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Protein Synthesis During Fungal Spore Germination

IV. Transfer Ribonucleic Acid from Germinated and Ungerminated Spores[1]

JAMES L. VAN ETTEN, R. KENT KOSKI, AND MAHMOUD M. EL-OLEMY

Department of Plant Pathology and Department of Pharmacognosy, University of Nebraska, Lincoln, Nebraska 68503

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Transfer ribonucleic acid (tRNA) fractions isolated from germinated and ungerminated spores of *Botryodiplodia theobromae* and *Rhizopus stolonifer* had acceptor activity for all 20 amino acids commonly found in protein, when tested with an enzyme fraction from germinated spores. Accordingly, it is unlikely that the absence of tRNA for a particular amino acid limits protein synthesis in fungal spores.

One of the most striking features of fungal spore germination is the initiation of and rapid increase in the synthesis of ribonucleic acid (RNA) and protein (2, 13, 15, 22), Therefore, an investigation comparing the protein-synthesizing components of germinated and ungerminated spores may provide insight into cellular control mechanisms that are involved in spore germination (e.g., see the recent review on protein synthesis in fungal spores, 19). Previous papers in this series have demonstrated the presence of active ribosomes (18), aminoacyl-transfer RNA (tRNA) synthetases (20), and transfer enzymes (J. L. Van Etten and R. M. Brambl, *in press*) in ungerminated conidiospores of *Botryodiplodia theobromae*. Although these components were more active if they were obtained from germinated spores, the requirements for biological activity were similar for the components from the two spore states. One possible means of control of protein synthesis in ungerminated spores could be the lack of tRNA for a particular amino acid. Previous investigators reported that the physical characteristics and the nucleotide composition were similar with unfractionated tRNA isolated from germinated and ungerminated spores of *Neurospora crassa* (3, 4) and *Aspergillus oryzae* (17). However, the tRNA prepared from these spores was not assayed for biological activity with individual amino acids. Although no data were presented, Staples (14) mentioned that tRNA prepared from uredospores of the bean rust fungus *Uromyces phaseoli* was capable of accepting all of the amino acids found in protein. While this manuscript was in preparation, Horikoshi et al. (5) reported that conidia of *A. oryzae* contained tRNA which was active for the 13 amino acids which they tested. Furthermore, the tRNA from the spores had activities similar to those obtained from germinated conidia. The present investigation presents evidence that cytoplasmic tRNA isolated from ungerminated spores of two fungi *B. theobromae* and *Rhizopus stolonifer*, has acceptor activities for the 20 amino acids commonly found in protein, when assayed with an enzyme fraction from germinated spores.

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MATERIALS AND METHODS

Materials. Radioactive amino acids (^{14}C-labeled with specific activities of 20 to 410 mc/mmole) were purchased from New England Nuclear Corp. (Boston, Mass.), Nuclear-Chicago Corp. (Des Plaines, Ill.), and Schwarz BioResearch (Orangeburg, N.Y.). The techniques for the growth and harvesting of *B. theobromae* spores were identical to those previously described (18). The *B. theobromae* spores were germinated in 7-liter quantities in glucose-yeast extract medium (1% glucose, 0.2% yeast extract) at 34 C in a Microferm fermentor (model MF-114, New Brunswick Scientific Co., New Brunswick, N.J.). The culture of *R. stolonifer* (mating strain +) was obtained from W. Gauger of the Botany Department, University of Nebraska. After 7 to 10 days of growth at 28 C, the *R. stolonifer* spores were harvested from 500-ml Erlenmeyer flasks containing 100 ml of V-8 juice agar medium (18). The agar surfaces were flooded with sterile, distilled water, and the culture was gently scraped with an inoculating needle; mycelial fragments and the spores were separated by filtering the suspension through a Rapid-Flo milk filter (Johnson & Johnson, Chicago, Ill.). The spores were collected on Whatman no. 1 filter paper and washed repeatedly with sterile, distilled water. *R. stolonifer* spores were germinated for 6 to 7 hr at 25 C in baffled 500-ml Erlenmeyer flasks containing 100 ml of a medium composed of 20 g of glucose, 2 g of asparagine, 0.5 g of KH_2PO_4, 0.26 g of $MgSO_4$, and water to 1 liter. Germinated

spores from both fungi were harvested by either centrifugation or filtration and washed several times with distilled water. The tRNA fractions of both fungi were prepared from ungerminated or germinated spores which had been stored at −20 C; enzyme extracts from germinated spores of both fungi were prepared either from fresh material or from material stored under liquid nitrogen.

