STUDIES ON THE NATURE OF MESSENGER RNA IN GERMINATING WHEAT EMBRYOS

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During the last decade many thorough investigations were carried out on the mechanism of transcription and translation in the living organism. These have clarified the main features of nucleic acid replication and of protein biosynthesis in the cell, and have enabled the study of the early stages of differentiation at a molecular level.[1-3] Such studies are at present mainly concerned with the structure, function, and changes in the molecular pattern of animal eggs before and after fertilization,[4-6] and of spores and seeds before and after germination.[7] Seed germination transfers the embryo from the latent stage into an active developmental stage, and thus represents an interesting phase in the life cycle of the plant. Marcus *et al.*[8, 9] have shown that protein synthesis is inactivated both *in vivo* and *in vitro* in the ungerminated wheat embryo, which was found to contain ribosomes that could be activated[10] and yielded soluble RNA (sRNA) and supernatant capable of supporting amino acid incorporation. It was thus suggested that messenger RNA (mRNA) is the limiting factor.

In the present work, evidence is forwarded for the presence of conserved mRNA in the dry wheat embryo. It is suggested that the masked mRNA is activated upon germination, and supports early protein biosynthesis. Transcription of new mRNA starts at a later stage of development.

Materials and Methods.—Germination procedure: Wheat embryos (*Triticum durrum* var. *Nursit*) were prepared according to Johnston and Stern,[11] and stored over $CaCl_2$ at 4°. Viable embryos (500 mg) were germinated in the dark at 23° in a Petri dish containing 5 ml of a germinating medium (GM) containing 0.01 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.6, 0.02 *M* KCl, sucrose (10 mg/ml), chloramphenicol (50 μg/ml), and mycostatin (200 μg/ml). Fresh germinating medium was added after 6, 24, 48, and 72 hr germination.

Preparation of DNA: DNA was prepared from wheat embryos (3 gm) germinated for 24 hr according to the procedure of Marmur.[12]

Preparation of RNA: Wheat embryos were suspended in 0.05 *M* KCl (50 ml) for 2 min at room temperature. Homogenization was performed at 0° in 50 ml TM solution (0.01 *M* Tris buffer, pH 7.8, 0.01 *M* $MgCl_2$, and 0.02 *M* KCl), and the RNA was extracted by the phenol method.[13] The RNA obtained was dissolved in TM solution and digested for 4 hr at 28° with deoxyribonuclease (30 μg/ml Worthington Biochemical Corp., electrophoretically purified). Predigested pronase was then added (200 μg/ml, Calbiochem) and incubation prolonged for 12 hr at 23° in a dialysis bag against 1 liter of TM solution. The final RNA digest was extracted and dissolved in TM solution as above. The DNase and pronase treatments were repeated in the dialysis bag to ensure removal of any DNA and protein contaminants. Final purification of the RNA preparation obtained was effected by methylated albumin kieselguhr (MAK) column chromatography[14] and filtration through a membrane filter to remove traces of DNA. The purified RNA obtained was 99% sensitive to RNase digestion and insensitive to DNase.

Preparation of P^{32}-labeled RNA: Wheat embryos were germinated in GM as described above for the time periods specified in the *Results*, and then transferred into a germinating medium containing 100 μc/ml carrier-free P^{32}-orthophosphate for 24 hr, unless other-

wise stated. P^{32} incorporation was stopped by washing the embryos thoroughly six times with 50-ml portions of GM solution with 0.1 *M* unlabeled orthophosphate.

P^{32} ribosomal RNA and P^{32} sRNA were isolated from pulse-chased embryos as follows. The embryos were germinated for 24 hr in unlabeled GM solution, then transferred for another 24 hr into a GM solution containing 500 μc/ml carrier-free P^{32}-orthophosphate, and finally chased for 24 hr with 0.05 *M* unlabeled orthophosphate in GM solution. Total RNA was extracted and fractionation was performed on a MAK column using an NaCl concentration gradient (0.2–1.2 *M*). The results obtained are given in Figure 1. sRNA was eluted at 0.45–0.55 *M* NaCl, whereas ribosomal RNA (rRNA) was eluted at 0.95–1.05 *M* NaCl. The sRNA gave a sedimentation coefficient of $s_w^\circ = 3.2S$, whereas the rRNA gave two peaks with the sedimentation coefficients of $s_w^\circ = 16S$ and $24S$, in 0.05 *M* Tris buffer, pH 7.8, 0.15 *M* KCl, using a Spinco model E analytical ultracentrifuge with UV optics attachment.

