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Messenger RNA is conserved during drying of the drought-tolerant moss *Tortula ruralis*

[protein synthesis/double labeling/conserved poly(A)-rich RNA/*in vitro* translation]

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ABSTRACT **On rehydration after complete drying, the drought-tolerant moss *Tortula ruralis* (Hedw.) Gaertn., Meyer, and Scherb reforms its polyribosomes and can resume protein synthesis without new RNA synthesis. Results obtained with the double-label ratio technique coupled with polyacrylamide disc gel electrophoresis show that (*i*) the proteins synthesized on rehydration are largely similar to those synthesized at the time of dehydration and (*ii*) inhibition of RNA synthesis does not alter the rate or the pattern of protein synthesis during rehydration of slowly dried moss. Poly(A)-rich RNA has been isolated from fresh and dried moss by chromatography on oligo(dT)-cellulose and has been translated *in vitro* in the cell-free wheat germ system. It is concluded that mRNA is conserved during complete drying of *T. ruralis* and supports protein synthesis on subsequent rehydration. This ability to conserve mRNA is a characteristic held in common by vegetative drought-tolerant tissue of *T. ruralis* and air-dried seeds of higher plants.**

Plants experience water stress frequently in nature. One of the earliest biochemical effects of increasing water stress is a reduction in the ability of plant tissues to synthesize proteins (1, 2). This effect appears to be correlated with a stress-induced decline in polyribosome population (3, 4). The drought-tolerant moss *Tortula ruralis* (Hedw.) Gaertn., Meyer, and Scherb loses polyribosomes partially during rapid drying but completely during slow drying (5) or under an imposed steady-state water potential of less than −40 bars (−4000 MPa) (4). We have previously reported a lack of quantitative correlation and of temporal coincidence between the increasing levels of ribonuclease and declining levels of polyribosomes during stress. Consequently, we suggested that the stress-induced polyribosome loss was not due to ribonuclease activity but to ribosome run-off from mRNA coupled with a failure to reform the initiation complex (4, 6). It has also been reported (7) that cytoplasmic RNA is stable during drying. Here we describe experiments that provide evidence that mRNA is conserved during drying of *T. ruralis* and that this conserved messenger supports protein synthesis on subsequent rehydration.

MATERIALS AND METHODS

Plant Material. Apical 1-cm pieces of the gametophyte of *Tortula ruralis* were used as experimental material. Procedures for collecting and preparing the plants for experiments have been described (8).

Administration of Drought. Fresh moss of known weight was slowly or rapidly dried as described (9). For slow drying, the moss was placed over a stirred saturated solution of ammonium nitrate (relative humidity 65%) at room temperature. In about 9 hr, moss dried to a constant 20% of its original fresh weight, although it remained over the solution for 24 hr. Rapid drying to the same constant weight was achieved in 20–30 min by placing fresh moss on a single layer of cheese cloth over activated silica gel particles in a closed dish (relative humidity 0%) at room temperature.

Analysis of Polyribosomes. The procedures for extracting polyribosomes and for analyzing their sedimentation profiles were as described (10) except that dry moss equivalent to 500 mg fresh weight was used per sample.

Measurement of Protein and RNA Synthesis. Incorporation of [4,5-^{3}H]leucine into proteins was used as a measure of protein synthesis. After incorporation, moss proteins were extracted; protein content and radioactivity were determined as described (1, 9). Further specific details are given in appropriate figure legends.

Incorporation of [5,6-^{3}H]uridine into RNA was used as a measure of RNA synthesis. After incorporation, RNA was extracted by the phenol/chloroform method. RNA content was determined spectrophotometrically by the absorbance at 260 nm, and radioactivity was measured by liquid scintillation spectrometry.

