Ribosomal Activity in Uredospores Germinated on Membranes

Zohara Yaniv and Richard C. Staples

Boyce Thompson Institute, Yonkers, New York 10701, U.S.A.

Work supported in part by a Grant No. GB 17003 from the U.S. National Science Foundation. Part 3 in a series entitled "Spore Germination and Ribosomal Activity in the Rust Fungi."

Accepted for publication 14 March 1974.

ABSTRACT

Ribosomes from bean rust uredospores germinated on collodion membranes were about three times more active in assays for amino acid incorporation than ribosomes from spores germinated on water. Ribosomes from spores induced to differentiate by addition of oil to the membrane had a small additional increment of activity. All ribosomes were equally active with polyuridylic acid regardless of mode of germination. The increased activity of ribosomes from spores germinated on membranes is correlated with an increased formation of polyribosomes.

Phytopathology 64:1111-1114

Additional key words: ribosomes, differentiation, infection structures, protein synthesis, transferases.

Infection structures are required for invasion of host tissue by the rust fungi. Development of infection structures begins with formation of the appressorium. A peg then pushes past the stomatal guard cells, and a vesicle develops which fills the substomatal cavity. Finally, haustoria penetrate host cells.

A detailed study of infection structure formation on membranes was carried out by Maheshwari et al. (6) using uredospores of the bean rust fungus (*Uromyces phaseoli*). They found that nitrocellulose membranes containing mineral oil promoted formation of infection structures including appressoria, vesicles, and haustorial mother cells. Chemicals and heat shock were ineffective. The induced infection structures were morphologically similar to those produced during infection of the host, and nuclear division was induced just prior to formation of the appressorium. This was followed by a second nuclear division in the appressorium, and a third such division in the vesicle. Eventually the haustorial mother cells contained two to five nuclei. In contrast, nuclear division did not occur in germ tubes formed on membranes without mineral oil. Other work by this group with wheat stem rust uredospores (*Puccinia graminis* f. sp. *tritici*) showed that actinomycin D blocked differentiation, whereas cycloheximide prevented both germination and differentiation (4).

Earlier studies in this series (10, 11) have emphasized changes in the activities of ribosomes from uredospores germinated on water surfaces. It was established that ribosomes from germinated bean rust uredospores were less stable and less active than ribosomes from nongerminated spores. The present studies were made to determine the influence of a membrane surface on ribosome activity. It was found that germination on membranes increased ribosomal activity significantly, and that induction of the appressorium by addition of mineral oil to the membrane had a small additional effect.

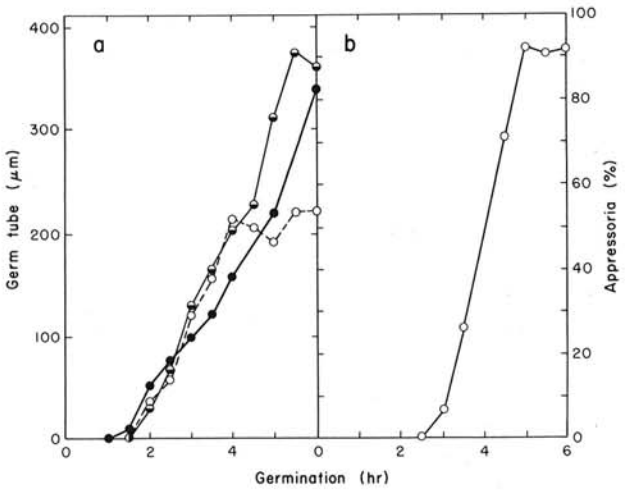

Fig. 1-(a, b). Kinetics of spore development on collodion membranes. **a)** Rate of germ tube elongation, (O-O), differentiated spores on membranes containing paraffin oil; (●-●), nondifferentiated spores on collodion membranes without oil; (●-●), nondifferentiated spores on water. **b)** Rate of appressorial formation on membranes containing oil.

MATERIALS AND METHODS.–*Spores.*–The collection and germination of urēdospores of *Uromyces phaseoli* (Pers.) Wint. were described previously (11, 13). Spores were prepared for germination by floating them on water at 4 C for 16 h. After hydration, 200 mg of spores were dusted onto two baking dishes (18 × 30 cm) which contained water, collodion membranes, or collodion membranes with 1% paraffin oil to induce formation of appressoria. Germination was carried out in the dark at 20 C. The agar slide technique of Schein (9) was used to monitor germination.

