Developmental Regulation in Cotton Seed Germination: Polyadenylation of Stored Messenger RNA†

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ABSTRACT: Evidence that RNA preexisting in the cotyledons of mature cotton seed (stored mRNA) is polyadenylated during the first day of germination is presented, based on three different experimental data sets. First, actinomycin D is found to inhibit $^{32}PO_4$ incorporation into mRNA-poly(A) by 62%, into mRNA by 70%, but into poly(A) only 30%. Second, far more $^{32}PO_4$ and [2-^{3}H]adenosine are incorporated into the poly(A) portion of mRNA-poly(A) than into the mRNA portion as would be expected from their relative sizes and base composition. This underlabeling of the mRNA moiety is enhanced when cotyledons are germinated in actinomycin D. However, an expected distribution of the isotopes between the mRNA and poly(A) moieties is found in cotyledons labeled later in germination. Third, spectral measurements of the absolute amount of mRNA-poly(A) accumulated during the first day of germination in cotyledons germinated in actinomycin D are larger than would be expected from the 70% inhibition of mRNA labeling caused by the drug. The three sets of data suggest that over 50% of the total mass of mRNA polyadenylated during early germination exists in the mature seed. Its complexity, however, has not been measured. These data may explain the sensitivity of much of germination enzyme synthesis to inhibition by 3′dAdo during early germination and its insensitivity to actinomycin D during this period.

Over the past several years we have collected data that suggest the existence of "stored" mRNA[1] in the cotyledons of mature cotton seed, that is, mRNA that is transcribed in embryogenesis but not translated until germination (Ihle & Dure, 1969, 1970, 1972a,b). This stored mRNA is conceptually distinct from "residual" mRNA that most seeds probably contain which presumably functions during embryogenesis, survives the desiccation phase of seed maturation, and resumes functioning during early germination (Dure, 1977). Evidence for this distinction in cotton cotyledons has been the identification of an enzyme activity (carboxypeptidase C) unique to germination whose de novo synthesis during this period is insensitive to actinomycin D. The time in embryogenesis when the mRNA for this enzyme appears to be synthesized along with a description of the developmental parameters involved in a gross sense with the regulation of its synthesis and the prevention of its translation in embryogenesis have been reported (Ihle & Dure, 1970, 1972b). It is likely that this enzyme represents a class of germination enzymes whose appearance in the germination of many dicot seeds is governed in this manner.

It was subsequently observed that the advent and increase in this enzyme activity, which is not demonstratable prior to 24 h of germination and reaches a maximum at 4 days, are sensitive to 3′dAdo (cordycepin), but only if the tissue is exposed to the inhibitor during the first day of germination (Walbot et al., 1974). Conversely, enzyme appearance is not sensitive to the pyrimidine analog of cordycepin, 3′dCyd. These latter observations are consistent with the proposition that the putative stored mRNA requires polyadenylation in early germination for its expression. Presented here is evidence that RNA transcripts extant in cotyledons of the dry cotton seed are polyadenylated during the first day of germination, along with transcripts newly synthesized during this period.

Experimental Procedures

Germination of Seeds. Mature cotton embryos (*Gossypium hirsutum,* variety Coker 201) were removed from their seed coats and soaked 0.5 h in water containing the bacteriostatic agent gramicidin D, 0.5 mg/mL, and other components where indicated. When used, the concentration of actinomycin D was 20 μg/mL, and 3′dAdo was 5×10^{-3} M. The embryos were transferred to Petri dishes and allowed to germinate for 8 h at 25 °C in darkness between layers of filter paper wetted with water containing the same concentrations of solutes as the soaking mixtures. In the basic germination regime embryos were transferred at the 8th hour to fresh filter paper moistened with the same mixtures as before and containing $^{32}PO_4$, 100 μCi/mL, and [2-^{3}H]adenosine, 100 μCi/mL, and germinated for an additional 12 h. This labeling period was chosen because previous experiments had shown that the subsequent development of germination enzyme activity is most sensitive to inhibition by 3′dAdo during this period (Walbot et al., 1974). (About 6 h is required for the embryo to become fully hydrated.) The embryos were removed after this 20-h period and rinsed well, the embryonic axis (rudimentary root and stem) was removed, and the cotyledons, which comprise 95% of the embryo volume and weight, were used for the isolation of RNA.

In some experiments (chase experiments) embryos were removed at 20 h, rinsed, and returned to their germination media on filter paper without radioactive isotopes and germinated an additional 12 h. In other experiments, in order to compare molecular sizes of RNA and poly(A) synthesized during the 8–20 h with that synthesized during the chase, [2-^{3}H]adenosine was supplied during the 8–20-h period and $^{32}PO_4$ supplied during the 20–32-h period. In still other ex-

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[1] Abbreviations used: mRNA, tRNA, and hnRNA, messenger, transfer, and heterogeneous nuclear RNA, respectively; poly(A) and poly(U), poly(adenylic acid) and poly(uridylic acid), respectively; AMP, adenosine monophosphate; $NaDodSO_4$, sodium dodecyl sulfate.