Preparation of tRNA. The tRNA fraction was prepared from both spore states of *B. theobromae* by a slightly modified method of that used by Tanaka et al. (17) for the extraction of tRNA from *A. oryzae* spores. The fungal material was disrupted by a Braun model MSK mechanical homogenizer as previously described (20), in buffer A containing 0.2 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 7.8, 0.4 M sucrose, 0.01 M magnesium acetate, and 0.06 M KCl. This was followed by centrifugations at 20,000 × *g* for 20 min, 30,000 × *g* for 30 min, and 105,000 × *g* for 150 min. Sodium dodecyl sulfate was added to the supernatant fluid to give a final concentration of 0.2% (w/v), and the mixture was shaken with an equal volume of water-saturated phenol for 20 min. The aqueous layer was separated from the phenol layer by centrifugation, and the phenol layer was extracted with buffer A minus sucrose. The nucleic acid contained in the combined aqueous fractions was precipitated at −20 C for 3 hr by the addition of 2.5 volumes of cold ethyl alcohol. The crude RNA was washed two times with ethyl alcohol and dissolved in 0.02 M acetate buffer, *p*H 5.4. This material was applied to a diethylaminoethyl (DEAE) cellulose column and washed with 0.4 M KCl in 0.02 M acetate (*p*H 5.4) until no more 260-nm absorbing material could be eluted. The salt concentration was then increased to 1.0 M KCl in acetate buffer, and the tRNA fraction was eluted from the column. The RNA was precipitated by the addition of 2.5 volumes of ethyl alcohol. The material was centrifuged and the precipitate was dissolved in 1 M Tris (*p*H 9.0) and incubated at 37 C for 30 min to remove amino acids attached to the tRNA. The RNA was precipitated once more with ethyl alcohol and dissolved in water. Transfer RNA fractions were obtained from *R. stolonifer* by the procedure of Shearn and Horowitz (12), except that the cells were disrupted with the mechanical homogenizer and then centrifuged at 20,000 × *g* for 20 min and 30,000 × *g* for 30 min to remove whole mitochondria before adding water-saturated phenol.

Preparation of enzyme extracts. The enzyme extracts were prepared from germinated spores of both fungi as previously described (20), except that, after the protamine sulfate treatment, the soluble fraction was brought to 65% saturation with solid $(NH_4)_2SO_4$ and allowed to mix for 1 hr. The resultant $(NH_4)_2SO_4$ precipitate was dissolved in a 20% solution of glycerol containing 0.02 M Tris (*p*H 7.8), 0.01 M magnesium acetate, 0.06 M KCl, 0.02 M 2-mercaptoethanol, and 0.003 M reduced glutathione before applying to a Sephadex G-25 column equilibrated with the same buffer. The protein fraction was either assayed immediately or stored in portions under liquid nitrogen.

Assay for aminoacyl-tRNA formation. Several assay mixtures were used, since no one assay system was found which gave activity for all 20 amino acids. The following assay mixtures, expressed as micromoles per milliliter, were used and are noted in Tables 1 and 2 for the various amino acids. Mixture A contained 50 μmoles of Tris (*p*H 7.5), 10 μmoles of magnesium acetate, 1 μmole of adenosine triphosphate (ATP), and 5 μmoles of 2-mercaptoethanol. Mixture B was the same as mixture A, only 50 μmoles of cacodylate buffer (*p*H 7.0) was used instead of the Tris buffer. Mixture C contained 100 μmoles of Tris (*p*H 7.8), 12 μmoles of magnesium acetate, 10 μmoles of KCl, 10 μmoles of ATP, and 5 μmoles of 2-mercaptoethanol. Mixture D contained 100 μmoles of cacodylate buffer (*p*H 8.0), 12 μmoles of magnesium acetate, 60 μmoles of KCl, 10 μmoles of ATP, and 5 μmoles of 2-mercaptoethanol. Mixture E contained 50 μmoles of Tris-maleate buffer (*p*H 6.6), 10 μmoles of magnesium acetate, 30 μmoles of NH_4Cl, 1 μmole of ATP, and 5 μmoles of 2-mercaptoethanol. Assays were run in 1-ml portions under conditions in which the tRNA fraction was rate-limiting. This was usually accomplished with 50 to 100 μg of RNA plus 400 to 600 μg of protein per assay tube. All assay mixtures contained 0.6 μc of ^{14}C-amino acid plus 0.05 μmole each of the remaining 19 unlabeled amino acids. The tRNA fractions from the ungerminated and germinated spores were always assayed together in the same experiment with the same enzyme fraction. The formation of aminoacyl-tRNA was determined in 0.05-ml fractions at several time intervals up to 1 hr by the filter paper disc procedure as described by Bøllum (1). Control reactions did not contain tRNA. In addition, a sample on a paper disc was placed in boiling 5% trichloroacetic acid for 15 min and then treated as the others. Glass fiber discs (Whatman GF/A) were used for assaying tryptophan and cysteine acceptor ability. The filter discs were dried under an infrared lamp and placed in counting vials containing 10 ml of counting solution (4 g of 2,5-diphenyloxazole, 50 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, and toluene to 1 liter) and counted in a scintillation counter. The results are expressed as nanomoles of aminoacyl-tRNA formed per milligram of RNA.

