In Vitro Translation of Polyadenylate-Containing RNAs from Dormant and Germinating Spores of the Fungus *Botryodiplodia theobromae*

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Polyadenylated RNA isolated by oligodeoxynucleotide-cellulose chromatography from spores of the fungus *Botryodiplodia theobromae* was translated in a cell-free protein-synthesizing system derived from wheat embryo. Reaction conditions which would yield efficient and accurate in vitro translation of the spore RNA were established. Dual isotopically labeled mixtures of in vitro translation products from germinated and dormant spore polyadenylated RNA, as well as polyadenylated RNAs from intermediate stages of germination, produced qualitatively similar gel electrophoresis patterns, with polypeptides of 10,000 to 55,000 molecular weight. Proteins synthesized in vivo and extracted from germinating spores at three different stages possessed a greater size range, with molecular weights up to 85,000, although the in vitro synthesis apparently did yield the lower-molecular-weight proteins which were synthesized in vivo. Tryptic digest patterns of proteins translated in vitro from polyadenylated RNA of dormant and germinated spores were found to be identical in positions in only 40% of the spots. Furthermore, a dual-label comparison by isoelectric focusing of proteins translated from polyadenylated RNA of germinated and dormant spores also showed qualitative and quantitative differences among the in vitro translation products. We conclude that there are differences between the in vitro translation products of mRNA from dormant and germinated spores and that the mRNA preserved in the dormant spores contains genetic information which is qualitatively different from that of the germinated spores.

The dormant spores of the fungus *Botryodiplodia theobromae* contain latent mRNA which is preserved during cell dormancy and which is translated immediately upon the initiation of spore germination in the absence of new mRNA synthesis (9, 16). These early translational products have not been identified, and it would be of considerable interest to learn what genetic information is encoded within this mRNA and which of the proteins translated from this mRNA are essential for completion of spore germination.

Our previous studies have shown that early translation of the latent mRNA by cytoplasmic ribosomes is required for subsequent nucleic acid synthesis and for germination as well as for cyanide-sensitive spore respiration (5, 9). Indirect kinetic evidence showed that translation of the mRNA during germination also may be required for synthesis or assembly of part of the cytochrome c oxidase (6), an enzyme which is absent in the dormant-spore mitochondria (6, 8). It seems plausible, therefore, that translation of the preserved mRNA could yield (among other products) proteins required for organization or assembly of one or more enzyme systems involved in mitochondrial respiration.

The products of translation of the dormant-cell mRNA which are essential for initiation of growth have not been identified for any eucaryotic cell. We describe here a first step toward identifying those proteins whose syntheses are required for fungal spore germination. We have translated in vitro the polyadenylate-containing [poly(A)+] RNAs (10) from dormant spores and from spores in different stages of germination (using the wheat embryo translation system) and compared their protein products. The major question in this study—whether qualitative differences exist between the in vitro translation products of mRNA from dormant and germinated spores—is answered affirmatively.

**MATERIALS AND METHODS**

Techniques for the growth, harvest, and germination of *B. theobromae* conidiospores have been described previously (5). Wheat embryos were isolated from Fletcher wheat seed by the flotation method (15). Brome mosaic virus was isolated by the method...
of Shih et al. (24), and the RNA was isolated from purified virus by phenol extraction (3). Maize protein body polyribosomal mRNA was prepared as described previously (11). Extraction of total RNA from dormant and germinated spores was by the procedure of Brakke and Van Pelt (4), with mechanical spore disruption accomplished as described previously (5). Poly(A)(+)-enriched RNA was prepared by affinity chromatography on oligodeoxynucleotidylate [oligo(dT)]-cellulose (grade T-3, Collaborative Research) as described by Aviv and Leder (1). The procedure of Marcus and Dudock (29) for preparation of the wheat embryo extract was modified to include in the extraction medium 10 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (pH 7.6), 1 mM dithiothreitol, 95 mM potassium acetate, and 1 mM magnesium acetate. In vitro protein synthesis was performed as described by Davies and Kaesberg (13). RNA concentrations were determined spectrophotometrically (E260nm = 24), and protein was determined by the method of Lowry et al. (18). Labeled proteins were extracted and prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (7). The products of the wheat embryo cell-free system were subjected to isoelectric focusing by the procedure of Wrigley (25). Urea-gasroyic acid horizontal slab gel electrophoresis of RNA was performed with the apparatus described by McDonell et al. (19) and the buffer solution described by Peacock and Dingman (21). Proteins translated in vitro were subjected to trypic digest mapping as described by Bennett (2).