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periments, the two isotopes were not supplied to the embryos until the 32nd hour and the cotyledons germinated until the 40th hour. No cell division occurs in the cotyledon tissue during the first days of germination.

Extraction and Fractionation of RNA. Ten cotyledons after germination for the specified period were homogenized in 0.1 M Tris-HCl (pH 7.6), 0.1 M NaCl, 0.001 M EDTA, and 0.5% $NaDodSO_4$ at 4 °C (2 mL/cotyledon pair) with a motor driven Duall homogenizer. An equal volume of a 1:1 mixture of phenol and chloroform was added to the uncentrifuged homogenate and the mixture shaken at room temperature for 20 min. The phases were separated by centrifugation, and the phenol–chloroform phase and interface material reextracted with 0.5 volume of a solution containing 0.1 M Tris-HCl (pH 9), 0.001 M EDTA, and 0.5% $NaDodSO_4$ by shaking 20 min at room temperature. The phases were again separated, the two aqueous phases were combined, the NaCl concentration was brought up to 0.2 M, and the nucleic acids were precipitated by the addition of 2.5 volumes of ethanol and overnight sto at −20 °C. The precipitate was resuspended in 0.1 M Tris-I (pH 7.6), 0.1 M NaCl, 0.001 M EDTA ' removed by centrifugation, and the nucl ... were reprecipitated with ethanol. The nucleic acid precipitate was dissolved in 0.1 M Tris-HCl (pH 7.6) and 0.001 M EDTA.

High molecular weight RNA was separated from 4 and 5S RNA and DNA by several methods. One involved se entation through CsCl after the addition of Sarkosyl to 0.2% by the method of Glisin et al. (1974). The other involved overnight precipitation from solution by either 2 M LiCl or NaCl. Both procedures rid the high molecular weight RNA of the final trace of protein and yielded the same A_{260} units of RNA. Frequently, the sedimentation through CsCl gave a pellet that was difficult to dissolve. In both cases the RNA was dissolved in 0.1 M Tris-HCl (pH 7.6), 0.001 M EDTA and precipitated with ethanol. The low molecular weight RNA and the DNA remaining in solution were precipitated with ethanol after the appropriate dilution of the salts, and aliquots electrophoresed (see below) to test for the completeness of the separation of high molecular weight RNA.

To separate mRNA–poly(A) from other RNA, the precipitated RNA was dissolved in 0.01 M Tris-HCl (pH 7.4), 0.4 M NaCl, 0.01 M EDTA, and 0.2% $NaDodSO_4$ and chromatographed on oligo(dT)-cellulose by the procedure of Aviv & Leder (1972). (When the RNA that did not bind to the column in 0.4 M NaCl was reapplied to a second column, no additional material bound to the column.) After extensive washing of the column with the high salt buffer without $NaDodSO_4$, the material bound to the column was eluted with 0.01 M Tris-HCl (pH 7.4). This fraction exhibited small but identifiable peaks of rRNA on $NaDodSO_4$–polyacrylamide gel electrophoresis, which was removed by a second passage through the affinity column. In this step the bound fraction was made 0.4 M NaCl and reapplied to the column. The fraction that did not bind constituted about 10% of the radioactivity and about 40% of the A_{260} units of the bound fraction. The material that rebound on the second passage was eluted with 0.01 M Tris-HCl (pH 7.4), lyophilized, dissolved in 0.1 M Tris-HCl (pH 7.4), 0.2 M NaCl, 0.001 M EDTA, and precipitated with ethanol with the aid of carrier yeast nucleate. The long labeling times employed are considered to preclude a significant contribution of hnRNA–poly(A) radioactivity to this preparation. The affinity chromatography was monitored at 254 nm with an ISCO column monitor. In early experiments mRNA–poly(A) was purified by chromatography on poly(U)–Sepharose as described by Harris & Dure (1974). Very little difference was found in the yield of mRNA–poly(A) in comparison with oligo(dT)-cellulose chromatography. However, the possibility exists that RNA containing very short poly(A) tracts was not bound to the oligo(dT)-cellulose column, but our data indicate that this would represent a negligible portion of the mRNA–poly(A) fraction (see Results).

The absolute amounts of mRNA–poly(A) in cotyledons germinated normally and in the presence of actinomycin and 3′dAdo were measured by the absorption spectrum of mRNA–poly(A) prepared as given above. However, in these instances, 40 cotyledon pairs were used as the starting material, no radioisotopes were present in the germination media, and no carrier RNA was used in the ethanol precipitation of mRNA–poly(A).

Purification of Poly(A). The RNA twice bound to oligo(dT)-cellulose was digested with RNases A and T_1 as described by Adesnik & Darnell (1972). Poly(A) was separated from the digested material by another passage over oligo(dT)-cellulose. The digested material was lyophilized and subjected to alkaline hydrolysis and its molar base composition ...ctrophoresis. The composition of this ma... ...e distribution of ^{32}P, is considered to represent that of the mRNA portion of newly synthesized mRNA–poly(A).

