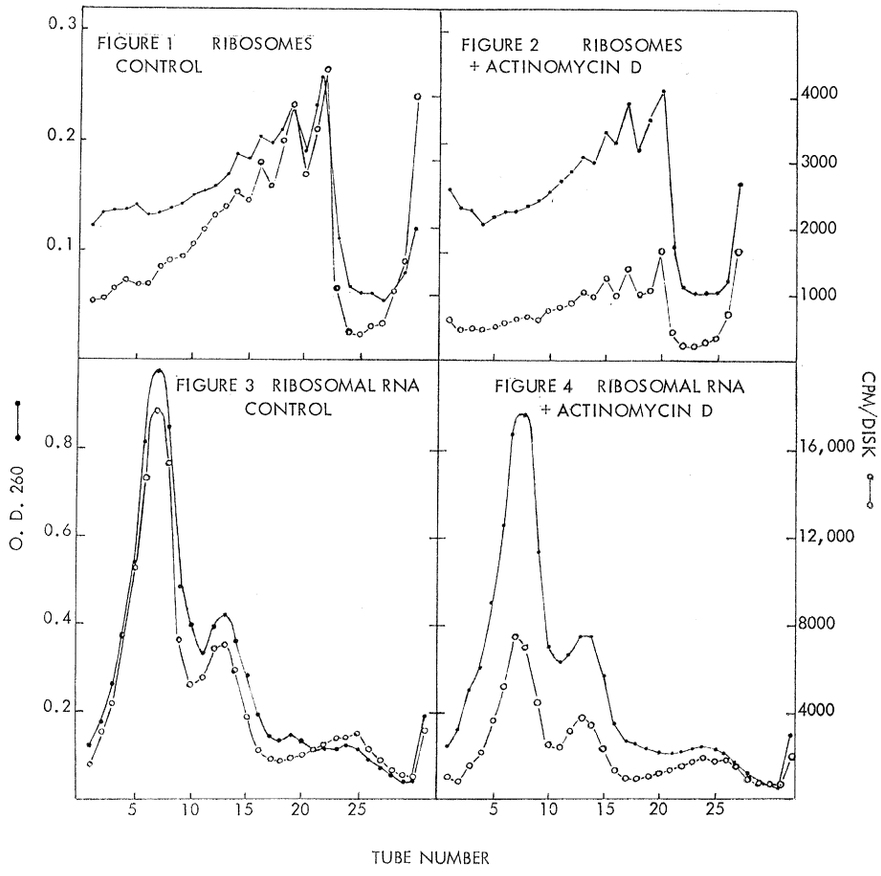
Long-Lived Messenger RNA: Evidence from Cotton Seed Germination

Abstract. *In germinating cotton embryos the partial inhibition of RNA synthesis by actinomycin D does not inhibit the incorporation of leucine-^{14}C into soluble protein nor cause a loss of polyribosomes during the first 16 hours of germination. This suggests that the protein synthesis observed during this period is directed by messenger RNA which exists in the mature seed and which is not rapidly degraded and resynthesized.*

Evidence is accumulating that rapid degradation and resynthesis is not a characteristic of all messenger RNA (mRNA). Observations on the high turnover rate of mRNA have been made only on bacteria (*1*) and HeLa cells (*2*). Observations that some mRNA is relatively stable and not rapidly degraded have come from studies of the synthesis of hemoglobin by reticulocytes (*3*) and studies of the synthesis of feather protein (*4*). In these instances the cells taking part synthesize one or several very specialized proteins and hence may represent unique situations.

However, sea urchin embryos retain the capacity to synthesize protein in the absence of RNA synthesis (*5*), as do rat liver tissue (*6*) and lens tissue (*7*). These observations suggest that a portion of mRNA in many tissues may in fact be stable and have a relatively long lifetime; and we now present supporting evidence gathered from our studies on RNA synthesis during the germination of cotton seeds.