Other determinations. Protein was determined by the method of Lowry et al. (9) with bovine serum as a standard. RNA was measured at 260 nm, and it was assumed that 1 mg of RNA is equivalent to 24 optical density units.

RESULTS

We readily extracted tRNA from *B. theobromae* by using the DEAE-cellulose method. Roughly 1 mg of tRNA was obtained from 5 g (wet weight) of starting material of either spore state. The RNA extracted from both spore states of *B. theobromae* gave identical ultraviolet (UV) profiles with a 260 to 230 nm ratio of 2.3 and a 260 to 280 nm ratio of 2. Determinations of the dry weights of the tRNA fractions indicated that approximately 35 to 40% of the material was RNA. Several attempts at extracting tRNA from *B. theobromae* by using the method of

Shearn and Horowitz (12) led to very poor recovery. In the case of *R. stolonifer*, 1 mg of tRNA could be obtained from 1 to 2 g of starting material for both spore states. The tRNA from germinated and ungerminated spores of *R. stolonifer* gave identical UV profiles with 260 to 230 nm and 260 to 280 nm ratios of about 2. Determinations of the dry weights of the tRNA fractions indicated that approximately 12 to 20% of the material was RNA.

The acceptor activities of tRNA from germinated and ungerminated spores of *B. theobromae* and *R. stolonifer* are reported in Tables 1 and 2. The values reported are for the maximum acceptor activity of tRNA for a particular amino acid. Acceptor activity was obtained for all of the amino acids with tRNA from both spore states with both fungi. Furthermore, plots of the amount of aminoacyl-tRNA formed as a function of time gave essentially similar curves for tRNA from both spore states for a particular amino acid. The ratio of acceptor activity of the tRNA

TABLE 1. *Specific acceptor activity of tRNA from germinated and ungerminated spores of Botryodiplodia theobromae*

Amino acid	Acceptor activity[a]		Ratio of A to B	Assay conditions[b]
	Ungerminated spore (A)	Germinated spore (B)		
Alanine	1.03	0.75	1.4	A
Arginine	0.64	0.44	1.5	A
Asparagine	0.17	0.18	0.9	B
Aspartic acid	0.45	0.25	1.8	B
Cysteine	0.11	0.08	1.4	A
Glutamic acid	0.73	0.65	1.1	B
Glutamine	0.38	0.23	1.7	B
Glycine	0.95	0.52	1.8	A
Histidine	0.10	0.12	0.8	B
Isoleucine	0.80	0.44	1.8	A
Leucine	0.66	0.66	1.0	C
Lysine	0.77	0.54	1.4	A
Methionine	0.73	0.46	1.6	A
Phenylalanine	0.58	0.41	1.4	C
Proline	0.19	0.07	2.6	C
Serine	1.15	0.82	1.4	A
Threonine	0.67	0.20	3.4	A
Tryptophan	0.77	0.60	1.3	A
Tyrosine	0.34	0.15	2.3	A
Valine	1.12	0.71	1.6	A

[a] Expressed as nanomoles of aminoacyl-tRNA formed per milligram of RNA. Totals for acceptor activity are as follows: ungerminated spore (A), 12.34; germinated spore (B), 8.28. Average ratio of A to B, 1.5.

[b] Assay mixtures are listed in Materials and Methods.

TABLE 2. *Specific acceptor activity of tRNA from germinated and ungerminated spores of Rhizopus stolonifer*

Amino acid	Acceptor activity[a]		Ratio of A to B	Assay conditions[b]
	Ungerminated spore (A)	Germinated spore (B)		
Alanine	0.42	0.36	1.2	A
Arginine	1.39	0.70	2.0	A
Asparagine	0.60	0.31	1.9	A
Aspartic acid	0.82	0.91	0.9	A
Cysteine	0.36	0.28	1.3	A
Glutamic acid	0.27	0.26	1.0	E
Glutamine	0.53	0.43	1.2	A
Glycine	1.83	1.27	1.4	C
Histidine	0.54	0.47	1.1	A
Isoleucine	1.54	1.14	1.4	C
Leucine	2.78	2.98	0.9	C
Lysine	1.53	1.22	1.3	A
Methionine	1.29	1.57	0.8	C
Phenylalanine	0.97	0.60	1.6	A
Proline	0.12	0.11	1.1	C
Serine	2.26	2.06	1.1	A
Threonine	1.84	1.42	1.3	A
Tryptophan	1.54	1.57	1.0	C
Tyrosine	0.09	0.06	1.5	D
Valine	2.40	2.00	1.2	A

[a] Expressed as nanomoles of aminoacyl tRNA formed per milligram of RNA. Totals for acceptor activity are as follows: ungerminated spore (A), 23.12; germinated spore (B), 19.72. Average ratio of A to B, 1.2.