The purity of the poly(A) was determined by the electrophoresis of aliquots after alkaline hydrolysis. Over 99% of the ^{32}P migrated as AMP. The mass average chain length of poly(A) was determined by comparing the ^{3}H that migrated as adenosine and as AMP in the electrophoresis. In addition the poly(A) preparations were sized in 10% polyacrylamide gels using cotton 5S and tRNA as markers.

For the spectral measurements of absolute amounts of poly(A) in cotyledons, poly(A) was separated from mRNA as given above and its purity determined spectrally by comparison to commercial poly(A). Absolute amounts of poly(A) were also measured by the amount of [^{14}C]poly(U) that was made RNase resistant by the method of Bishop et al. (1974). Here, poly(A) was used as a standard and yeast nucleate (cleaned of possible poly(A) sequences by passage over oligo(dT)-cellulose) was used to measure background values.

Analytical Procedures. Aqueous gel electrophoresis in 0.2% $NaDodSO_4$ utilizing 10% polyacrylamide gels was performed by the method of Loening (1967); nonaqueous 3.0% polyacrylamide gels (99% formamide) were prepared as described by Duesberg & Vogt (1973), and electrophoresis was carried out as prescribed by Haines et al (1974). Gels were scanned at 260 nm on a Cary 15 recording spectrophotometer adapted for gel scanning and sliced into 1-mm sections to determine the distribution of radioactivity.

Molar base ratios of the various RNA fractions were determined by either the paper chromatographic method of Lane (1963) or by electrophoresis at pH 3.7 of aliquots hydrolyzed in 0.3 N KOH for 18 h at 37 °C. Base composition was based on the distribution of ^{32}P.

Each of the various germination regimes, RNA extractions, mRNA–poly(A) and poly(A) purifications and analytical measurements were repeated 5–10 times and the data, which in some measurements varied ±15%, were averaged.

Since these experiments compare the yield of radioactivity and A_{260} units between cotyledons germinated under different conditions, precise and detailed accounting of the recovery of radioactivity and A_{260} units was employed and the integrity of the RNA species monitored by gel electrophoresis throughout the fractionation. It was found necessary to siliclad and autoclave all glassware and pipets. Treatment of solutions and glassware with diethyl pyrocarbonate was not found necessary provided all manipulations were carried out rapidly.

TABLE I: Distribution of ^{32}P in RNA as Percentage of Total RNA.

	germinated 20 h[a]	germinated 20 h in Act D[a,b,e]	germinated 20 h, 12 h chase[c,e]	germinated 40 h[d]
total RNA	100	100 (16.5)	100 (150)	100
18S, 25S RNA	83	72 (14)	84.5 (150)	85.3
5S, tRNA	10	12 (20)	12 (150)	13
mRNA-poly(A)	7	16 (38)	3.3 (80)	1.77
mRNA	5.7	10.3 (30)	3.0 (87)	1.65
poly(A)	1.3	5.7 (70)	0.3 (58)	0.12

[a] Germinated 20 h, exposed to $^{32}PO_4$ between 8 and 20 h. [b] Exposed to actinomycin D at 20 μg/mL throughout 20 h. [c] As in *a* followed by additional 12-h germination without $^{32}PO_4$. [d] Germinated 40 h, exposed to $^{32}PO_4$ between 32 and 40 h. [e] Values in parentheses are percentage of 20-h control.

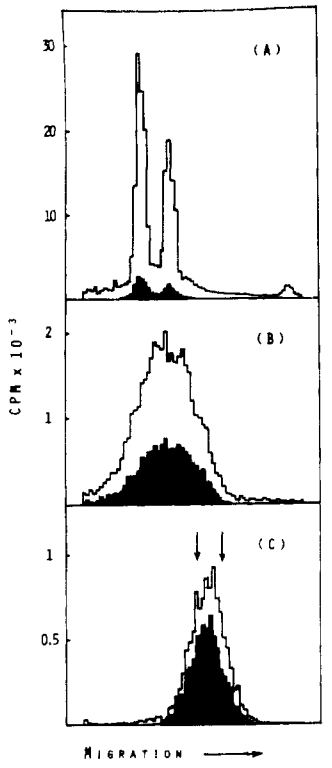

FIGURE 1: Histogram profiles of ^{32}P from polyacrylamide electrophoresis. (A) LiCl precipitated RNA electrophoresed in 99% formamide, 3% polyacrylamide. (B) RNA twice bound to oligo(dT)-cellulose electrophoresed as in A. (C) Poly(A) fraction separated from RNA in B (by nuclease digestion) electrophoresed in 0.2% sodium dodecyl sulfate in 10% polyacrylamide. Arrows represent migration of 5 and 4S RNA. Open histograms are of RNA from cotyledons germinated 20 h; shaded histograms are from an identical aliquot (per cotyledon basis) from cotyledons germinated in actinomycin D.