RNA-DNA hybridizations: The hybridization experiments were carried out according to Gillespie and Spiegelman[15] and used filters loaded with alkaline-denatured wheat H^3-DNA.

Template activity assay: A cell-free ribosome and supernatant system similar to that described by Marcus and Feeley[8] was utilized in these studies. The ribosomes obtained from wheat embryos according to the above authors were layered over a 5–20% sucrose gradient and the 74*S* fraction was pooled after centrifugation for 45 min at 36,000 rpm in the SW 39 Spinco rotor. The ribosomes thus obtained were dialyzed against 0.1 *M* Tris buffer, pH 7.8, 0.05 *M* NaCl, and 1×10^{-4} *M* $MgCl_2$ for 4 hr at 4°. The $MgCl_2$ level was then raised to 1×10^{-2} *M* and the ribosomes were sedimented in $100{,}000 \times g$ for 1.0 hr and resuspended in TM solution (1.0 ml). The above treatment strips the ribosomes of endogenous message, which permits a response to exogenous message. The cell-free system used was saturated with respect to sRNA to ensure the dependence of the amino acid incorporation on the mRNA and not on the sRNA present in the exogenous RNA added.

Results.—Characterization of the RNA of the wheat embryo: When the embryos were germinated for 24 hr in carrier-free P^{32}, the specific activity of the total RNA extracted did not exceed 40 to 60 cpm/μg RNA. However, when the embryos were allowed to germinate for 24 hr in nonlabeled GM and then transferred to P^{32}-labeled GM for another 24 hr, they yielded an RNA preparation with a specific activity of about 30,000 cpm/μg RNA.

The fractionation of the highly labeled RNA on a MAK column is given in Figure 1*A*. The OD pattern obtained reveals the presence of sRNA (tubes 30 to 50) and of light and heavy rRNA (tubes 80 to 110). The radioactivity pattern shows that the P^{32} label is unevenly distributed with respect to optical density at 260 mμ, and that RNA species of high specific activity are present in the heavy RNA fraction. To determine the nature of the fractions possessing high P^{32} incorporation, a chase experiment was designed. Twenty-four-hour wheat embryos were labeled with P^{32} by germination for another 24 hours in P^{32} containing GM and then transferred to GM containing unlabeled phosphate for an additional period of 24 hours. The chased P^{32}-RNA of the embryos was isolated and fractionated on a MAK column as above. The results obtained (Fig. 1*B*) show clearly that the high-specific-activity material was chased out, and that the P^{32} label is now evenly distributed along the sRNA and rRNA regions.

The above findings suggest the presence of mRNA, transcribed during 24 to 48 hours of germination, mainly in the heavy RNA fraction. Furthermore, it can be concluded that sRNA and rRNA are also synthesized in the embryo after 24 hours of germination.