Double-Label Ratio Technique. This was used to compare the pattern of protein synthesis before and after desiccation, and to examine the effects of new RNA synthesis on the pattern of protein synthesis during rehydration of slowly dried moss. Five hundred milligrams of fresh moss or 100 mg of dry moss was used. Dry moss was rehydrated in water for 10 min before the addition of radioactive label. When actinomycin D and cordycepin were used, they were present during the 10-min rehydration period as well as during incubation in radioactive precursor. For comparison of patterns of protein synthesis before and after desiccation, the fresh moss was incubated with [*U*-^{14}C]leucine whereas rapidly or slowly dried moss was incubated with [4,5-^{3}H]leucine. When the effect of new RNA synthesis on the pattern of protein synthesis during rehydration of slowly dried moss was studied, moss rehydrated in the presence of inhibitors of RNA synthesis was labeled with [4,5-^{3}H]leucine whereas that rehydrated in water alone was labeled with either [*U*-^{14}C]leucine or [4,5-^{3}H]leucine. Incubations for amino acid incorporation were for 75 min, and the soluble protein extracts were then prepared as described (1). Double-label mixtures were prepared by mixing equal volumes of ^{14}C-labeled extract from fresh moss with ^{3}H-labeled extract from rapidly or slowly dried moss, and equal volumes of ^{14}C-labeled extract from slowly dried moss rehydrated in water alone with ^{3}H-labeled extract from moss rehydrated in the presence of actinomycin D or cordycepin. A control double-label mixture was prepared by mixing ^{14}C-labeled and ^{3}H-labeled proteins, both from slowly dried moss rehydrated in water alone. A 100-μl aliquot of each double-label mixture was separated on 7.5% polyacrylamide gel as described (1, 9). After electrophoresis, gels were stained with Coomassie brilliant blue, scanned at 560 nm, and cut into 1-mm slices. Each slice was

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solubilized in 0.5 ml of NCS (Amersham/Searle), and the $^3H/^{14}C$ ratio (cpm/cpm) was determined with a Nuclear-Chicago liquid scintillation spectrometer.

Isolation of Total RNA. Two grams of fresh moss or 400 mg of dry moss was ground in 20 ml of 0.1 M Tris-acetate buffer (pH 9.0) containing 0.1 M NaCl, 2 mM Na_2EDTA, and 1.0% sodium dodecyl sulfate (11). The homogenate was centrifuged at 15,000 × *g* for 10 min. RNA in the supernatant was extracted twice with an equal volume of a phenol/chloroform/isoamyl alcohol (50:50:1, vol/vol) according to Aviv and Leder (12). RNA was precipitated overnight with 2.5 volumes of ethanol at −20° and pelleted by centrifugation at 15,000 × *g* for 10 min. The pellet was washed twice with 70% ethanol in 0.1 M potassium acetate (pH 6.5), and dissolved in binding buffer [10 mM Tris-acetate (pH 7.6)/0.5 M KCl].

Oligo(dT)-Cellulose Chromatography of RNA. Total cellular RNA dissolved in binding buffer was applied to an oligo(dT)-cellulose column equilibrated with the same buffer. The column was washed with the binding buffer to remove nonadsorbed RNA. RNA was precipitated from the effluent with ethanol and was considered to be non-poly(A)-containing RNA, or poly(A)$^-$ RNA. Bound RNA was eluted with 10 mM Tris-acetate (pH 7.6) and precipitated with ethanol, and was considered to be poly(A)-rich or poly(A)$^+$ RNA. Both poly(A)$^-$ and poly(A)$^+$ RNA fractions were dissolved in water and stored at −70°.

Preparation of Wheat Germ RNA. Wheat germ tRNA was prepared from commercial wheat germ (General Mills, California) by the procedures of Marcus *et al.* (13).

Preparation of Wheat Germ S-23. This was prepared by grinding 5 g of wheat germ with 5 g of sand in 25 ml of 1 mM Tris-acetate (pH 7.6). To the 14,000 × *g* supernatant, 1/50 volume of 1 M Tris-acetate (pH 7.6) was added. After recentrifugation at 14,000 × *g* for 10 min, 14 ml of supernatant was collected, avoiding the top lipid-containing layer. The supernatant was passed through a Sephadex G-25 column having a void volume of 18.5 ml and equilibrated with 1 mM Tris-acetate (pH 7.6)/50 mM KCl/3 mM magnesium acetate/4 mM mercaptoethanol. After 19 ml was voided, a 12-ml fraction was collected. This was centrifuged at 14,000 × *g* for 10 min and stored in 1-ml aliquots at −70°.