To determine germ tube length, germ tubes longer than twice the spore diam. were measured using a ocular micrometer. Percentage of appressoria formed was monitored on pieces of membrane stained with 0.02% trypan blue in lactophenol (12). An appressorium was considered to have formed when the cytoplasm had accumulated within it.

Special chemicals.–(^{14}C)*L*-phenylalanine (sp act 375 mCi/mmole) was purchased from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Poly(^{3}H)uridylic acid from Miles Laboratories, Kankakee, Illinois, U.S.A., had a molecular weight greater than 50,000 daltons (gel filtration) and a specific activity of 34 mCi/mmole P. Polyuridylic acid from Miles Laboratories had a molecular weight greater than 100,000 daltons. Yeast tRNA was purchased from General Biochemicals Corporation, Chargin Falls, Ohio, U.S.A. Phenylalanyl-tRNA was prepared as described previously (13).

Preparation of ribosomes.–Procedures for the preparation of ribosomes, and postribosomal supernatant solutions were described previously (11, 13). Briefly, 1.0 g spores germinated on water or membranes was frozen in liquid N_2, hand-ground in a mortar, and after thawing stirred with grinding buffer (pH 7.6) composed of 0.45 M sucrose, 50 mM Tris [tris (hydroxymethyl) aminomethane], 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. The homogenate was centrifuged for 15 min at 20,000 *g*.

Ribosomes were obtained from the supernatant solution by centrifugation for 60 min at 151,000 *g*. The pellet was resuspended in TKM buffer (pH 7.6) composed of 50 mM Tris, 15 mM KCl, 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol, and centrifuged at 20,000 *g* to remove aggregates. The ribosomes were then further purified by centrifugation in a 10-30% sucrose density gradient prepared according to Brakke & Van Pelt (2) in TKM buffer. The gradients were centrifuged for 75 min at 40,000 rpm and 4 C in a Spinco SW 41 Ti rotor (Beckman Instruments, Palo Alto, Calif., U.S.A.), and the ribosomes collected from gradient fractions by centrifugation at 151,000 *g* for 60 min.

The upper half of the postribosomal supernatant solution from nongerminated spores was used as the source of transferases (13). The solution was passed

through a 2- X 20-cm column of Sephadex G25 before use.

Transferase assay.—Ribosomes in TKM buffer were assayed for transferase activity using the polymerization assay adapted previously (14). Briefly, the reaction mixture (0.55 ml) contained 27.5 μmoles Tris (pH 7.6), 13 μmoles KCl, 2.25 μmoles magnesium acetate, 0.019 μmoles GTP, 1.5 μmoles PEP, 2 μg pyruvate kinase, 0.1 μmoles (^{12}C)*L*-phenylalanine, 40 μg polyuridylic acid (when used), and 6 μmoles 2-mercaptoethanol. (^{14}C)phenylalanyl-tRNA (100 μg containing 6,000 cpm) was used with 100 μg of ribosomes. After incubation at 30 C for 30 min, the reaction was stopped by adding trichloroacetic acid to a final concn of 5%. Then 1.0 mg of bovine serum albumin was added and the mixture was incubated for 15 min at 90 C. The precipitates were washed, plated, and counted by liquid scintillation procedures.

Polyribosomes.—The polyribosome content of the ribosome suspensions was determined by centrifugation on sucrose density gradients. Aliquots of ribosomes washed with 0.25% sodium deoxycholate and containing two A_{260} units were layered onto 10 to 30% sucrose density gradients prepared in TKM buffer as described by Brakke & Van Pelt (2). The gradients were centrifuged at 40,000 rpm and 4 C in a Spinco SW 41 rotor (Beckman Instruments, Palo Alto, Calif., U.S.A.) for 75 min, and fractionated with an ISCO Model D gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebr., U.S.A.).

