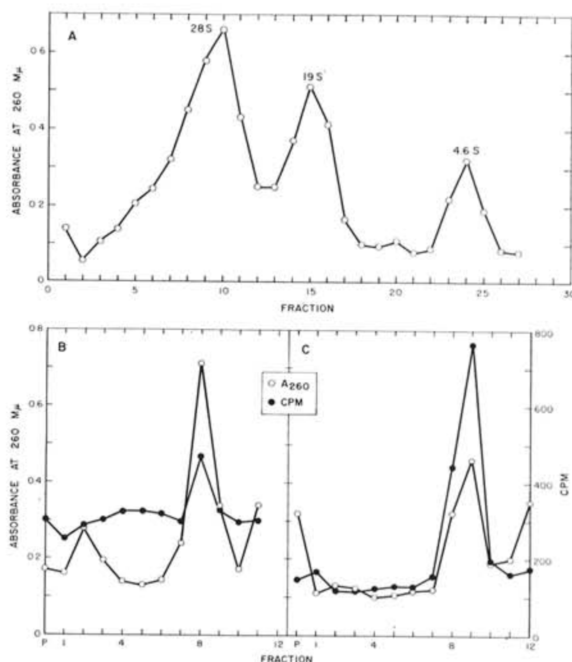

Fig. 1. Sucrose density-gradient analyses of ribonucleic acid (RNA) and ribosomes extracted from *Uromyces phaseoli* uredospores which were germinated for 4 hr. **A)** Analysis of RNA using a 5-20% linear gradient which was centrifuged for 150 min at 65,000 rpm in a Spinco SW65 rotor. Each fraction contained 14 drops. Sedimentation was from right to left. Ribosomal RNA is contained in the fractions labeled 28 S and 19 S. **B)** Analysis of ribosomes using a linear 15-30% gradient which was centrifuged for 25 min at 65,000 rpm. After collection, each fraction was divided into two parts. One part was analyzed for absorbance at 260 mμ; the other part was assayed for capacity to incorporate aminoacyl-tRNA. Open circles represent absorbance at 260 mμ, while closed circles represent radioactivity. The pellet is labeled P. Centrifugation was from right to left. **C)** Same as (B) except fractions were assayed for capacity to incorporate phenylalanyl-tRNA with polyuridylic acid.

A sedimentation velocity analysis of the soluble fraction is shown in Fig. 1-A, B. When DOC was omitted, polyribosome profiles were not resolved, and only the monosome boundary could be seen (Fig. 1-A). In contrast, if the soluble fraction were washed once with 0.25% DOC, abundant polyribosomes could be observed (Fig. 2-B). An average of 57 ± 2% of the ribosomes was polymerized. The weight classes heavier than 82-S monosomes had sedimentation constants ($S_{20,w}$) of 129 ± 1, 164 ± 1, 184 ± 4, and 203 ± 1 corresponding to dimers through pentamers (11). All boundaries heavier than 82 S were destroyed by a trace of ribonuclease.

If this soluble fraction was then further purified by using the step gradient (17), it was found that the monomers and dimers were depleted as expected (Fig. 1-C). In addition, classes heavier than hexamers were also present. Uncorrected sedimentation constants of