Mature cotton embryos (*Gossypium hirsutum*, var. Coker 100) were removed from their seed coats and germinated in the presence of carrier-free $KH_2{}^{32}PO_4$ (50 μc/ml) for varying lengths of time. After this time the embryos were homogenized in 0.1*M* tris-succinate buffer, *p*H 7.8, 0.25*M* sucrose, 0.0025*M* $MgCl_2$, 0.001*M* spermidine, and 1 percent sodium deoxycholate in a loose-fitting Duall homogenizer, motor-driven at 100 rev/min. Ribosomes and polyribosomes were isolated from this homogenate by the method of Staehelin *et al.* (*8*). One half of the mixture of ribosomes and polyribosomes was sedimented for 2 hours through a sucrose density gradient (10 to 34 percent) containing 0.1*M* tris-succinate buffer, *p*H 7.5, 0.005*M* $MgCl_2$, and 0.001*M* spermidine. The other half was treated briefly with sodium dodecyl sulfate (SDS), final concentration 0.5 percent (*9*), and sedimented for 16 hours through a sucrose density gradient (5 to 20 percent) containing 0.05*M* KCl and 0.001*M* potassium acetate, *p*H 6.0. Fractions from both gradients were collected, and the ultraviolet absorbancy and radioactivity of each fraction were measured. The radioactivity of a portion of each fraction was determined by the paper disk method (*10*) adapted from the original method of Bollum (*11*). The fractions from the SDS-treated ribosomes having the peaks of absorbancy at 260 mμ showed the characteristic spectra of ribosomal RNA (the ratios of 280/260 and 230/260 being below 0.5 and of 220/260 being less than 1.0).



Figs. 1–4. Sucrose density-gradient profiles of absorbancy and radioactivity from embryos incubated with ^{32}P for 12 hours. CPM, counts per minute. Fig. 1. Ribosomes from untreated embryos. Fig. 2. Ribosomes from embryos treated with actinomycin D (20 μg/ml). Fig. 3. The SDS-released RNA from ribosomes of untreated embryos. Fig. 4. The SDS-released RNA from ribosomes from embryos treated with actinomycin D (20 μg/ml).

There was a substantial rate of incorporation of isotope into ribosomal RNA (rRNA); this rate increased during the first 16 hours of germination. In order to determine if this high amount of incorporated radioactivity represented a substantial contribution of newly synthesized RNA to the total RNA present in the germinating embryos, the embryos were incubated on wet filter paper with actinomycin D but without isotope for 4 hours. During the 4-hour period the desiccated embryos imbibe considerable amounts of water and swell to approximately the same weight as that which they have after 16 hours. The embryos were then transferred to wet filter paper containing $KH_2{}^{32}PO_4$ (25 μc/ml), leucine-^{14}C (10 μc/ml) (uniformly labeled) and the same concentration of actinomycin D as used in the earlier incubation; they were allowed to germinate for an addi-

tional 12 hours. Controls were handled identically except for the omission of actinomycin D in both incubation mixtures.

Ribosomes and polyribosomes were isolated, a portion was treated with SDS, and both portions were subjected to sucrose density-gradient centrifugation and characterized with respect to ultraviolet absorbancy and radioactivity. It was anticipated that the extent to which isotope incorporation into RNA was inhibited by actinomycin D would be reflected in a decrease in the total RNA absorbancy and perhaps by a decrease in the amount of polyribosomes relative to the monomeric ribosomes, *provided* the high extent of isotope incorporation in nontreated embryos represented a substantial contribution of new RNA to the total RNA.