Translation of Moss mRNA. The reaction mixture with a total volume of 125 μl contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes)/KOH at pH 7.6, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 4 μg of creatine phosphate kinase, 2.5 mM dithiothreitol, 2.3 mM magnesium acetate, 20 mM KCl, 130 mM potassium acetate, 30 μM total amino acid mixture lacking leucine, 0.25 μCi of [*U*-^{14}C]leucine (298 Ci/mol), 1.5 μg of wheat germ tRNA, 25 μl of S-23, and mRNA as indicated in the experiments. Brome mosaic virus RNA was used as control messenger. Amino acid incorporation was for 60 min (unless otherwise stated) at 25° and the radioactivity incorporated was then determined according to Marcus *et al.* (14).

RESULTS

In order to determine whether synthesis of new RNA is essential for restoration of polyribosomes, we rehydrated slowly dried moss (equivalent to 500 mg fresh weight) in water (control), actinomycin D (100 μg/ml), or cordycepin (250 μM). After 75 min of rehydration, ribosomes were extracted and separated on a 16–32% linear sucrose gradient, and their sedimentation profiles were determined with an ISCO gradient fractionator coupled to a strip chart recorder. Polyribosome profiles from the ISCO chart were copied by xerography onto a uniform weight paper. Monoribosome and polyribosome peaks were cut out and weighed. Relative percentages of monoribosomes and polyribosomes were calculated and the percentage of polyribosomes obtained from an RNase-treated control (16%) was subtracted. Moss rehydrated in water alone had 24% polyribosomes, in 100 μg of actinomycin D per ml, 20%, and in 250 μM cordycepin, 22%. Thus, inhibitors of RNA synthesis did not inhibit this reformation of polyribosomes.

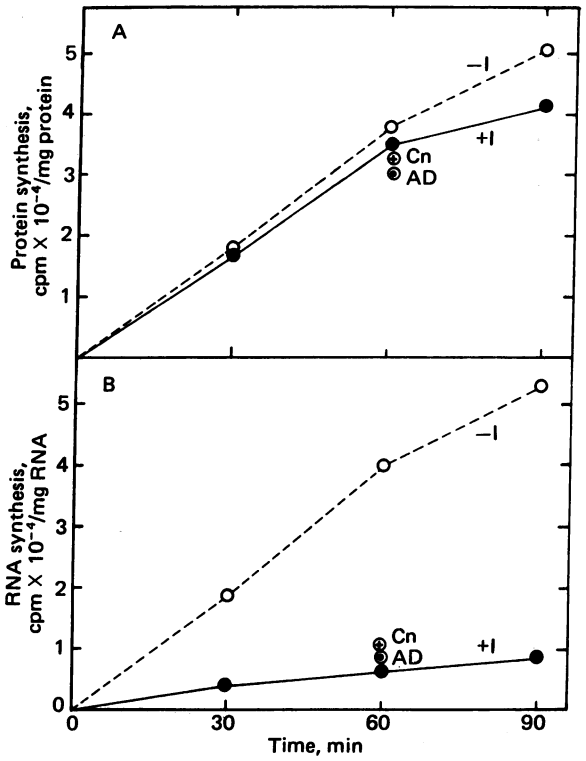

FIG. 1. Rates of protein and RNA synthesis during rehydration of slowly dried moss in the presence and absence of inhibitors (I) of RNA synthesis. Each sample consisted of 275 mg of fresh moss dried slowly to 55 mg. Each value plotted is a mean of two replicates. (*A*) Moss was rehydrated in water (−I), 100 μg of actinomycin D per ml + 250 μM cordycepin (+I), actinomycin D alone (AD), or cordycepin alone (Cn). After 10 min, 5 μCi of [*U*-^{14}C]leucine (298 Ci/mol) was added and incorporated for various times; then samples were homogenized in 10 ml of 0.1 M Tris·HCl (pH 8.0). Proteins were precipitated from the 10,000 × *g* supernatant with 5% trichloroacetic acid, pelleted at 15,000 × *g*, and hydrolyzed in 1 M NaOH overnight at 37°. Aliquots of hydrolysate were used to determine protein content and radioactivity. (*B*) After rehydration of moss as described in *A*, 20 μCi of [5,6-^{3}H]uridine (45 Ci/mmol) was added. After incorporation, samples were homogenized in 5 ml of 0.1 M Tris-acetate (pH 9.0) and extracted twice with equal volumes of a phenol/chloroform/isoamyl alcohol (50:50:1, vol/vol). RNA from the aqueous phase was precipitated with ethanol at −20° overnight, pelleted, and dissolved in water. Aliquots were used to determine radioactivity by liquid scintillation spectrometry and RNA content by A_{260}. It was assumed that 1 mg of RNA per ml gives A_{260} = 20.