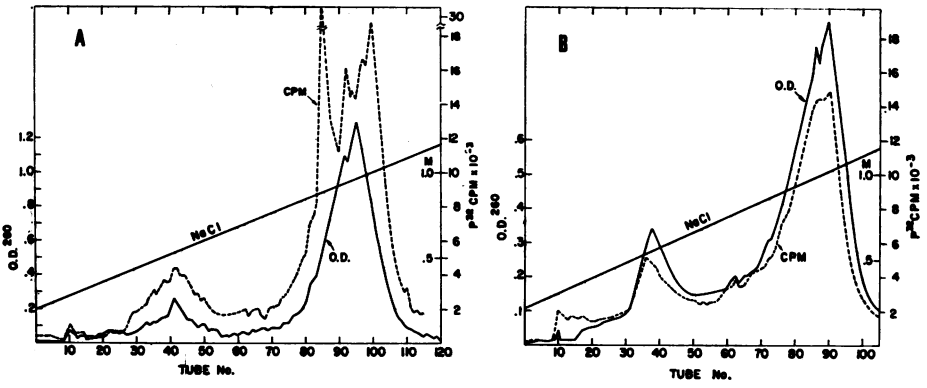

FIG. 1.—MAK chromatography of total P^{32}-labeled RNA from wheat embryos. (*A*) Germinating wheat embryos were pulsed for 24 hr with carrier-free P^{32} (100 μc/ml) between 24 and 48 hr after germination. Total P^{32}-RNA was then extracted as described in *Methods*, and 3 mg RNA was loaded on the column. Fractionation of the RNA was carried out with 0.05 *M* phosphate buffer pH 6.9 and a NaCl gradient from 0.2 to 1.2 *M*. Fractions of 3 ml were collected, the optical density was recorded at 260 mμ, and radioactivity was assayed in the Packard Tri-Carb liquid scintillator. (*B*) Wheat embryos were pulsed as described in (*A*) and chased with unlabeled phosphate-GM solutions (see *Methods*) for an additional time period of 24 hr. RNA was extracted and fractionated on MAK as in (*A*).

Template activity of the RNA of the dry wheat embryo: In order to test for a possible template activity of the RNA extracted from dry wheat embryo, a cell-free amino acid-incorporating system derived from wheat embryos was used.[8] The data presented in Table 1 show that the stripped monosomes prepared are practically devoid of endogenous message.

When the total RNA derived from ungerminated wheat embryos was added in increasing amounts to the cell-free system used, a corresponding increase in amino acid incorporation was observed (Fig. 2). Practically no amino acid incorporation took place in response to ribosomal RNA derived from the stripped ribosomes employed. Since the incorporation system was saturated with respect to sRNA and the wheat ribosomal RNA was found to have practically no template activity, one may conclude that the template activity of the RNA of the dry wheat embryo is most likely due to mRNA.

TABLE 1. *Properties of ribosomal preparations in a cell-free incorporation system of wheat embryos.*

Preparation	C^{14}-Phenylalanine Incorporated (cpm/mg rRNA)	
	Endogenous activity	+Poly U (10 μg)
Monosomes	5,340	23,200
Stripped monosomes	300	27,900
Polysomes	12,600	13,200
Control (ribosomes omitted during incubation)	154	160

The cell-free incorporation system was that described in *Methods*. Ribosomes (100 μg) were added to each tube and the reaction was stopped after 45 min at 30°. Active polysomes were obtained from 48-hr germinated embryos on a 5–20% sucrose gradient as described in *Methods*. Monosomes were isolated from the same gradient, recentrifuged at 105,000 *g* for 2 hr, and resuspended in TM buffer. Stripped monosomes were prepared from ordinary monosomes according to *Methods*.