Because of the extensive conversion of AMP to GMP in the cotyledon tissue (40% in the 8–20-h labeling period as measured with [U-^{14}C]Ado), it was imperative that [2-^{3}H]Ado be used rather than [2,8-^{3}H]Ado since the 2-^{3}H is lost on the conversion of AMP to GMP.

Results

Evidence for the Polyadenylation of Preformed RNA from Isotope Distribution. The rationale for these experiments is simply that, should preexisting RNA along with newly synthesized RNA become polyadenylated during early germination, it should be demonstratable by measuring the relative amounts of incorporation of radioactive precursors into mRNA and poly(A) during this period; that is, the poly(A) component of mRNA-poly(A) should contain an inordinate proportion of radioactive precursors relative to the mRNA component (after allowing for their relative size) since some of the mRNA exists prior to the exposure of the tissue to the isotopes and would not contain radioactive components. Furthermore, actinomycin D should enhance this apparent underlabeling of mRNA chains by decreasing the amount of newly synthesized mRNA (radioactive) relative to stored mRNA (nonradioactive) that becomes polyadenylated. A comparison of the amounts of radioactivity in the mRNA and poly(A) components of mRNA-poly(A) should provide an estimate of the amount of stored mRNA relative to newly synthesized mRNA during the labeling period.

To obtain these measurements, total RNA was extracted from cotyledons germinated in the presence of $^{32}PO_4$ and [2-^{3}H]adenosine, and high molecular weight RNA was separated from 5S and tRNA by several methods and fractionated into poly(A)-containing and non-poly(A)-containing RNA by chromatography on oligo(dT)-cellulose. Poly(A) was separated from mRNA by nuclease digestion of the mRNA-poly(A) and readsorption of the poly(A) on oligo(dT)-cellulose. The yield in A_{260} units, the base composition, electrophoretic profile and specific radioactivity for both isotopes were determined for all fractions.

Table I shows the distribution of ^{32}P in the various RNA fractions obtained from cotyledons germinated for varying time periods. Several points emerge from these numbers. Much more radioactivity is incorporated into mRNA-poly(A) relative to other RNA in cotyledons labeled early in germination than in those labeled further along in germination. Actinomycin D is found to inhibit total RNA synthesis about 87% in early germination but inhibits mRNA-poly(A) synthesis only 62%. However, the drug prevents $^{32}PO_4$ incorporation into mRNA far more (70%) than into poly(A) (30%). Figure 1 shows this difference in degree of inhibition of isotope incorporation into the different RNA fractions caused by actinomycin D by way of electrophoretic profiles of radioactivity. If it is assumed that the actinomycin D mediated decrease in radioactivity in poly(A) reflects an inhibition of mRNA accumulation and not a direct effect on polyadenylation, then this differential effect of the drug on mRNA and poly(A) synthesis indicates that the amount of presumptive mRNA available for polyadenylation is decreased only 30% in the drug-treated tissue by the 70% inhibition of mRNA synthesis. This in itself suggests that nonradioactive RNA (stored RNA) becomes polyadenylated during this period, and the differential inhibition values allow an estimate to be made of the amount of putative stored mRNA polyadenylated relative to newly synthesized mRNA. If x represents the amount of stored mRNA polyadenylated during this period and y represents the amount of newly synthesized mRNA polyadenylated during

TABLE II: Calculation of Apparent mRNA Chain Length Based on Distribution of Radioactivity.

	experimental data		
	germinated 20 h[c]	germinated 20 h in Act D[c]	germinated 40 h[c]
1. average poly(A) length[a]	110	110	110
2. % [^{32}P]AMP in mRNA[b]	22	22	28.4
3. % [^{32}P]AMP in mRNA-poly(A)[b]	38.3	43.1	33
4. % ^{32}P of mRNA-poly(A) in poly(A)	18.8	35.7	6.7
5. % [^{3}H]AMP of mRNA-poly(A) in poly(A)	45.4	55	20
	calcd av mRNA chain length		
from 1 and 4	475	200	1530
from 1, 2, and 3	420	300	1620
from 1, 2, and 5	600	410	1550
av	500	300	1570

[a] Determined by ratio of [^{3}H]Ado:[^{3}H]AMP after hydrolysis. [b] Determined by distribution of ^{32}P among nucleotides after hydrolysis. [c] Cotyledons exposed to isotopes as given in Table I.

this period, then $x + y = 100\%$ of the mRNA polyadenylated. Since actinomycin D reduces $x + y$ to 70% and reduces y to 30%, then $x + 0.3y = 70\%$. Solving these two equations gives the value of x as 57% and y as 43%.