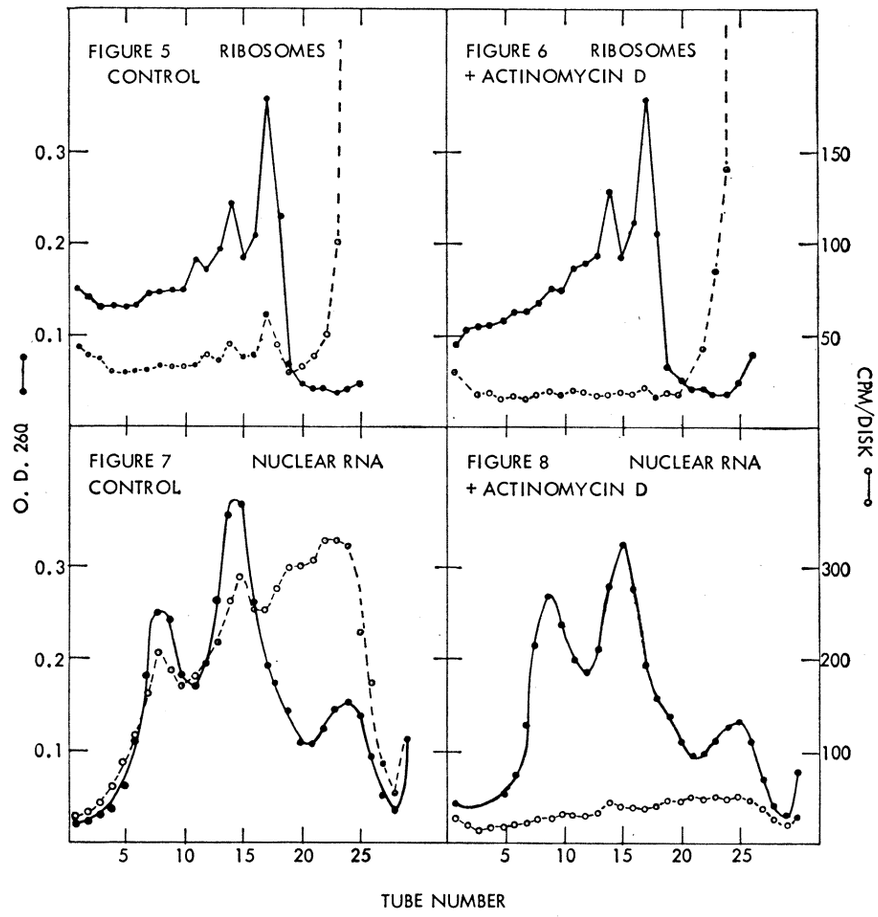
In addition, the soluble protein remaining in the supernatant after precipitation of ribosomes and polyribosomes was precipitated with trichloroacetic acid (TCA) and resuspended in KOH at *p*H 13, and the contaminating RNA was subjected to alkaline hydrolysis for 16 hours at 37°C. The protein was then precipitated with TCA, washed with a mixture of alcohol and ether (3:1), dissolved in dilute KOH, and dialyzed for 48 hours against dilute KOH. The amount of leucine-^{14}C incorporated into this soluble protein was determined by the paper disk method (*10*) and expressed as specific activity (counts of ^{14}C per minute per milligram of protein). The radioactivity in this acid-precipitable fraction was made acid-soluble when the fraction was subjected to trypsin digestion.

It was also anticipated that an inhibition of mRNA synthesis by actinomycin D would be reflected in a lower specific activity of the soluble protein of the antibiotic-treated embryos, again provided that the mRNA synthesized during this 16-hour period constituted a substantial amount of the total mRNA in these embryos.

Two concentrations of actinomycin D were used; the extent of the inhibition of ^{32}P-incorporation into RNA obtained with each concentration is presented in Table 1. Higher concentrations of this antibiotic, which possibly would have given greater inhibition, cause a necrosis of the cells exposed directly on the filter paper to the antibiotic. The inability to inhibit complete incorporation of ^{32}P into RNA with actinomycin D (20 μg/ml) may reflect an uneven distribution of this inhibitor throughout the embryo. Table 1 also gives for the control and treated embryos the total amount of radioactivity incorporated into the supernatants from which mitochondria had been removed. There is no decrease in the absorption of either isotope as a result of treatment with actinomycin D. Figure 1 shows the sedimentation pattern of ribosomes and polyribosomes from cotton embryos after 16-hour germination, and also the extent of incorporation of ^{32}P after exposure to the isotope for the final 12 hours. The distribution of radioactivity coincides with the absorbancy peaks which represent the monometric ribosome and polyribosomes of various sizes. A sedimentation rate for the monomeric ribosome in cotton has not yet been established. Although the antibiotic at a concentration of 20 μg/ml has inhibited ^{32}P-incorporation by 63 percent (Fig. 2), there is no diminution of the total ribosomal population and, furthermore, there is no shift of polyribosomes to ribosomes.