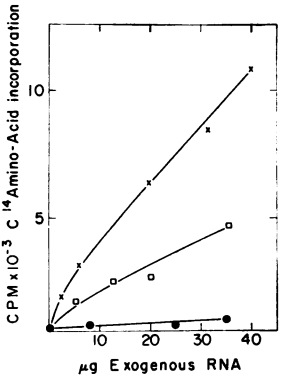

FIG. 2.—Response of the cell-free protein synthesizing system to exogenous template-active RNA. Reaction mixture of 0.5 ml contained 24 μM Tris buffer pH 7.8, 2.2 μM $MgCl_2$, 22 μM KCl, 3 μM guanosine 5′-triphosphate, 1 μM adenosine 5′-triphosphate, 20 μg phosphoenolpyruvic kinase, 130 μg phosphoenolpyruvic acid, 1 μM mercaptoethanol, 50 μg wheat sRNA, and 50 μg stripped monosomes (see *Methods*). One μg reconstituted protein hydrolysate (1.0 μc/μg, RPH, New England Corporation) and 105,000 × *g* purified supernatant containing 0.5 mg protein. Exogenous RNA was added and the reaction mixture was incubated for 45 min at 30°. The reaction was stopped with 1 ml cold 20% trichloroacetic acid (TCA) and 0.5 ml unlabeled RPH (1 mg/ml) was added. The resulting precipitate was washed with 9 ml 5% TCA, heated with 5 ml 5% TCA for 15 min at 90°, cooled and filtered through a Millipore filter, and counted in a Packard Tri-Carb scintillation counter. The exogenous RNA used: purified total RNA from dry ungerminated wheat embryos (—□—□—); purified rRNA from stripped monosomes (—●—●—); and mRNA prepared from polysomes of 48-hr germinated wheat embryos (—×—×—).

Hybridization experiments with sRNA, rRNA, and mRNA: The hybridization of total P^{32}-RNA transcribed during a 24-hour time interval, between 24 and 48 hours of germination, is recorded in Figure 3. The newly synthesized RNA at this stage of germination was found to be complementary to 1.45 per cent of the wheat embryo DNA. A typical saturation curve with total ribosomal RNA (18*S* + 24*S*) is given in Figure 4*A*. Saturation occurred when 0.28 per cent of the DNA was hybridized. Saturation with sRNA occurred when 0.025 per cent of the DNA was hybridized (Fig. 4*B*). One is thus left with 1.15 per cent of the genome which was active in the transcription of complementary RNA molecules other than sRNA and rRNA, namely mRNA.

The specificity of the hybridization reaction was tested by measuring the degree of sequence homology between heterologous RNA and wheat embryo DNA. Figure 5 gives the results of a competition experiment where wheat P^{32}-RNA was challenged with cold T2-mRNA or hamster cell RNA. The heterologous RNA's were found to compete for no more than 5–9 per cent of the DNA sites hybridized even at a ratio of wheat RNA to heterologous RNA of 1:12.

Evidence for message conservation in the dry embryo: The presence of mRNA conserved in the dry embryo could be detected by hybridization competition experiments, provided that the mRNA of the dry embryo is similar to that of 24- to 48-hour P^{32}-labeled mRNA. The results of a hybridization-competition experiment in which unlabeled RNA, derived from dry wheat embryo, was allowed to compete with the 24- to 48-hour P^{32}-labeled RNA, are given in Figure 6. Data for self-competition with cold RNA, isolated from embryos after 48-hr germination, are included. From the data presented it is evident that nearly all of the species of RNA transcribed during 24–48 hours of germination can be displaced by the RNA present in the ungerminated embryo. It is thus plausible to assume that mRNA is conserved in the ungerminated embryo and that practically no new species of RNA are transcribed during the first 48 hours of germi-

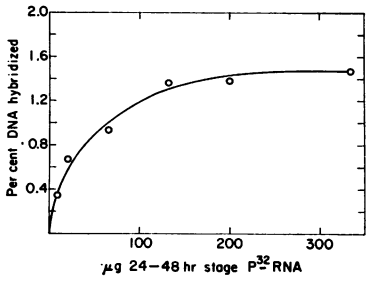

FIG. 3.—Saturation curve with 24–48 hr P^{32}-RNA. Each sample contained 29 μg of DNA and increasing amounts of P^{32}-RNA (11,150 cpm/μg), transcribed between 24 and 48 hr of germination. Since only one strand of the DNA is active in RNA transcription, the 1.5% of the DNA hybridized indicates that about 3% of the total information was transcribed between 24 and 48 hr of germination.

nation. The competition experiments do not exclude the possible presence in the ungerminated embryo of mRNA species in addition to those transcribed during 48 hours of germination.

Changes in the population of mRNA during germination: The results of a hybridization experiment of wheat embryo DNA with RNA labeled with P^{32} during 48 to 72 hours of germination are given in Figure 7. The data presented show that 1.45 per cent of the DNA is complementary to the RNA transcribed at this stage of germination.