Table I also shows that about 19% of the ^{32}P of mRNA-poly(A) is in the poly(A) component itself in the 20-h germinated cotyledons. This value is about 36% in the actinomycin-treated cotyledons. When cotyledons are germinated an additional 12 h in isotope-free medium, radiolabeling of rRNA and tRNA continues, presumably from intracellular and free space pools of $^{32}PO_4$, but the radioactivity of the mRNA-poly(A) decreases, indicating considerable turnover of this fraction. Yet continued radiolabeling of this fraction during the chase period is also indicated by the change in ratio of ^{32}P in the mRNA and poly(A) moieties. The poly(A) component after this chase contains only 9% of the ^{32}P in the mRNA-poly(A) fraction. In cotyledons labeled much later in germination, the ^{32}P in the poly(A) component is reduced to less than 7%.

The unusually large amount of isotope in the poly(A) moiety relative to mRNA in the 20-h germinated cotyledons again suggests that nonradioactive RNA is polyadenylated during early germination. A careful analysis of the distribution of ^{32}P and [^{3}H]AMP between the two components of mRNA-poly(A) allows for calculations of the apparent mass average chain length of mRNA by three independent computations. If preexisting RNA is polyadenylated during early germination, the values for the apparent mass average chain length based on isotope content should be lower than the real mass average chain length, and the difference should reflect the ratio of preexisting to newly synthesized RNA polyadenylated. Again, germination in the presence of actinomycin D should increase this disparity between calculated and intrinsic mRNA chain length. Finally, there should be no disparity between the calculated and intrinsic mass average chain length in mRNA-poly(A) obtained from the tissue at a later time in germination when the polyadenylation of the putative stored mRNA is complete.

The arithmetic computations of apparent chain length are based on the following experimentally determined values: (1) the mass average [^{3}H]poly(A) chain length; (2) the mole percent [^{32}P]AMP in mRNA; (3) the mole percent [^{32}P]AMP in mRNA-poly(A); (4) the distribution of ^{32}P between mRNA and poly(A); (5) the distribution of ^{3}H between mRNA and poly(A). These data are presented in Table II. The mass average poly(A) chain length was found to be 110 AMP residues (±10) by electrophoretically measuring the ratio of [^{3}H]-Ado:[^{3}H]AMP after alkaline hydrolysis, which value is supported by the migration of unhydrolyzed poly(A) on 10% acrylamide gels as shown in Figure 1C. This value does not vary with germination time.

The first computation of apparent chain length is based on the fact that, if the poly(A) chain averages 110 residues and contains x fraction of the ^{32}P of the mRNA-poly(A), then the mRNA-poly(A) chain length average would be equal to

$$\frac{110}{x}$$

This value, less the 110 AMP residues in poly(A), would yield the apparent average mRNA length.

The second computation is based on the mole fraction of AMP in mRNA, the poly(A) chain length of 110 and the mole fraction of AMP in mRNA-poly(A), and is expressed by the following equation:

$$\frac{\text{total [}^{32}\text{P]AMP in mRNA-poly(A)}}{\text{total }^{32}\text{P nucleotides in mRNA-poly(A)}} = \text{mole fraction AMP in mRNA-poly(A)}$$

Allowing x to represent the number of AMP residues in mRNA, then $110 + x$ = total AMP residues in mRNA-poly(A) (numerator). The total nucleotides in mRNA-poly(A) (denominator) is given by

$$\left(\frac{x}{\text{mole fraction [}^{32}\text{P]AMP in mRNA}}\right) + 110$$

For example, if AMP comprises 22% of mRNA, then $4.5x$ would give the total number of nucleotides in mRNA. Adding the 110 AMP residues of the poly(A) chain to $4.5x$ would give the total nucleotides in mRNA-poly(A). Thus for the data obtained from 20-h germinated cotyledons, the equation would be:

$$\frac{110 + x}{4.5x + 110} = 0.383$$

Solving for x gives the total number of AMP residues in mRNA and $4.5x$ gives the total nucleotides in mRNA.

The third computation is based on the % [^{3}H]AMP of mRNA-poly(A) that is contained in poly(A) alone, the mole

fraction [^{32}P]AMP in mRNA and the poly(A) chain length. The relationship used here is expressed by the ratio % [^{3}H]-AMP of mRNA-poly(A) in poly(A):110[poly(A) chain length] = 100% [^{3}H]AMP of mRNA-poly(A):x[total number of AMP residues in mRNA-poly(A)]. Thus, $x - 110$ is equal to the total AMP residues in mRNA and $(x - 110)$/mole fraction AMP in mRNA is equal to the total nucleotides in mRNA.

For example, should the mass average mRNA length be 1500 nucleotides, then, using the experimental values [^{32}P]-mRNA = 22% [^{32}P]AMP and poly(A) = 110 nucleotides, the poly(A) portion of mRNA-poly(A) should contain only 6.8% of the ^{32}P and 25% of the [^{3}H]AMP. Furthermore, AMP should comprise 27% of the mRNA-poly(A). As shown in Table II the experimental values obtained for mRNA-poly(A) synthesized in early germination show far more radioactivity of both isotopes in the poly(A), and the inordinate amount of radioactivity in poly(A) is accentuated by germination in actinomycin D. Yet a distribution of radioactivity between mRNA and poly(A) closer to expectation is found in mRNA-poly(A) synthesized later in germination.