RESULTS

In Table 1 are shown the characteristics and component requirements of the in vitro translation system using RNAs from germinated spores of Botryodiplodia. Absolute requirements existed for the wheat embryo extract and for exogenous mRNA; only very low levels of activity were detected when RNA was deleted or when the mRNA was replaced with purified rRNA fractions prepared from either Botryodiplodia or wheat embryo. Furthermore, reaction mixtures supplied with optimum quantities of poly(A)(+) RNA showed no significant stimulation of activity by the simultaneous inclusion of purified tRNA. Therefore, the stimulation of amino acid incorporation cannot be explained by nonspecific activity of contaminating tRNA as others have reported (22). RNA washed from the oligo(dT)-cellulose column with 0.5 M KCl showed some mRNA activity, which could be due to authentic poly(A)(-) mRNA in this fraction and/or to poly(A)(+) mRNA not retained by the affinity column. After poly(A)(+) enriched RNA was recycled through oligo(dT)-cellulose, the second unretained poly(A)(-) RNA fraction showed a higher specific activity than the first poly(A)(-) RNA fraction, demonstrating that the poly(A)(+) RNA likely was incompletely retained on the column bed and that it appeared also in the poly(A)(-) fraction. The activity of the system was sharply dependent upon an energy-generating system and on ATP as well as spermidine. The inclusion of ribonuclease A abolished activity, and puromycin also inhibited activity. The system was sensitive to cycloheximide but insensitive to chloramphenicol. Inclusion of unfractonated brome mosaic virus RNA stimulated very large amounts of protein synthesis in this in vitro system, as has been reported (23).

Several reaction conditions were optimized by using poly(A)(+) RNA obtained from one passage through the oligo(dT)-cellulose column. A time course measurement of the reaction demonstrated linear incorporation of [3H]leucine into protein for 40 to 50 min, and a standard incubation interval of 90 min was selected for all further experiments. The optimum amount of wheat embryo extract protein required for protein synthesis was found to be about 600 μg in a reaction volume of 100 μl. Incubation temperatures higher and lower than the optimum 30°C sharply inhibited activity. The magnesium concentration curve was sharp, with a maximum at 3.5 mM, whereas a broad range of potassium was tolerated. Inclusion of 0.25 mM spermidine in the incubation mixture doubled the incorporation of [3H]leucine into protein at all poly(A)(+) RNA concentrations tested and simultaneously reduced the magnesium concentration optimum from 4 to 3.5 mM. The optimum concentration of poly(A)(+) RNA was found to be 18 μg, provided that the RNA was eluted from the column once; however, if the poly(A)(+) RNA was tested after two cycles of affinity chromatography, the optimum reaction concentration of RNA was 5 μg.

We sought to establish that the system was
capable of translating mRNA efficiently and without premature peptide chain termination, and we translated the mRNA of brome mosaic virus, whose sole in vitro product is a 20,000-molecular-weight coat protein (23). The gel electrophoresis radioactivity pattern of the brome mosaic virus mRNA in vitro translation product was identical in electrophoretic mobility to the solubilized viral coat protein, with only trace quantities of peptides with lower molecular weights. In other experiments, we translated the poly(A)(+) mRNA isolated from polyribosomes of the protein bodies of maize endosperm. The results showed that our in vitro translation system accurately translated this mRNA into only two radioactive proteins which were electrophoretically congruent with Z₁ and Z₂ components of authentic zein, as others have reported (17).

The germinated-spore RNA fractions tested in this study were subjected to agarose-urea horizontal slab gel electrophoresis to examine the relative purity of the fractions obtained from the oligo(dT)-cellulose column. The poly(A)(+) and poly(A)(-) fractions each contained large quantities of ethidium bromide-stained rRNA after a single passage through the column. However, after a second cycle of chromatography of the poly(A)(+) fraction obtained in the first step, nearly all the rRNA was found in the poly(A)(-) fraction, and the poly(A)(+) fraction of the second cycle contained only a trace amount of the rRNA. Almost all of the poly(A)(+) RNA applied to the gel migrated in a heterogeneous manner under these denaturing conditions in 5 M urea.

A major objective of this study was to compare the in vitro translation products of the poly(A)(+) RNA isolated from the dormant and germinated spores to establish whether major differences exist in the genetic information encoded within these molecules. In Fig. 1 is shown a comparison of the SDS-polyacrylamide gel dual-label radioactivity patterns of these labeled translation products of poly(A)(+) mRNA from

![Fig. 1. Dual-isotope, SDS-polyacrylamide gel co-electrophoresis of [14C]leucine-labeled in vitro translation products of poly(A)(+) mRNA from 300-min spores (○) and [3H]leucine-labeled in vitro translation products of poly(A)(+) mRNA from dormant spores (○). In this and all subsequent gel patterns, the direction of migration is from left to right.](http://jb.asm.org/)

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both the dormant and germinated spores. Similar comparisons (data not shown) also were made of the translation products of poly(A)(+) mRNA from germinated spores and from spores at intermediate stages of germination (60 and 195 min). In these experiments, translation products of the mRNA from germinated 300-min spores were labeled with [³⁴C]leucine, whereas all others were labeled with [³H]leucine. In each electrophoresis experiment, therefore, a precise comparison was possible between the germinated spore poly(A)(+) mRNA products and those from the other stages of germination. The electrophoretic distribution of polypeptides translated from the poly(A)(+) mRNA fraction from the dormant spores was nearly identical to the counterpart polypeptides from the poly(A)(+) mRNA of germinated spores, and few differences could be detected between the polypeptides translated from the poly(A)(+) mRNA from the intermediate-stage germinated spores. The molecular weights of these translation products ranged from about 10,000 to 55,000.