A set of representative results of hybridization-competition experiments, in which P^{32}-labeled RNA transcribed at 48 to 72 hour of germination was challenged with cold RNA derived from embryos germinated for 0, 24, 48, and 72 hr, is given in Figure 8. The cold RNA of a 72-hour germinated embryo displaced approximately 80 per cent of the bound P^{32}-RNA, whereas the cold RNA of 0-, 24-, and 48-hour germinated embryos displaced only approximately 50 per cent of the bound P^{32}-RNA. The experimental findings thus suggest that during 48 to 72 hours of germination new species of mRNA molecules appear. Furthermore, since the size of the genome transcribed did not change during 72 hours of germination, one has to assume that the activation of the new parts of the genome after 48 hours of germination is accompanied by a concomitant switching off of a similar fraction of the genome which was active during the first 48 hours of germination.

Discussion.—The presence of conserved mRNA in the ungerminated dry wheat embryo was proved by (*a*) the ability of the RNA of the dry embryo to

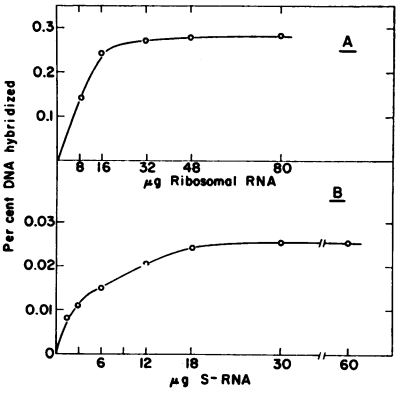

FIG. 4.—(*A*) Size of the DNA region complementary to wheat embryo ribosomal RNA. DNA (150 μg) was saturated with increasing amounts of chased and purified ribosomal P^{32}-RNA (3707 cpm/μg). The average size of the DNA region complementary to ribosomal RNA obtained with various preparations is 0.26–0.29%. (*B*) Size of the DNA region complementary to wheat embryo sRNA. DNA (150 μg) was saturated with increasing amounts of chased and purified P^{32}-sRNA (5790 cpm/μg). The average size of the DNA region complementary to sRNA with the various preparations is 0.023–0.026%.

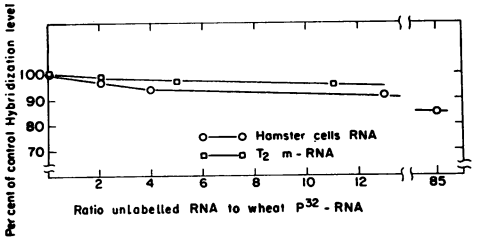

FIG. 5.—Competition experiment in which increasing amounts of heterologous unlabeled RNA were allowed to compete with saturating amounts of wheat P^{32}-RNA for the complementary binding sites of wheat DNA. 72 μg P^{32}-RNA (11,700 cpm/μg) prepared for 72-hr germinated wheat embryos was allowed to compete with increasing amounts of hamster-cell RNA (—O—O—). 10 μg of wheat DNA was used in an experiment. 48-hr P^{32}-RNA (200 μg, 12,800 cpm/μg) was allowed to compete with increasing amounts of cold T_2 mRNA (—□—□—). 29 μg of wheat DNA was used in an experiment.

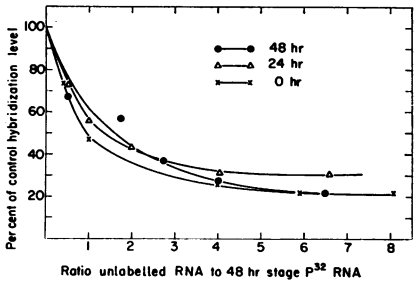

FIG. 6.—Evidence for the presence of mRNA in the ungerminated wheat embryo. 134 μg of total P^{32}-RNA, (12,800 cpm/μg) prepared from 48-hr germinated wheat embryos was challenged with unlabeled RNA prepared from ungerminated embryos, and from 24-hr germinated embryos. The DNA was 40 μg in an experiment.

enhance amino acid incorporation in a cell-free system, and (*b*) the ability of the RNA of the dry embryo to compete with P^{32}-labeled mRNA formed at the early stages of germination. Since wheat seed preserve their viability for prolonged periods of time even at extreme environmental conditions,[16] its embryonal mRNA should possess high stability. Marcus *et al.*[8, 9] have shown that the entire apparatus necessary for protein synthesis is present in the ungerminated wheat embryo, and that activation[10] or formation of mRNA[17] occurs during imbibition and seed germination. Our findings strongly support the alternative of the presence in the ungerminated wheat embryo of a masked form of mRNA synthesized during seed maturation.