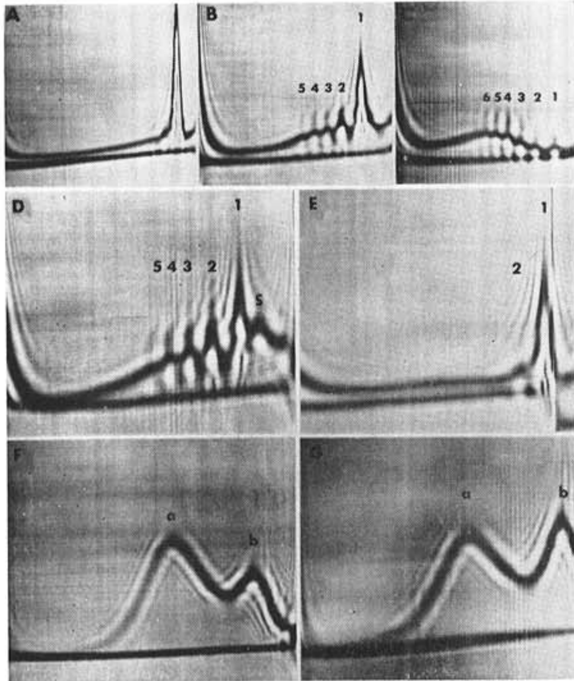

Fig. 2. Sedimentation velocity analyses of ribosomes from *Uromyces phaseoli* uredospores which were germinated for 4 hr. Centrifugation was at 20,410 rpm for 16 min at 16.5 C. The boundaries which contain the monosomes are labeled 1. Sedimentation is from right to left. **A)** Ribosomes not washed with deoxycholate (DOC), bar angle 50°, concentration 8.4 mg/ml. **B)** Ribosomes washed with DOC, bar angle 40°, 4.4 mg/ml. **C)** Polyribosomes washed with DOC and passed through step gradient, bar angle 40°, 4.4 mg/ml. **D)** Ribosomes recovered from the pellet of aggregates by treatment with trypsin. The aggregates were produced after removal of DOC during ribosome preparation by centrifugation. Bar angle was 40°, 5.0 mg/ml. The position of the large subunit is marked by S. **E)** Same as (D), but treated with a trace of ribonuclease. **F)** Step gradient shown in (C), but after centrifugation had proceeded for only 4 min. Boundary a is the heavy aggregate; boundary b contains the unresolved polyribosomes. **G)** Same as (F), but treated with 2.5 μg/ml of trypsin for 1 hr at 0 C before centrifugation.

peaks 1 to 6 were 82, 122, 154, 179, 200, and 220, respectively.

The polyribosomes obtained by treatment with DOC actively incorporated amino acids from tRNA are shown in Fig. 1-B. Only the monoribosomes were active with polyuridylic acid (Fig. 1-C). A rigorous identification of the gradient fractions was carried out previously (15).

After treatment of the ribosome suspensions with DOC, the ribosomes recovered by ultracentrifugation were always contaminated with aggregates which were conveniently removed by centrifugation at low speed (13). Further extraction of these aggregates with additional DOC did not remove significant amounts of ribosomes. Use of Triton X-100 as suggested by Blobel & Potter (2) produced an extract that was too contaminated with lipids to be used. However, treatment of the aggregates with 2.5 μg/ml of trypsin, according to the procedure of Chefurka & Hayashi (3), caused a large release of polyribosomes (Fig. 2-D). The average polymerization of three of these preparations was 69 ± 5%. All of the trypsin-extracted polyribosomes were destroyed by approximately 0.001 μg/ml of ribonuclease (Fig. 2-E). The large subunit (S) was fairly abundant (Fig. 2-D), indicating that some degradation of the ribosomes occurred during trypsin extraction. Raising the trypsin concentration to 25 μg/ml caused some polyribosome disaggregation to occur. This process was quite marked at 50 μg/ml, and nearly complete at 100 μg/ml.

The polyribosomes shown in Fig. 2-C were prepared using DOC, and purified on a step gradient. During the early stages of polyribosome resolution in the analytical ultracentrifuge before the polyribosome boundaries were resolved, a very heavy and fast-moving boundary was present in the polyribosome suspension (boundary a, Fig. 2-F). The polyribosomes are present in boundary b. Boundary a could be seen only during the first 4 min after reaching speed, and was completely sedimented and out of view in 8 min. Attempts to identify the material in boundary a were not conclusive, but trypsin treatment of the polyribosome suspension obtained from the step gradient caused an incease in polyribosome boundary b relative to boundary a (Fig. 2-G).

Ribosomes prepared by the use of trypsin had little capacity to incorporate amino acids. Tests showed that ribosomes prepared with trypsin incorporated 8% of the phenylalanine that was incorporated by ribosomes prepared with DOC.