[b] Assay mixtures are listed in the Materials and Methods.

preparation from the ungerminated spores to that of the germinated spores is also shown in the two tables. The tRNA preparations from the ungerminated spores of both fungi equalled or excelled the acceptor activity obtained with the tRNA fraction from the germinated spores for 18 of 20 amino acids with *B. theobromae* tRNA and 17 of 20 for *R. stolonifer* tRNA. The biggest deviation from the average ratio was for threonine and proline in the *B. theobromae* system and arginine and asparagine in the *R. stolonifer* system.

As has been noted by many investigators (10, 11), it was necessary to use several reaction mixtures to get activity for all 20 amino acids. Problems with the acylation of tRNA for certain amino acids were consistently noted. These difficulties were probably caused by the enzyme preparations rather than by the tRNA. Prolyl-tRNA synthetase from both fungi was only active if a fresh enzyme preparation was used, and even then some enzyme preparations were

inactive. In addition, the formation of aminoacyl-tRNA with glutamine, glutamic acid, and tyrosine was somewhat inconsistent in the systems derived from *B. theobromae*. The formation of aminoacyl-tRNA from tyrosine and glutamic acid gave inconsistent results in the system from *R. stolonifer*. It is also possible that ideal assay conditions were not found for these amino acids even though many combinations were tried.

All of the tRNA preparations were acylated with their amino acids within 20 min at 25 C with most of them reaching a maximum by 10 min. Although the amount of aminoacyl-tRNA formed with most amino acids would level off once they had reached a maximum, others reached a peak and then declined, probably because the aminoacyl-tRNA formed was unstable. With *B. theobromae* tRNA, this latter characteristic was observed for aminoacyl-tRNA formed with leucine, tryptophan, alanine, isoleucine, methionine, arginine, and cysteine. With *R. stolonifer* tRNA, this characteristic was observed for glutamine, alanine, and arginine.

The total acceptor activity, i.e., the sum of the specific acceptor activities for the 20 amino acids, can be used to estimate the amount of tRNA in the RNA preparation. If one assumes an average molecular weight of 30,000 for tRNA, then there is approximately 33.3 nmoles of tRNA per mg of pure tRNA. Therefore, roughly 37 and 25% of the RNA from ungerminated and germinated spores of *B. theobromae*, respectively, accept amino acids (Table 1). The same calculation indicates that roughly 69 and 59% of the RNA from ungerminated and germinated spores of *R. stolonifer*, respectively, accept amino acids (Table 2).

DISCUSSION

One of the possible means of controlling protein synthesis in fungal spores could be the lack of tRNA for a particular amino acid. However, this seems unlikely since the tRNA fractions isolated from spores of the two fungi have acceptor activity for all amino acids commonly found in protein. These data are compatible with previous reports which have demonstrated that the physical and chemical properties of a tRNA fraction from spores of *N. crassa* (3, 4) and *A. oryzae* (17) are similar to those of germinated spores. Furthermore, tRNA fractions from ungerminated and germinated conidia of *A. oryzae* have essentially identical activities for 13 amino acids (5).

Another possibility is that a specific tRNA molecule(s) controls protein synthesis during spore germination. It has been suggested that certain tRNA molecules may be involved in the regulation of protein synthesis and cell differentiation at the level of translation (16). Changes in the distribution of aminoacyl-tRNA during differentiation have been reported by Kaneko and Doi (6) for valyl-tRNA when *Bacillus subtilis* undergoes sporulation. Also, a species of lysyl-tRNA was found in spores of *B. subtilis* which was not present in vegetative cells of the bacterium (7), although the appearance of this tRNA species was subsequently shown to be dependent on the medium in which the cells were grown (8). A comparison of aminoacylated tRNA from wheat embryos with that from wheat seedlings also indicates that at least three aminoacyl-tRNA species in the embryo are different from those in the seedling (21). Since the ratio of acceptor activity of the tRNA from spores to germinated spores for some of the amino acids deviates quite widely from the average ratio for both fungi, this may indicate that there are different molecular species of tRNA for a particular amino acid in the two spore states. However, at the present, we do not have any evidence to indicate whether these differences are physiologically significant.

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