We also determined the effect on protein synthesis of inhibiting RNA synthesis during rehydration of slowly dried moss. For this purpose, duplicate samples of slowly dried moss (equivalent to 250 mg fresh weight) were rehydrated in 5 ml of water either in the absence or in the presence of one or both inhibitors of RNA synthesis. After 10 min of rehydration (to allow for uptake of the inhibitors) either 5 μCi of [4,5-^{3}H]leucine (43 Ci/mmol) or 20 μCi of [5,6-^{3}H]uridine (45 Ci/mmol) was added. At different times specific radioactivities of total protein and total RNA were determined. The results are shown in Fig. 1. There was little effect of actinomycin D and cordy-

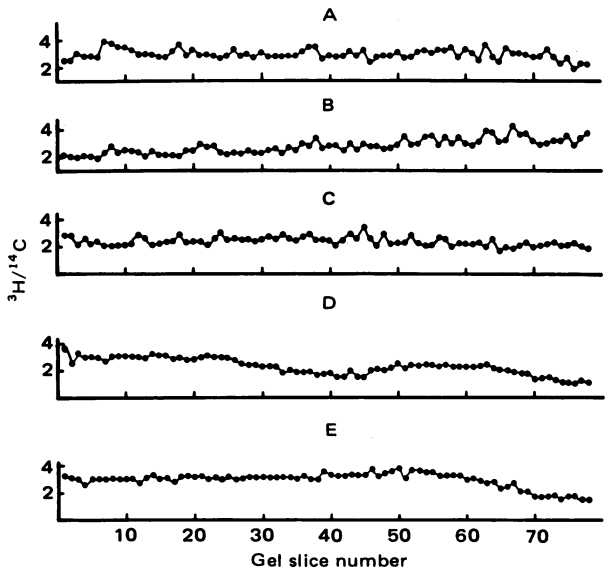

FIG. 2. Similarities between proteins synthesized by fresh moss and those synthesized by (*A*) rapidly dried or (*B*) slowly dried moss, and between proteins synthesized by slowly dried moss rehydrated in water and those synthesized by the same moss rehydrated in (*C*) actinomycin D or (*D*) cordycepin. Double-label ratio plot for the control double-label mixture is shown in *E*. (*A* and *B*) Fresh moss (500 mg) was incubated with 50 μCi of [*U*-^{14}C]leucine (298 Ci/mol) in 5 ml of water. One hundred milligrams of rapidly dried (*A*) or slowly dried (*B*) moss was rehydrated in 5 ml of water. After 10 min, 100 μCi of [*4,5*-^{3}H]leucine (43 Ci/mmol) was added. After incorporation for 75 min, each sample was homogenized in 2 ml of 0.1 M Tris·HCl containing 0.25 M sucrose, 6 mM ascorbic acid, 5 mM cysteine, and 5 mM dithiothreitol. The 30,000 × *g* supernatant was used as a soluble protein extract. An aliquot of ^{14}C-labeled extract from fresh moss was mixed with an equal volume of ^{3}H-labeled extract from rapidly dried moss (*A*) or that from slowly dried moss (*B*). A 100-μl aliquot of each of the two double-label mixtures was separated on 7.5% polyacrylamide gel, which was then cut into 1-mm slices. The $^3H/^{14}C$ ratio (cpm/cpm) for each slice was then determined. (*C*, *D*, and *E*) Slowly dried moss (100 mg) was rehydrated in 100 μg of actinomycin D per ml (*C*), in 250 μM cordycepin (*D*), or in 5 ml of water (control)(*E*). After 10 min, either 50 μCi of [*U*-^{14}C]leucine (298 Ci/mol) or 100 μCi of [*4,5*-^{3}H]leucine (43 Ci/mmol) was added to moss rehydrated in water. The moss rehydrated in actinomycin D or cordycepin was labeled with 100 μCi of [*4,5*-^{3}H]leucine. After 75 min of incorporation soluble protein extracts were prepared as described for *A* and *B* above. Double-label mixtures were made by mixing an aliquot of ^{14}C-labeled extract from moss rehydrated in water with an equal volume of ^{3}H-labeled extract from moss rehydrated in actinomycin D (*C*) or cordycepin (*D*). A control double-label mixture was prepared by mixing equal volumes of ^{14}C-labeled and ^{3}H-labeled extracts, both from moss rehydrated in water (*E*). A 100-μl aliquot of each of these double-label mixtures was separated on gels as described above.