Estimation of ribosome binding.—Analysis of the fractions of ribosomes extracted from uredospores is shown in Table 1. The total amount of ribosomes which remained soluble after removal of the aggregates by low-speed centrifugation was estimated to be 3.9 mg/g spore (expressed as ribosomal RNA). The amount of ribosomes in this fraction which was bound to membranes was determined using the step gradient technique of Loeb et al. (6). This procedure allows separation of free polyribosomes from polyribosomes attached to membranes, since only polyribosomes not attached to membranes would have passed through the bottom sucrose layer. Determinations on five different preparations of spores showed that 59 ± 6% of the polyribosomes in the soluble fraction were free, while the remainder required release by DOC to be sedimented through the gradient. Consequently, 2.3 mg RNA/g spore or 37% of the ribosomes were free. The remaining 63% of the ribosomes were bound in some way to membranes. Of this, 1.6 mg RNA/g spore were in the soluble fraction, and 2.3 mg RNA/g spore were in the aggregates and required trypsin to be solubilized. Total ribosomal RNA recovered from a g of spores was 6.2 mg, or 98% of that recovered using a phenol-SLS procedure.

Discussion.—In general, rapidly growing fungi contain free ribosomes, while in nongrowing fungi, most ribosomes are bound to cell membranes. For example, Moyer & Storck (9) found that ribosomes could easily be obtained from actively growing cultures of *Aspergillus niger*, but adequate yields were obtained from

TABLE 1. Recovery of *Uromyces phaseoli* ribosomes from uredospores by treatment with deoxycholate or trypsin[a]

Trial	Amount of ribosomes, Mg RNA / g spores				% Total ribosomal RNA		
	Soluble		Insoluble aggregates	Total recovery	Soluble		Insoluble aggregates
	Free	Bound			Free	Bound	
1	2.5	1.7	2.4	6.6	38	26	36
2	2.1	1.5	2.3	5.9	35	25	39
Avg	2.3	1.6	2.3	6.2	37	26	37

[a] The percentage of the soluble fraction which was not bound to membranes was determined using the step gradient technique of Loeb et al. (6), and averaged 59% of the total fraction. The final concentration of DOC was 0.25%. Ribosomes in the insoluble aggregates were released, using trypsin at a concentration of 2.5 μg/ml. Treatment with trypsin was carried out on ice for 1 hr.

Spores were germinated for 4 hr on membranes containing oil to induce differentiation. A different preparation of spores was used for each trial, and each trial was the result of two determinations. The data are based on total ribosomal RNA extracted by the phenol-SLS procedure, which was 6.3 mg/g spores.

mycelium in the stationary phase only if DOC was used during extraction. Similarly, DOC was required to extract ribosomes from nongrowing cultures of *Saccharomyces cerevisiae* (4, 8) and *Neurospora crassa* (5), but the detergent was not required if the cultures were growing rapidly. Fukuhara (4) clearly showed that bound ribosomes were the only ribosomes active in nongrowing yeast cultures undergoing respiratory adaptation. Of great interest was the fact that polyribosomes were present in the bound fraction, although the degree of polymerization was not determined.

The demonstration with rust fungi that at least 63% of the polyribosomes are bound to cellular membranes shows that nongrowing uredospores fit the general pattern to be expected of fungi in a stationary phase of growth, even though their germ tubes are developing rapidly. The rapid growth of the germ tubes may account for the large number of ribosomes which were polymerized (57%) just as they were in nongrowing yeast which was developing a respiratory apparatus (4). Recent studies showed that uredospores while not growing have a carefully regulated mechanism for converting food reserves into glucans, presumably for production of the germ tube (18). The membrane-bound ribosomes probably provide the apparatus for utilization of stored reserves after germ tube formation is induced by hydration, but the details of this relationship have not been studied.

DOC does not release ribosomes as efficiently as trypsin. However, the difficulty in controlling tryptic activity will probably prevent its use for routine preparation of ribosomes. The partial separation of the ribosomes into subunits during treatment (Fig. 2-D) could result from a proteolytic attack on ribosomal proteins, as suggested by Nair et al. (10), and may cause the loss of ability to incorporate amino acids. Larger amounts of trypsin (100 μg/ml) completely disaggregated the ribosomes.