Consequently, as shown in the bottom portion of Table II, when these data are used to calculate the apparent mass average mRNA chain length using the computations outlined above, absurdly short mRNA is arrived at for early germination, even shorter mRNA for germination in actinomycin D, but mRNA averaging over 1500 nucleotides is arrived at for the later period in germination. The actual mass average ^{32}P or [^{3}H]mRNA size found for mRNA from all of these germination regimes by electrophoresis under denaturing conditions is about 2000 nucleotides (see Figure 1B) and the number average size is about 1500 nucleotides.

As mentioned before, our interpretation of these data is that preexisting (nonradioactive) RNA is polyadenylated during early germination along with newly synthesized RNA. Since the calculated chain length for mRNA synthesized early in germination is roughly one third the size calculated for that made later, this suggests that roughly two-thirds of the RNA polyadenylated during the early period is stored RNA.

Evidence from Optical Measurements of mRNA-Poly(A). The interpretation that both the differential inhibition of mRNA and poly(A) synthesis by actinomycin D (Table I) and the underlabeling of mRNA (Table II) indicate the polyadenylation of stored mRNA during early germination assumes that both mRNA synthesis and polyadenylation use precursor pools that reach the same specific radioactivity during the labeling period. This, of course, may not be true in this case. Also relevant here are the recent observations that nuclear polyadenylation of mRNA is followed by an additional round of polyadenylation that takes place in the cytoplasm (Sawicki et al., 1977) and that the poly(A) chain may undergo terminal turnover in the cytoplasm (Brawerman & Diez, 1975). It is conceivable that the radioactivity of the ATP pool feeding this cytosol synthesis is greater in both ^{32}P and ^{3}H than the nuclear pool. The fact that the distribution of isotopes between mRNA and poly(A) in mRNA-poly(A) synthesized later in germination approaches that expected may indicate that this consideration is not a problem in interpreting these data. Nevertheless, to further substantiate the indication that preexisting RNA is polyadenylated during early germination, the absolute amounts of mRNA-poly(A) present in the cotyledons of dry seeds and in cotyledons germinated 24 h ± actinomycin D and ±3′dAdo were measured optically. The point of these measurements is that, if measurable amounts of stored mRNA are polyadenylated in early germination, the accumulation of mRNA-poly(A) in actinomycin should be greater than predicted from the 70% inhibition of mRNA synthesis caused by actinomycin. That is, the increase in mRNA-poly(A) found in actinomycin would be greater than 30% of that found in the untreated cotyledons. Again, the degree to which the 30% is exceeded is a measure of the relative amounts of stored to newly synthesized mRNA polyadenylated. The amount of mRNA-poly(A) found in cotyledons germinated in 3′dAdo in which no polyadenylation takes place (Harris & Dure, 1974) is used to determine the net increse in mRNA-poly(A) that occurs in normal germination.

These determinations involved measuring spectrally the amount of RNA that is bound to oligo(dT)-cellulose (based on that which is rebound on a second passage through the column (see Experimental Procedures)). When high molecular weight RNA (produced by precipitation with LiCl) is first passed through the column, the bound material shows small but distinct peaks at 25 and 18 S when electrophoresed under denaturing conditions. When this bound material is recovered and passed through the column again in high salt, about 60% rebinds. This twice bound fraction shows no rRNA peaks on electrophoresis and its gel A_{260} profile is essentially identical with the radioactivity profile shown for [^{32}P]- or [^{3}H]-mRNA-poly(A) in Figure 1B. To further substantiate that this fraction is predominately mRNA-poly(A), the amount of poly(A) it contained was measured after digesting away the mRNA moiety (see Experimental Procedures). In every instance the poly(A) represented about 5% of the total A_{260} units of this fraction. In view of the 110 nucleotide average length of poly(A) and the average mRNA-poly(A) length of 1500–2000 nucleotides, this percentage indicates that the fraction is principally mRNA-poly(A). As a further criterion, the poly(A) content of the fraction was also measured by its extent of hybridization with [^{14}C]poly(U). These data were consonant with the spectral data. Hybridization with [^{14}C]-poly(U) was also carried out with unfractionated high molecular weight RNA and the amount of poly(A) measured in this manner was the same as that obtained by oligo(dT)-cellulose (data not shown). This fact also suggests that any mRNA-poly(A) not sequestered by the affinity column because of very short poly(A) tracts must represent a small component of this fraction.

The results of these measurements are presented in Table III. No corrections have been made for the difference in E_{260} between poly(A) and mRNA-poly(A) which conceivably could give a small overestimation of the amount of poly(A). The data in Table III show that any change during the first 24 h of germination in total high molecular weight RNA, which is predominantly rRNA, is within experimental error even when germination occurs in actinomycin D or 3′dAdo. The fact that protein synthesis is carried out in early germination on ribosomes carried over from embryogenesis has been documented for other seed cotyledons that, like cotton, do not undergo cell division during early germination (Walbot, 1971).