cepin, alone or in combination, on protein synthesis (Fig. 1*A*) until after 90 min of rehydration. The inhibitors separately or in combination inhibited RNA synthesis by more than 80% (Fig. 1*B*). Thus, on rehydration, most protein synthesis was resumed whereas most RNA synthesis was inhibited.

We also compared the electrophoretic pattern of protein synthesis in fresh moss with that in moss rehydrated after desiccation. We argued that if mRNA is conserved during desiccation and used on subsequent rehydration, then the proteins made before and after drying should be similar, assuming of course that there is no preferential utilization of message. Moreover, we compared the patterns of protein synthesis in the presence or absence of concomitant RNA synthesis during rehydration of slowly dried moss. If newly synthesized RNA is not essential for resumption of protein synthesis on rehydration (after desiccation), then similar patterns of protein synthesis should be observed whether or not RNA synthesis is occurring. Results from our studies with the double-label ratio technique are shown in Fig. 2. Gel scans at 560 nm showing separation of protein bands were identical to those published earlier (9) and are therefore not included in Fig. 2. The double-label ratio plot for the control mixture containing both ^{14}C-labeled and ^{3}H-labeled proteins made by slowly dried moss rehydrated in water alone is shown in Fig. 2*E*. There is a more or less constant ratio along the length of the gel, as expected, since both ^{14}C-labeled and ^{3}H-labeled proteins were made under identical conditions. Fig. 2 *A* and *B* represents double-label ratio plots of mixtures of ^{14}C-labeled proteins from fresh moss and ^{3}H-labeled proteins from rapidly dried and slowly dried moss, respectively. In both cases there were minor variations in double-label ratios at some places along the length of the gel. Apparently small differences exist between proteins made by fresh moss and those made by rapidly dried or slowly dried moss. Fig. 2 *C* and *D* shows the double-label ratio plots of mixtures containing ^{14}C-labeled proteins made by slowly dried moss when rehydrated in water alone and ^{3}H-labeled proteins made by slowly dried moss rehydrated in actinomycin D and cordycepin, respectively. It appears that imbibition in actinomycin D resulted in very small differences in the synthesis of a few proteins by slowly dried moss on rehydration. Cordycepin, however, does not appear to have caused any noticeable differences in the pattern of protein synthesis. Thus, proteins made when most RNA synthesis is inhibited do not differ substantially from those made when RNA synthesis occurs.