LITERATURE CITED

1. APP, A. A., & M. M. GEROSA. 1966. A soluble fraction requirement in the transfer reaction of protein synthesis by rice embryo ribosomes. Plant Physiol. 41: 1420-1424.
2. BLOBEL, G., & V. R. POTTER. 1967. Studies on free and membrane-bound ribosomes in rat liver. II. Interaction of ribosomes and membranes. J. Mol. Biol. 26:293-301.
3. CHEFURKA, W., & Y. HAYASHI. 1966. The effect of trypsin on rough endoplasmic membranes. Biochem. Biophys. Res. Commun. 24:633-638.
4. FUKUHARA, H. 1967. Protein synthesis in non-growing yeast. The respiratory adaptation system. Biochim. Biophys. Acta 134:143-164.
5. HENNEY, H. R., JR., & R. STORCK. 1964. Polyribosomes and morphology in *Neurospora crassa*. U.S. Nat. Acad. Sci. Proc. 51:1050-1055.
6. LOEB, J. N., R. R. HOWELL, & G. M. TOMKINS. 1967. Free and membrane-bound ribosomes in rat liver. J. Biol. Chem. 242:2069-2074.
7. MAHESHWARI, R., A. C. HILDEBRANDT, & P. J. ALLEN. 1967. The cytology of infection structure development in urediospore germ tubes of *Uromyces phaseoli* var. *typica* (Pers.) Wint. Can. J. Bot. 45:447-450.
8. MARCUS, L., H. RIS, H. O. HALVORSON, R. K. BRETTHAUER, & R. M. BOCK. 1967. Occurrence, isolation, and characterization of polyribosomes in yeast. J. Cell. Biol. 34:505-512.
9. MOYER, R. C., & R. STORCK. 1964. Properties of ribosomes and RNA from *Aspergillus niger*. Arch. Biochem. Biophys. 104:193-201.
10. NAIR, K. G., R. ZAK, & M. RABINOWITZ. 1966. Studies of the effect of proteolytic enzymes on ribosomes and polysomes from reticulocytes and rat liver. Biochemistry 5:2674-2680.
11. PFUDERER, P., P. CAMMARANO, D. R. HOLLADAY, & G. D. NOVELLI. 1965. A helical model for the polysome in solution. Biochem. Biophys. Res. Comm. 18: 355-358.
12. STAPLES, R. C. 1968. Protein synthesis by uredospores of the bean rust fungus. Netherlands J. Plant Pathol. 74 (Suppl. 1):25-36.
13. STAPLES, R. C., A. A. APP, W. J. MCCARTHY, & M. M. GEROSA. 1966. Some properties of ribosomes from uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. 23:159-164.
14. STAPLES, R. C., & D. BEDIGIAN. 1967. Preparation of an amino acid incorporation system for uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. 23:345-347.
15. STAPLES, R. C., D. BEDIGIAN, & P. H. WILLIAMS. 1968. Evidence for polysomes in extracts of bean rust uredospores. Phytopathology 58:151-154.
16. TAYLOR, M. M., & R. STORCK. 1964. Uniqueness of bacterial ribosomes. U.S. Nat. Acad. Sci. Proc. 52: 958-965.
17. WETTSTEIN, F. O., T. STAEHELIN, & H. NOLL. 1963. Ribosomal aggregate engaged in protein synthesis:

characterization of the ergosome. Nature 197:430-435.
18. WYNN, W. K., & C. GAJDUSEK. 1968. Metabolism of glucomannan-protein during germination of bean rust spores. Contrib. Boyce Thompson Inst. 24:123-138.
19. YANIV, Z., & R. C. STAPLES. 1968. Study of the transfer of amino acids from aminoacyl transfer ribonucleic acid into protein by ribosomes from uredospores of the bean rust fungus. Contrib. Boyce Thompson Inst. 24:103-108.