RESULTS.—*Kinetics of germination.*—Bean rust uredospores germinated on moist collodion membranes produce infection structures if the membranes contain paraffin oil (12). As shown in Fig. 1-a, germ tubes of differentiated spores essentially cease to elongate within 4 h after the appressorium is formed. The vesicle, initiated by 6 h in this system, elongates considerably in the subsequent 12 h. Until 4 h, however, the rate of germ tube elongation by spores which will differentiate is essentially similar to the rates of elongation of nondifferentiated germ tubes formed on oilless collodion membranes or water.

The first infection structure formed is the appressorium, some of which appear as early as 3 h (Fig. 1-b). Most appressoria have appeared by 4 h, and 90 to 95% of the germ tubes have formed appressoria after 5 h.

Ribosome activity.—Ribosomes were assayed for their capacity to incorporate amino acids in the presence and absence of polyuridylic acid. When polyuridylic acid was added to the system as a synthetic messenger RNA, ribosomes were found to be equally active in the first 6 h of germination regardless of the surface on which the spores were germinated. However, when tested in the absence of polyuridylic acid, ribosomes from spores germinated on membranes incorporated approximately three times more (^{14}C)phenylalanyl-tRNA than ribosomes from spores germinated on water (Table 1). The activity of ribosomes from spores germinated on water was not elevated during germination. Ribosomes from differentiated spores were always somewhat more active than those from nondifferentiated spores germinated on membranes, but the differences were small.

Polyribosome content.—To study polyribosome content of ribosomes prepared from uredospores, ribosomes were centrifuged on sucrose density gradients as shown in Fig. 2. Fig. 2 illustrates the different polyribosome profiles found for spores germinated on water (a) and membranes (b). The profiles of ribosomes from spores germinated on membranes with oil (actually shown) and membranes without oil were similar.

TABLE 1. Incorporation of phenylalanyl-tRNA by ribosomes from germinated bean rust uredospores

Hours of germination	% Phe-tRNA incorporation[a]		
	Water	Differentiated	Nondifferentiated
0	100	100	100
2	98	350	280
4	96	340	260
6	96	350	280

[a]The incorporation was expressed as a percentage of the (^{14}C)phenylalanine incorporated by 100 μg of ribosomes prepared from nongerminated spores (1,300 counts/min). The reaction mixture included transferases prepared from nongerminated spores and 2.25 μmoles magnesium acetate in a volume of 0.55 ml. Polyuridylic acid was omitted. The ribosomes were purified by centrifugation on sucrose density gradients.

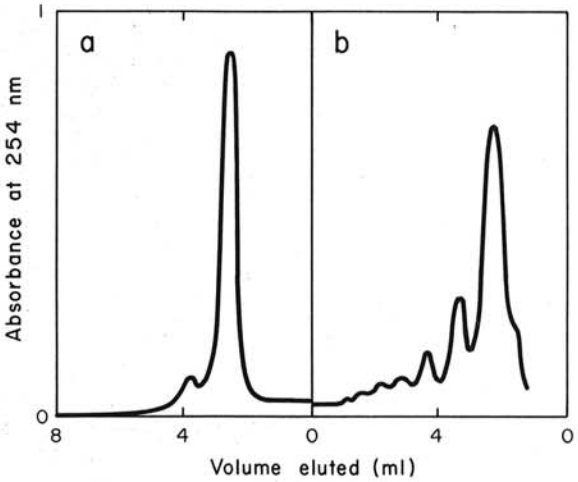

Fig. 2-(a, b). Density-gradient analyses of ribosomes from suspensions having an absorbancy of 2.0 at 260 nm. Spores were germinated for 4 h on **a)** water, **b)** membranes with oil. The 10 to 30% gradients were centrifuged at 40,000 rpm for 75 min in a Spinco SW 41 rotor, and scanned with an ISCO gradient fractionator. The 77S monoribosomes are the large peaks on the graphs. Centrifugation was from right to left.

The quantity of polyribosomes found was small in proportion to the total ribosome population. Nevertheless, repeated trials have always revealed a larger amount of polyribosomes from spores germinated on membranes than from spores germinated on water. Added ribonuclease destroyed all boundaries heavier than the 77S monoribosomes in agreement with earlier results (8). Integration of the curves in Fig. 2. by weighing revealed that the ribosome preparation from spores germinated on membranes contained 22 ± 1% polyribosomes, while spores germinated on water had a dimer content of 7%. Other techniques were tested to improve polyribosome yield (1, 3, 5, 7), but these were less satisfactory than the procedures used here.