The P^{32}-labeling experiments, as well as the RNA-DNA hybridization data, have shown that practically no new mRNA is synthesized during the first 24 hours of germination. Since germination during this period is accompanied by marked protein synthesis,[7–9] one has to assume that the masked mRNA has already been activated for a short while after imbibition. The mRNA molecules formed in the time interval of 24 to 48 hours of germination seem, however, to resemble closely the conserved mRNA molecules present in the ungerminated embryo. New species of mRNA molecules appeared after a germination period of 48 hours. However, about 60 per cent of the original mRNA molecules were still transcribed during the germination time interval of 48 to 72 hours. The size of the active genome did not change markedly during the germination period of 24 to 72 hours. Hence, during this interval there has been both a turning off of some gene transcription and a turning on of other gene transcription.

The value of 0.28 per cent for the DNA ribosomal RNA cistron is in agreement with the corresponding value for pea seedlings,[18] *Drosophila*,[19] HeLa cells,[20] and bacteria.[21, 22] The value of 0.026 per cent for the DNA region complementary

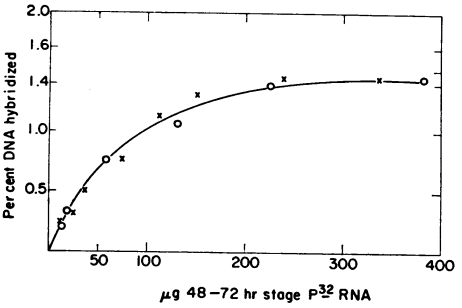

FIG. 7.—A representative saturation curve with P^{32}-RNA, prepared from 72-hr germinated embryos labeled between 48 and 72 hr of germination. Each sample contained 10 μg DNA and increasing amounts of 72 hr P^{32}-RNA (22,400 cpm/μg RNA) (—O—O—). Each sample contained 29 μg DNA and increasing amounts of 72-hr P^{32}-RNA (6,700 cpm/μg RNA) (—×—×—).

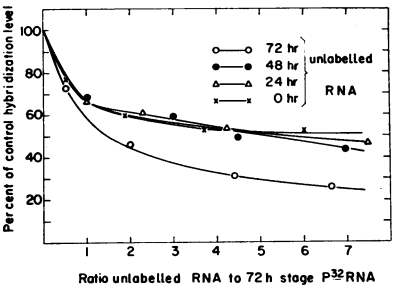

FIG. 8.—Competition experiment in which RNA from various stages of germination was allowed to compete with P^{32}-RNA transcribed between 48 and 72 hr of germination. Each sample contained 10 μg DNA, 125 μg of 72 hr P^{32}-RNA, (11,730 cpm/μg), and increasing amounts of cold RNA prepared from 0-, 24-, 48-, and 72-hr germinated wheat embryos.

to soluble RNA resembles values reported for *Drosophila*[23] and *E. coli*.[24, 25] The finding that the size of sDNA and rDNA is the same in such different organisms suggests that the number of these cistrons is roughly proportional to the length of the DNA molecule.

In conclusion, it might be deduced that imbibition triggers the activation of the conserved mRNA of the dry wheat embryo, and thus induces the synthesis of the enzymes and the other proteins which permit germination to proceed at a stage at which the genome is yet inactive. The control mechanism involved in wheat embryo germination resembles in its pattern the corresponding control mechanisms involved in the early stages of the development of the fertilized eggs of invertebrates.[4–6, 26, 27]

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