Further, dry seed cotyledons are found to contain mRNA-poly(A) which probably constitutes "residual" mRNA-poly(A) carried over from embryogenesis. Protein synthesis has been shown to commence within minutes of water uptake in several seed systems, and this synthesis requires neither concomitant RNA synthesis nor polyadenylation (Spiegel & Marcus, 1975). As shown in Table III, mRNA-poly(A) appears to double in amount during the first 24 h of germination, becoming ~1.6% of the high molecular weight RNA. It appears to increase by only 50% when germination is carried out in actinomycin D. Since the thrust of these measurements is to determine if more mRNA-poly(A) ac-

TABLE III: A_{260} Units of High Molecular Weight RNA per 100 Cotyledon Pairs (±15%).

	dry seed	germinated 24 h		
		untreated	+Act D[e]	+3′dAdo[f]
total[a]	500	500	500	500
mRNA-poly(A)[b]	4	8	6	<0.1
mRNA[c]	3.8	7.6	5.7	<0.1
poly(A)[b]	0.2	0.4	0.3	<0.1
poly(A)[d]	0.17	0.45	0.35	<0.1

[a] Prepared from total nucleic acid by precipitation with LiCl. [b] Measured spectrally. [c] Estimated by subtracting poly(A) from mRNA-poly(A). [d] Estimated by hybridization of mRNA-poly(A) to [^{14}C]poly(U). [e] Germinated in actinomycin D, 20 μg/mL. [f] Germinated in 3′-deoxyadenosine, 5×10^{-3} M.

cumulates in actinomycin than would be expected from the 70% inhibition of mRNA synthesis caused by actinomycin (based on isotope incorporation), the difference between 50% and 70% is not compelling in view of the limitations of the techniques employed. However, the data in Table III show that, when cotyledons are germinated in 3′dAdo, no polyadenylated RNA can be recovered. This suggests that the mRNA-poly(A) extant in the dry seed (residual mRNA-poly(A)) is either degraded or deadenylated during the 24-h germination period. This, in turn, suggests that all the mRNA-poly(A) found after 24-h germination in both actinomycin-treated and untreated cotyledons is adenylated during this 24-h period. Thus cotyledons germinated in actinomycin accumulate 75% of the mRNA found in untreated cotyledons. Since new mRNA synthesis in actinomycin is only 30% that of untreated cotyledons, the 70% inhibition of newly synthesized mRNA leads only to a 25% reduction in mRNA-poly(A). This allows for a third estimate of the relative amounts of stored to newly synthesized mRNA polyadenylated in early germination which is expressed in the following equations

$$x + y = 8A_{260}; x + 0.3y = 6A_{260}$$

where $x = A_{260}$ units of stored RNA and $y = A_{260}$ units of newly synthesized RNA. The value of x is 5.14 A_{260} units which is 64% of the total mRNA-poly(A).

Table IV summarizes the values for the relative amounts of preexisting to newly synthesized RNA polyadenylated during early germination as arrived at by the different methods.

Turnover of mRNA-Poly(A) in Early Germination. The data in Table I show that a portion of the mRNA-poly(A) synthesized during the first 20 h of germination is degraded or deadenylated during the subsequent 12 h. Isotope continues to be incorporated into rRNA during this chase from inner or intracellular pools, its specific radioactivity increasing by 50%. The amount of ^{32}P in mRNA-poly(A) decreases, however, and the amount in poly(A) alone decreases markedly. Isotope incorporation into mRNA-poly(A) presumably continues during this period as indicated by the fact that the amount of ^{32}P in poly(A) relative to mRNA is much reduced. Thus the turnover of mRNA-poly(A) during this period is even greater than that suggested by the values in Table I. The change in ratio of ^{32}P in poly(A) and in mRNA indicates that most of the mRNA polyadenylated during this period is newly synthesized. This is also suggested by the fact that the computation of the mass average chain length derived for the mRNA from these chase experiments is about 1000 nucleotides (data not shown), which is intermediate between that found at 20 h and that found at 40 h. This may explain why the synthesis of the indicator enzyme (carboxypeptidase C), thought to arise from stored mRNA, becomes insensitive to 3′dAdo inhibition during this period (Walbot et al., 1974).

TABLE IV: Summary of Estimates of the Relative Amounts of Stored and Newly Synthesized RNA Polyadenylated during Early Germination.

experimental basis	% of total RNA polyadenylated	
	stored	newly synthesized
1. differential inhibition of isotope incorp into mRNA and poly(A) by actinomycin	57	43
2. low level of isotope incorp into mRNA relative to poly(A)[a]	67	33
3. Increase in mRNA-poly(A) in presence of actinomycin as measured spectrally	64	36

[a] from the average of three computations based on isotope distribution between mRNA and poly(A).