DISCUSSION.—Germination of uredospores on membranes increases the number of ribosomes attached to messenger RNA which suggests that protein synthesis is more vigorous in spores germinated on membranes than in spores germinated on water. Ramakrishnan and Staples (8) have previously shown that messenger RNA is not synthesized in uredospores until after formation of the appressorium which occurs between the 4th to 5th hour of germination, nor does template activity of RNA increase until then. This suggests that one early effect of germination on membranes, either with or without oil, is to increase the number of protein chains initiated. Both the increased attachment of ribosomes to messenger RNA, and their increased activity with transferase, reflect this. We conclude that an increased synthesis of protein by ribosomes is a necessary prelude to differentiation of germ tubes, and a membrane is required to stimulate this, at least for bean rust uredospores. With wheat rust uredospores, a membrane was not required to enhance ribosomal transferase activity (11), nor is a membrane required for differentiation (6).

While a membrane is required for differentiation of bean rust uredospores, appearance of the infection structures requires a response by the germ tube to additional signals such as oil in the membrane (12). Apparently, while the membrane stimulates protein synthesis above a critical level necessary for differentiation, the secondary signals induce the fungus to synthesize a new messenger RNA required to guide completion of the infection structures.

LITERATURE CITED

1. APP, A. A., and R. R. GRANADOS. 1972. Effect of nuclear polyhedrosis virus infection on polyribosome content of Gypsy moth larvae (Porthetria Dispar). Exp. Cell Res. 74:519-524.
2. BRAKKE, M. K., and N. VAN PELT. 1970. Linear-log sucrose gradients for estimating sedimentation coefficients of plant viruses and nucleic acids. Anal. Biochem. 38:56-64.
3. DAVIES, E., B. A. LARKINS, and R. H. KNIGHT. 1972. Polyribosomes from peas. Plant Physiol. 50:581-584.
4. DUNKLE, L. D., R. MAHESHWARI, and P. J. ALLEN. 1969. Infection structures from rust urediospores: effect of RNA and protein synthesis inhibitors. Science 163:481-482.
5. LONSDALE, D. M., and D. BOULTER. 1973. Protection of polyribosomes by attachment to membranes and by diethylpyrocarbonate during their extraction from Vicia faba. Phytochemistry 12:39-41.
6. MAHESHWARI, R., A. C. HILDEBRANDT, and 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.
7. RAMAGOPAL, S., and T. C. HSIAO. 1973. Polyribosomes from maize leaves. Isolation at high pH and amino acid incorporation. Biochem. Biophys. Acta 299:460-467.
8. RAMAKRISHNAN, L., and R. C. STAPLES. 1970. Changes in ribonucleic acids during uredospore differentiation. Phytopathology 60:1087-1091.
9. SCHEIN, R. D. 1962. Storage viability of bean rust uredospores. Phytopathology 52:653-657.
10. STAPLES, R. C., and Z. YANIV. 1973. Spore germination and ribosomal activity in the rust fungi. II. Variable properties of ribosomes in the Uredinales. Physiol. Plant Pathol. 3:137-145.
11. STAPLES, R. C., Z. YANIV, and W. R. BUSHNELL. 1972. Spore germination and ribosomal activity in the rust fungi I. Comparison of a bean rust fungus and a culturable wheat rust fungus. Physiol. Plant Pathol. 2:27-35.
12. WYNN, W. K., and C. GAJDUSEK. 1968. Metabolism of glucomannan-protein during germination of bean rust spores. Contrib. Boyce Thompson Inst. 24:123-138.
13. YANIV, Z., and R. C. STAPLES. 1969. Transfer activity of ribosomes from germinating uredospores. Contrib. Boyce Thompson Inst. 24:157-163.
14. YANIV, Z., and R. C. STAPLES. 1971. The purification and properties of the aminoacyl-tRNA binding enzyme from bean rust uredospores. Biochem. Biophys. Acta 232:717-725.