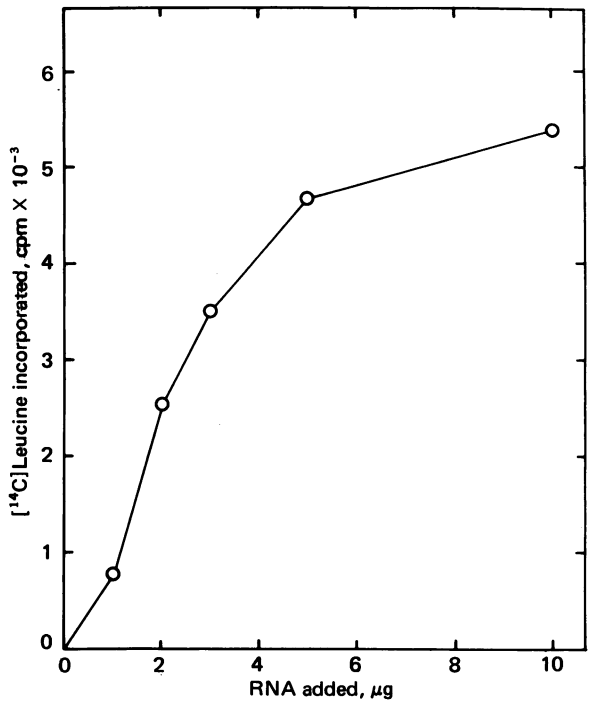

FIG. 3. Dependence of *in vitro* translation of poly(A)-rich mRNA from slowly dried moss on the amount of RNA added to the reaction mixture. Each value is a mean of two replicates. Experimental details were the same as described in *Materials and Methods* except that an increasing amount of poly(A)-rich mRNA was added to the reaction mixture.

Translation of Extractable mRNA from Fresh and Dried Moss. Stimulation of *in vitro* protein synthesis in the wheat germ system by poly(A)$^+$ RNA from slowly dried moss as a function of its concentration is shown in Fig. 3. [*U*-^{14}C]Leucine incorporation into polypeptides increased linearly with the

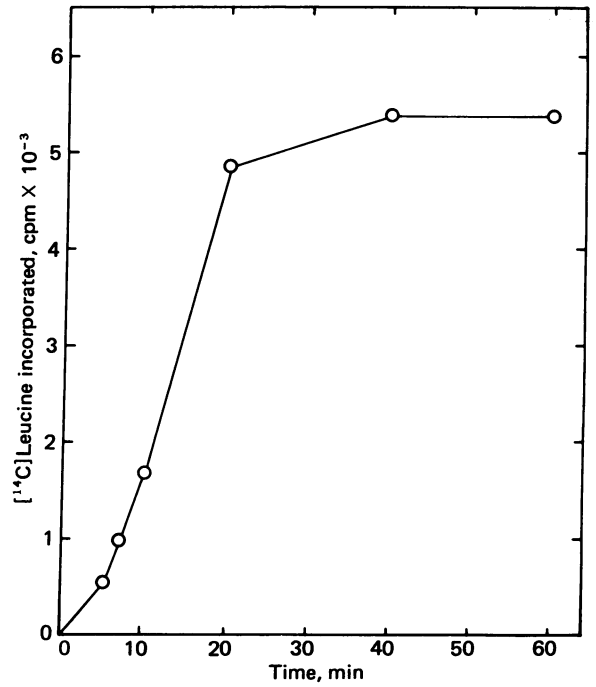

FIG. 4. Time course of *in vitro* translation of 10 μg of poly(A)-rich mRNA from slowly dried moss. Each value is a mean of two replicates. Experimental details were the same as in *Materials and Methods* except that incorporation was allowed for increasing time p ods.

amount of RNA added up to 5 μg. Doubling the amount of RNA to 10 μg resulted in a relatively minor increase in total incorporation.

A time course of *in vitro* protein synthesis supported by 10 μg of poly(A)$^+$ RNA from slowly dried moss is shown in Fig. 4. Incorporation increases linearly with time up to 20 min and reaches a maximum at 40 min.

A comparison of stimulation of *in vitro* protein synthesis by poly(A)$^+$ RNA from fresh, rapidly dried, and slowly dried moss is shown in Table 1. Poly(A)$^+$ RNAs from fresh, rapidly dried, and slowly dried *T. ruralis* were capable of supporting *in vitro* protein synthesis, although the template activity of poly(A)$^+$ RNA from dried moss was a little lower than that of poly(A)$^+$ RNA from fresh moss. Template activity of brome mosaic virus RNA, used as control messenger, was comparable to that observed by other workers (15). Also, poly(A)$^-$ RNA from fresh and dried moss possessed little template activity. The activity of the endogenous messenger alone contained in S-23 was only 693 cpm, while incorporation in the absence of S-23 was 85 cpm and was probably due to nonspecific binding of the radioactive amino acid.