The sucrose density-gradient profile of RNA released from ribosomes and polyribosomes after they are dissociated into subunits by SDS (Fig. 3) shows two large absorbancy peaks, characteristic of structural rRNA, and a small peak close to the origin that probably represents transfer RNA. The distribution of radioactivity reveals that, in this 12-hour exposure to the isotope, rRNA is highly labeled. There is no significant decrease in the amount of rRNA present in the antibiotic-treated embryos as revealed by absorbancy at 260 mμ, although its synthesis has been inhibited over 60 percent (Fig. 4).

This long incubation with ^{32}P does not allow for a preferential labeling of mRNA. In order to establish the degree of inhibition of mRNA synthesis by the antibiotic, attempts were made



Figs. 5–8. Sucrose density-gradient profiles of absorbancy and radioactivity from embryos incubated with ^{32}P for 30 minutes. Fig. 5. Ribosomes from untreated embryos. Fig. 6. Ribosomes from embryos treated with actinomycin D (20 μg/ml). Fig. 7. Nuclear RNA from untreated embryos. Fig. 8. Nuclear RNA from embryos treated with actinomycin D (20 μg/ml).

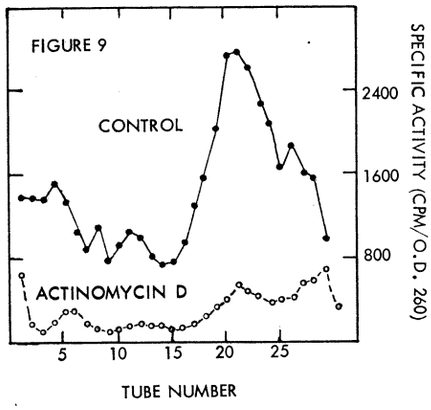


Fig. 9. Specific activity (count per minute/absorbancy) of data from Figs. 7 (●——●) and 8 (○- - -○). O.D., optical density.

to demonstrate the synthesis of a rapidly labeled RNA and to determine the effect of the antibiotic on this synthesis. Embryos were germinated for 12 hours in the presence of actinomycin D (20 μg/ml) and then incubated for 30 minutes more in the presence of $KH_2{}^{32}PO_4$ (100 μc/ml) and actinomycin D (20 μg/ml). After this short exposure to the isotope, the embryos were fractionated as before. The extent of isotope incorporation into the ribosome fraction of control embryos was very low (Fig. 5). There was negligible incorporation into the ribosomal fraction of treated embryos, although there was no loss of polyribosomes (Fig. 6). When these ribosome preparations were treated with SDS and then fractionated, the isotope was mainly associated with the transfer-RNA in the sucrose density gradient. Since there appeared to be no rapidly labeled RNA fraction associated with ribosomes, RNA from the nuclei was obtained by centrifuging the crude homogenate at 25,000g for 30 minutes, and the pellet extracted by means of the phenol-SDS method of Hiatt (*17*). This "nuclear" RNA was fractionated on a sucrose density gradient (5 to 20 percent), and the ultraviolet absorbancy and radioactivity of the fractions were determined (Figs. 7 and 8). An RNA with relatively high specific activity was found in tubes 18 to 22 of the control-embryo preparation, and virtually all nuclear RNA synthesis was inhibited by this concentration of actinomycin D. The high specific activity of tubes 18 to 22 is even more evident in Fig. 9. Other than its rapidity in incorporating isotope and its relative position on the sucrose density gradient, there is at present no evidence for considering this fraction mRNA. The ratio of its bases is intermediate between DNA from cotton and cotton rRNA.

Table 1. Incorporation of leucine-^{14}C into soluble protein by treated and untreated embryos.

Total radioactivity in supernatant*		Inhibition of ^{32}P-incorporation into RNA (%)	Specific activity of soluble protein (10^4 count/min)
10^4 ^{14}C count/min	10^4 ^{32}P count/min		
Control			
3.65	6.4		5.7
Actinomycin D (10 μg/ml)			
3.75	5.4	36	7.6
Control			
6	17.5		15
Actinomycin D (20 μg/ml)			
5.7	16	63	16

* After removal of mitochondria.