The absence of polypeptides of molecular weight higher than 55,000 in the preceding gel patterns suggested that the wheat embryo extract system could be translating exclusively mRNA of smaller molecular weight. To test whether the proteins synthesized in vitro accurately represented those synthesized in vivo, spores were labeled in vivo with [³H]leucine for 45-min intervals at three points (0, 135, and 255 min) during germination. After each labeling interval, the labeled spores were incubated further for 15 min with 1.1 mM unlabeled leucine. The total SDS-soluble spore proteins were extracted, and they were subjected to electrophoresis in a dual-label comparison of translation products of poly(A)(+) mRNA extracted from spores at 60, 195, and 315 min of germination so that the populations of proteins synthesized at different intervals of germination could be compared. The results (Fig. 2) demonstrated that the spores synthesized in vivo a considerably greater range of proteins than was translated in vitro from the respective poly(A)(+) mRNA fractions, including a number of higher-molecu-

![Fig. 2. Dual-isotope, SDS-polyacrylamide gel co-electrophoresis of [³⁴C]leucine-labeled in vitro translation products (●) and [³H]leucine-labeled in vivo translation products (○). (A) Sixty-minute poly(A)(+) mRNA in vitro translation products and 45-min in vivo translation products; (B) 195-min poly(A)(+) mRNA in vitro translation products and 180-min in vivo translation products; (C) 315-min poly(A)(+) mRNA in vitro translation products and 300-min in vivo translation products.](http://jb.asm.org/)
IN VITRO TRANSLATION OF Fungal spore mRNA

**B**

**C**
lar-weight proteins (45,000 to 85,000) which were not synthesized in vitro. However, the in vitro system appeared to translate the lower-molecular-weight proteins which were synthesized in vivo; a comparison of the proteins synthesized in vivo and in vitro in a dual-labeling experiment demonstrated (Fig. 2) that the lower-molecular-weight proteins synthesized in vitro migrated congruently with the counterpart low-molecular-weight proteins synthesized in vivo at the several stages of germination. Whereas no quantitative or qualitative differences were found in the low-molecular-weight proteins synthesized in vivo or in vitro in the early- or late-germinating spores, several quantitative differences did exist (with respect to germination stage) within the higher-molecular-weight proteins of the in vivo samples.

To test whether differences could exist between the lower-molecular-weight in vitro-synthesized proteins of dormant- and germinated-spore poly(A)(+) mRNA which may not be detected by gel electrophoresis, we hydrolyzed these polypeptides synthesized in vitro with trypsin and processed the products to obtain two-dimensional tryptic peptide maps. The separation patterns of [14C]-labeled polypeptide fragments were observed (after exposure to X-ray film) to contain at least 40 prominent spots in each of the digest patterns (shown diagrammatically in Fig. 3). Importantly, the peptides from the dormant- and germinated-spore poly(A)(+) mRNA in vitro translation products were identical in their map position in only about 40% of the spots. The remainder differed significantly in position of radioactivity, indicating a distinctive difference between the in vitro products of the poly(A)(+) mRNA from the dormant and germinated spores.

As another approach to test whether differences exist in composition of the dormant- and germinated-spore mRNA in vitro translation products, we subjected these [14C]-labeled proteins to electrophoresis to their isoelectric points in an acrylamide gel containing carrier ampholyte compounds with a pH range of 3.5 to 10. Parallel gels were subjected to electrophoresis to confirm establishment of a pH gradient throughout the length of the gel. This technique permitted an improved resolution of some of the in vitro-synthesized proteins over that obtained by the SDS-acrylamide gel electrophoresis. The results (Fig. 4) showed that several significant qualitative and quantitative differences in these proteins were detectable after isoelectric focusing. Free [14C]leucine at the anodic end of the gel masked the peptides which otherwise might have been observed, and proteins with isoelectric points above pH 9.5 were absent from both samples. However, the several differences which exist in the center region of the gel, as well as the presence of a few radioactive peaks near the cathodic end of the gel in the germinated spore sample, established that at least some of the in vitro translation products were in fact dissimilar.