Some of the stored mRNA that is polyadenylated during early germination persists, however, since the amount of ^{32}P in poly(A) relative to mRNA found after the chase period is not reduced to that found in cotyledons labeled later in germination (Table I). Furthermore, carboxypeptidase C activity increases until the 4th day of germination. No shortening of the poly(A) chain length based on the ratio of [^{3}H]Ado:[^{3}H]AMP is observed during the chase period (Table I).

Discussion

The validity of the evidence for the polyadenylation of stored mRNA that is based on isotope incorporation into mRNA and poly(A) (1 and 2 of Table IV) assumes that mRNA transcription and polyadenylation draw from nucleotide triphosphate pools of the same specific radioactivity. Should there be a second round of polyadenylation that takes place in the cytosol (Sawicki et al., 1977), and should the specific activity of the cytosol ATP pool be higher than that of the nuclear pool, these data must be reinterpreted. On the other hand, the long incubation time in the isotopes and the fact that labeling the tissue later in germination shows no underlabeling of mRNA suggest that pool disequilibria is not a problem in this case. Furthermore, if 10–20 additional AMP residues are added to mRNA-poly(A) from a cytosol ATP pool of higher specific radioactivity, the chain length of poly(A) based on the ratio of [^{3}H]Ado:[^{3}H]AMP would be shorter than that found by electrophoresis. That is, the 3′-terminal residues including the 3′Ado would have a higher specific radioactivity than the bulk of the poly(A) formed in the nucleus. No such disparity is found.

The third line of evidence for the polyadenylation of stored mRNA that is based on the increase in optically measurable or poly(U)-hybridizable mRNA-poly(A) in cotyledons ger-

minated in actinomycin is in turn based on the validity of the observed 70% inhibition of mRNA synthesis by the drug. An alternative explanation for this apparent reduction in the amount of isotope incorporated into mRNA would be the possibility of larger pools of phosphate and adenosine in actinomycin-treated tissue (caused by the almost total inhibition of rRNA synthesis) that lower the specific radioactivity of these pools. We have measured the phosphate and ATP pools in actinomycin-treated and untreated cotyledons and found no differences during the first day of germination (unpublished data). Furthermore, some enzyme activities unique to germination fail to develop in actinomycin-treated cotyledons (Radin & Trelease, 1976; Dilworth & Dure, (1978). Thus we feel that the 70% inhibition of mRNA synthesis by actinomycin is an accurate measurement.

Examples of the temporal separation of mRNA transcription and polyadenylation exist in the ontogeny of animal organisms as well, the best documented example being the maternal mRNA of sea urchin ova. Here, a substantial portion of the poly(A) sequences added to RNA immediately following fertilization is added to transcripts existing prior to fertilization (Slater et al., 1972; Wilt, 1973; Slater & Slater, 1974). However, in sea urchin ova a complex and dynamic situation involving the deadenylation and readenylation of existing mRNA appears to occur within several hours of fertilization (Dolecki et al., 1977; Wilt, 1977). Unfortunately germinating cotton cotyledons (which possess seven cell layers that must be diffusion-labeled) are not amenable to short time kinetic studies. Should the residual mRNA-poly(A) of dry seed cotyledons undergo deadenylation-readenylation, then our distinction between residual mRNA-poly(A) and stored mRNA is questionable. Particularly, our interpretation of the increase in mRNA-poly(A) in actinomycin-treated cotyledons measured spectrally, which is based on the disappearance of residual mRNA-poly(A) in cotyledons germinated in 3'dAdo (3 of Table IV) is questionable. However, our interpretation in part is based on the fact that a great many of the proteins synthesized in vivo after 20 h of germination in actinomycin are different electrophoretically from those synthesized in late embryogenesis (unpublished data) which suggests that a new population of mRNA-poly(A) replaces residual mRNA-poly(A) independent of RNA synthesis.

We cannot exclude the possibility that the presumptive stored mRNA contains a small poly(A) tract that is simply elongated during germination. The poly(A) tract would have to be too short to be sequestered by affinity chromatography. Such a possibility would not subvert the idea that the stored mRNA requires polyadenylation before it is translated in germination.

Although the data summarized in Table IV all suggest that over 50% of the mRNA polyadenylated during the first day of germination is stored mRNA, this in no way indicates the percentage of the total mRNA complexity existing by 24 h of germination that is derived from stored RNA. In fact we would submit that the complexity of stored mRNA will prove to be rather small, comprised of sequences for degradative, hydrolytic enzymes involved in mobilizing the storage nutrients of the cotyledons rather than enzymes involved in intermediary metabolism whose levels are likely regulated. This prediction is based on contrasting embryogenic development between dicot and monocot plants (Dure, 1977).

The possible requirement for the stored mRNA of dicot seeds like cotton to be further processed in germination provides a tentative basis for the role of the plant growth regulator, abscisic acid, whose occurrence in these seeds in the later stages of embryogenesis is somehow linked to the prohibition of the translation of the stored mRNA at the time of its transcription (Ihle & Dure, 1970, 1972b).

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