DISCUSSION

Vegetative tissues of most plants sustain irreversible damage during extreme water stress such as occurs during air drying. After dehydration beyond a certain point, most tissues fail to resume metabolic activities on subsequent rehydration. It is to be expected that plant tissues that are able to recover from complete drying should, on rehydration, resume metabolic activities, particularly those required in the restoration of normal cellular integrity. These may include repair to damage sustained during stress. Drought induces damage to membranes resulting in decompartmentation (16), ultrastructural changes in organelles (17, 18), and inability to retain solutes (9). Since synthesis of enzymes and structural proteins may be a prerequisite for repair to cellular damage, the conservation of components of the protein-synthesizing complex during drought could be of survival value to the plant.

Table 1. Translation *in vitro* of mRNA from fresh and dried *Tortula ruralis*

RNA	[14C]Leucine incorporation, total cpm*	
	Poly(A)$^-$ RNA	Poly(A)$^+$ RNA
Brome mosaic virus (5 μg)	72,580	—
Fresh moss (10 μg)	1,498	9,175
Rapidly dried moss (10 μg)	1,428	7,976
Slowly dried moss (10 μg)	1,288	7,185

* Incorporation was for 60 min. Each value is a mean of two replicates. Total incorporation in the absence of exogenous mRNA and of S-23 was, respectively, 693 and 85 cpm.

Species known to recover from desiccation are rare among both lower and higher plants. As a convenient model we have been investigating the physiology and biochemistry of the desiccation-tolerant moss *Tortula ruralis*. Although polyribosomes of *T. ruralis* are partially conserved during rapid drying, they are completely lost during slow drying (5). Thus, it is during slow drying that the conservation of translational components assumes the greatest importance. The conservation of active ribosomes in dehydrated tissue, as shown by their ability to carry out *in vitro* poly(U)-directed synthesis of poly(Phe) has been documented for slowly dried *T. ruralis* (19). Also, stability of total cytoplasmic RNA during rapid drying of this moss has been reported (7).

The present work provides both indirect and direct evidence for conservation of mRNA in the vegetative tissue of *T. ruralis* during rapid and slow drying. Indirect evidence comes from the observation that this moss resumes protein synthesis immediately on rehydration independently of concomitant RNA synthesis. Such results strongly suggest that mRNA conserved during drying is utilized for protein synthesis on subsequent rehydration. The apparently minor differences between proteins made by fresh moss and those made by moss rehydrated after drying may be due either to differential utilization of the mRNAs conserved during drying or to partial degradation of some mRNAs during drying. The patterns of protein synthesis exhibited on rehydration of both rapidly dried moss [which conserves some polysomes (8)] and slowly dried moss [which does not (5)] show similar variations from those of undesiccated moss, as shown by the double-label ratio method. These results are consistent with the suggestion that at least some mRNAs, if not the majority, may be conserved during drying.

Our work also provides direct and unequivocal evidence for the conservation of mRNA during rapid as well as during slow drying of *T. ruralis*. Poly(A)-rich mRNA is present in fresh, rapidly dried, and slowly dried moss, and is able to support *in vitro* protein synthesis in the wheat germ system. The template activity of poly(A)$^+$ mRNA is somewhat lower in dried moss, particularly after slow drying. This may be due to a limited degradation of the messages during homogenization of the tissue since dried moss has higher ribonuclease activity (6). Poly(A)$^-$ RNA shows little template activity in the *in vitro* protein-synthesizing system (Table 1). This does not, however, rule out the presence of non-poly(A)-rich messages in the moss. The apparent activities of such messages would be reduced because of the presence of bulk rRNA.

Not all mRNAs present in the fresh moss are necessarily conserved during rapid or slow drying. Indeed, the minor qualitative differences between the proteins synthesized by

moss rehydrated after rapid or slow drying and those made by undesiccated moss could be due to the loss of some mRNA during drying. The apparently reduced template activity of poly(A)$^+$ mRNA extractable from dried moss could be explained similarly. Nevertheless, such partial loss of mRNA does not impair the capacity of the moss to recover from desiccation and to resume considerable protein synthesis.

Dehydration of reproductive and perennating structures such as seeds and spores is not only common, but in many instances appears to be an essential part of their maturation process (20, 21). The presence of conserved messages in some of these structures has been documented (22, 23). We report here that a drought-tolerant vegetative tissue also exhibits the important characteristic of conserving active mRNA during total dehydration.

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