**DISCUSSION**

The dormant spores of *Botryodiplodia* con-

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**Fig. 3.** Tracing of autoradiograms obtained from tryptic digest maps of [14C]leucine-labeled proteins translated in vitro from dormant-spore or 300-min, germinated-spore poly(A)(+) mRNA. The origin of the two-dimensional map is indicated by ×; and the broken line and solid line represent the peptides translated from dormant-spore and germinated-spore mRNA, respectively.
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Fig. 4. Dual isotope, isoelectric focused gel co-electrophoresis of \([\text{\textsuperscript{14}}\text{C}]\)leucine-labeled in vitro translation products of poly(A)(+) mRNA from 300-min spores (●) and \([\text{\textsuperscript{3}}\text{H}]\)leucine-labeled in vitro translation products of poly(A)(+) mRNA from dormant spores (○). The pH gradient (□) was determined by measurement of pH values of the slices of a parallel gel.

Some mRNA which is translated upon initiation of spore germination in the absence of new mRNA synthesis. This early protein synthesis is essential for germination as well as for initiation of metabolic activities such as nucleic acid synthesis and respiration. However, as in other eukaryotes which share a similar period of cell dormancy, the products of this latent mRNA translation are not known, and the question of whether the cellular population of latent mRNA molecules codes for a group of proteins which is qualitatively different from that of the counterpart mRNA of physiologically active cells has not been answered.

In the present study we approached this question by isolating from dormant and germinating spores poly(A)(+) mRNA fractions and translating these mRNA fractions in vitro with a wheat embryo system. We found that no major qualitative differences could be detected between the in vitro translation products of the dormant-spore poly(A)(+) mRNA when the translation products were analyzed by SDS-polyacrylamide gel electrophoresis. While several minor (but reproducible) quantitative differences were noted, there appeared to be no substantial differences between the translation products. However, subsequent experiments, in which the in vitro translation products were analyzed by tryptic digest peptide mapping, indicated that the polypeptides translated from the two sources of poly(A)(+) mRNA were qualitatively dissimilar. Other dual-label experiments, in which the two types of translation products were subjected to isoelectric focusing, demonstrated clearly that the informational contents of the dormant- and germinated-spore poly(A)(+) mRNA fractions were qualitatively different. This finding justifies further experimentation to identify those proteins which are translated from the dormant-spore mRNA early in spore germination.

The proteins synthesized in vitro from the Botryodiplodia poly(A)(+) mRNA had a molecular weight range of about 10,000 to 55,000, and these in vitro products were compared with the
SDS-solubilized proteins extracted from labeled spores. The results showed that the proteins lower than 55,000 molecular weight synthesized in vitro and in vivo were approximately congruent electrophoretically; however, the proteins synthesized in vivo also contained a number of higher-molecular-weight species which were not translated from the poly(A)(+) mRNA in vitro. This difference could be explained either by a preference of the wheat embryo translation system for shorter mRNA in a heterogeneous population of mRNA or by a post-translational modification of the in vivo products (such as glycosylation) to yield the higher-molecular-weight proteins.

Other laboratories which have compared the in vitro translation products of poly(A)(+) mRNA isolated from cells in different stages of development have been unable to detect any differences in the electrophoretic gel patterns. In Xenopus the in vitro translational products of oocyte poly(A)(+) mRNA were found not to change during oogenesis, although changes in the patterns of proteins synthesized in vivo were observed (12). These authors proposed that this discrepancy could be due to a stage-specific translational control of protein synthesis which would otherwise not be detected in the in vitro translation experiments. The qualitative patterns of polypeptides synthesized by translation of poly(A)(+) mRNA fractions from dormant cysts and developing embryos of brine shrimp were also found to be similar (14). In addition, no differences could be detected in the patterns of the proteins synthesized in vitro from mRNA of dormant and germinated spores of Rhizopus stolonifer (J. L. Van Etten and S. N. Freer, Abstr. Second Int. Mycol. Congr. 1977, p. 702). It seems likely, however, that application of techniques other than SDS-polyacrylamide gel electrophoresis will provide more reliable and detailed comparisons of the mRNA translation products in these developing cells.

Our interest in the comparative in vitro translations of mRNA from dormant and germinated spores of B. theobromae was prompted in part by other experiments in this laboratory which showed that the dormant-spore cytoplasmic mRNA may code for certain proteins which are required for initiation of function of the mitochondrial respiratory system. A direct test of this hypothesis would involve immunoechemical identification of specific respiratory enzyme subunits which might be synthesized in vitro. Preliminary results from experiments now in progress show that the dormant-spore mRNA in fact does code for certain cytoplasmic polypeptides of the multiple-subunit enzymes F$_1$-adenosine triphosphatase and cytochrome c oxidase, as predicted earlier (6), and that these in vitro-synthesized subunits are the same subunits which we have found recently to be synthesized in vivo early in germination.

LITERATURE CITED


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