Further extraction of the pellet containing the nuclei with phenol and SDS yielded a trace of RNA but not with as high specific activity. Incorporation of isotope into this RNA was also negligible in antibiotic-treated embryos.

Thus all newly synthesized RNA evidenced by the incorporation of ^{32}P represents a negligible contribution to the total RNA. Furthermore, the fact that there is no decrease in polyribosome population even after 16 hours in the presence of the antibiotic suggests that either the mRNA maintaining the polyribosomal structure is not being degraded during this period or that its synthesis is not being inhibited by the antibiotic. In view of the almost total inhibition by actinomycin D of isotope incorporation into "nuclear" RNA with high specific activity, which occurs in embryos exposed briefly to the isotope, it is assumed that this concentration of the antibiotic also inhibits mRNA synthesis. In fact, evidence for the synthesis of a rapidly labeled RNA in nontreated embryos is at best sketchy (Fig. 7).

Further indication that the bulk of the embryo mRNA is stable during this period of germination is given in Table 1. There is no decrease in the specific activity of the soluble protein in embryos whose RNA synthesis has been inhibited by the antibiotic, indicating that the inhibition of rRNA and mRNA synthesis does not decrease protein synthesis during this period in germination.

These data suggest that, during the initial stages of cotton-embryo germination, protein synthesis is catalyzed by both mRNA and rRNA, which comes already formed in the mature seed, and that mRNA is not rapidly degraded and resynthesized during this period. Two other findings in this laboratory support this suggestion. (i) The number of polyribosomes relative to monomeric ribosomes is as high in the mature embryo as at any point during the first several days of germination. (ii) Embryos germinated for 36 hours in actinomycin D (20 μg/ml) exhibit the same rate of growth as do control embryos.

LEON DURE
LARRY WATERS
Department of Chemistry, Biochemistry Division, University of Georgia, Athens

References and Notes

1. F. Jacob and J. Monod, *J. Mol. Biol.* **3**, 318 (1961); F. Gros, H. H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough, J. D. Watson, *Nature* **190**, 581 (1961); C. Levinthal, A. Keynan, A. Higa, *Proc. Natl. Acad. Sci. U.S.* **48**, 1631 (1962); A. Kepes, *Biochim. Biophys. Acta* **76**, 293 (1963).
2. S. Penman, K. Scherrer, Y. Becker, J. E. Darnell, *Proc. Natl. Acad. Sci. U.S.* **49**, 654 (1963).
3. J. Bishop, G. Favelukes, R. Schweet, E. Russell, *Nature* **191**, 1365 (1961); E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *Proc. Natl. Acad. Sci. U.S.* **48**, 1238 (1962).
4. T. Humphreys, S. Penman, E. Bell, Biophysical Society Abstracts, 8th Annual Meeting, 26 February 1964.
5. P. R. Gross, L. A. Malkin, W. A. Moyer, *Proc. Natl. Acad. Sci. U.S.* **51**, 407 (1964).
6. G. Guidice and G. D. Novelli, *Biochem. Biophys. Res. Commun.* **12**, 383 (1963); M. Revel and H. H. Hiatt, *Proc. Natl. Acad. Sci. U.S.* **51**, 810 (1964).
7. R. B. Scott and E. Bell, *Science* **145**, 711 (1964).
8. T. Staehelin, C. C. Brinton, F. O. Wettstein, H. Noll, *Nature* **199**, 865 (1963).
9. T. Staehelin, F. O. Wettstein, H. Oura, H. Noll, *ibid.* **201**, 264 (1964).
10. R. J. Mans and G. D. Novelli, *Arch. Biochem. Biophys.* **94**, 48 (1961).
11. F. J. Bollum, *J. Biol. Chem.* **234**, 2733 (1959).
12. H. H. Hiatt, *J. Mol. Biol.* **5**, 217 (1962).
13. Supported by funds from NSF and AEC.

15 October 1964