

mRNA stabilization controls the expression of a class of developmentally regulated genes in *Dictyostelium discoideum*

(genomic library/hybrid release)

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ABSTRACT During the development of *Dictyostelium discoideum*, several thousand new mRNA species appear in the cytoplasm after the cells have formed stable aggregates. Here we show that six of these late mRNAs, corresponding to six clones randomly chosen from a genomic library, are synthesized from the very beginning of development at a rate comparable to that observed late in development but that transcripts do not accumulate until after aggregation. The early- and late-synthesized mRNAs are identical in size and compete with each other for hybridization to the genomic clones. The early-synthesized mRNAs do not accumulate in the cytoplasm in the preaggregation stage because they are very unstable. Their stability, estimated from the kinetics of incorporation during continuous labeling with ^{32}P , increases by perhaps an order of magnitude in the postaggregation stage. We conclude that mRNA stabilization is the major controlling factor of the expression of these genes.

Dictyostelium discoideum provides an ideal system for the analysis of gene expression during development. In a process induced by amino acid starvation and mediated by pulsatile changes in concentration of cAMP, individual cells aggregate to form mounds of about 10^5 cells. After 20–24 hr, they form a fruiting body composed mainly of two types of cells, spore and stalk cells (1, 2).

This process is accompanied by a striking change in the pattern of gene expression. Growing amoebae contain about 5000 unique polyadenylated RNA sequences in their polyribosomes (3). Most of these sequences, the “constitutive mRNAs,” continue to be present (4) and synthesized (5, 6) throughout development. About 3000 additional polyadenylated RNA sequences appear in the cytoplasm of developing cells (4, 7). Though modulation of the levels of mRNAs occurs throughout the differentiation cycle (8–12), the majority of developmentally regulated mRNAs appear in the cytoplasm only after the formation of tight cell–cell aggregates and hence are termed “late mRNAs” (4, 7).

For a large fraction of the late mRNA species, accumulation in the cytoplasm appears to be not only concomitant with but also dependent on cell–cell interactions (13–15), specifically on the formation of stable aggregates (16). This has been demonstrated by maintaining the starved amoebae as single cells in a vigorously shaken suspension (13, 15), by studying a variety of mutants deficient in aggregation (ref. 14 and unpublished data), and by seeding starved amoebae on gels derivatized with sugars—a condition that prevents the formation of stable aggregates but allows normal streaming of cells into mounds (16, 17). Under these various conditions a large number of late mRNA species fail to accumulate in the cytoplasm of the developing cells.

When the cellular aggregates formed during normal development are disrupted, transcription of one class of aggregation-dependent mRNAs ceases (13) and RNA molecules previously accumulated in the cytoplasm are destabilized (5) so that they disappear quickly from the cell. Addition of cAMP to the suspension of cells dispersed from the aggregates restores transcription (13) and prevents destabilization (6). This class of mRNAs appears to be preferentially expressed in pre-spore cells (18, 19). In contrast, dispersion of cell aggregates does not affect the synthesis and stability of the constitutive mRNAs and of a second, smaller class of developmentally regulated mRNA species that appears to be preferentially expressed in pre-stalk cells (refs. 13, 16, 18 and unpublished data).

The finding that most late mRNAs are selectively destabilized upon cell dispersion led us to investigate whether the converse mechanism, mRNA stabilization, might play a role in the control of late-gene expression during the normal aggregation process. Here we report that at least some of the late mRNAs are synthesized from the very beginning of development at a rate comparable to that observed in the postaggregation stage. However, they do not accumulate to a significant extent in the cytoplasm because they are highly unstable. Their accumulation occurs late in development when their half-life increases from somewhat less than 30 min to more than 4 hr.

MATERIALS AND METHODS

Conditions for growth and synchronous development of *D. discoideum* strain V12M2 have been described (5, 16). All procedures for the identification of single species of polyadenylated RNA and for the analysis of their metabolism are described in previous papers (5, 16, 20) as detailed in figure legends.

RESULTS

Synthesis and Accumulation of Late mRNAs. To analyze the synthesis and accumulation of individual species of mRNA, we used genomic clones each selected to contain a single gene that is expressed either constitutively (i.e., both in growth and development) or expressed only during development (16, 20).

The time course of accumulation of cytoplasmic poly(A)⁺ RNA complementary to these clones in the strain V12M2 is shown by blot-hybridization analysis in Fig. 1. Clones M1 and M2 are constitutively expressed, since a band can be detected in preparations of poly(A)⁺ RNA both from growing cells (lanes 1) and from cells at any stage of development (lanes 2–5). Transcripts of the other six clones cannot be detected in growing amoebae (lane 1) nor in early developing cells (lanes 2–4). They appear in significant amounts only

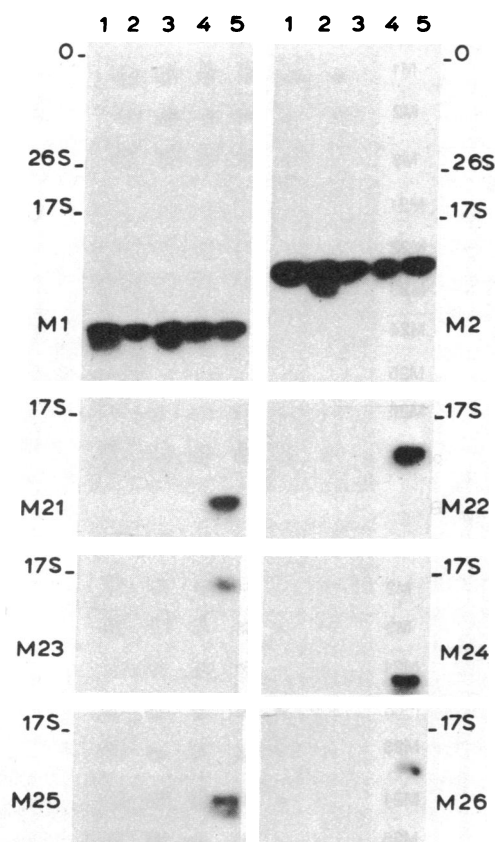


FIG. 1. Analysis of RNA accumulation during development. Cytoplasmic poly(A)⁺ RNA (2 μ g) from cells at 0 (lane 1), 2 (lane 2), 4 (lane 3), 6 (lane 4), or 8 hr (lane 5) of development was electrophoresed in formaldehyde-containing gels, blotted into nitrocellulose paper, and hybridized to ³²P-labeled DNA probes as described (20). The exposure time for autoradiography was 24 hr. For clones M1 and M2, the complete autoradiograms are shown; for M21–26, only the relevant areas are shown. O, origin.

after 7–8 hr of development, a time by which cellular aggregation is completed in this strain (21).

Although no mRNA complementary to the late-specific DNA clones accumulates, the mRNA corresponding to these clones is synthesized during the first 2 hr of development. Cellular RNA was labeled *in vivo* for 2 hr by adding [³²P]P_i to developing cells at 0 hr or 10 hr of development. Poly(A)⁺ RNA extracted from the cytoplasm was hybridized to each cloned DNA spotted on nitrocellulose paper. The hybridized RNA was then released from the hybrid and analyzed in an agarose gel under the same conditions of electrophoresis used for the blot analysis in Fig. 1.

Using early-labeled RNA, a band was detected for all of the clones (Fig. 2, lanes E), except clone D18. This clone was included as a control, since transcription of its corresponding RNA had been shown to be restricted to development by an *in vitro* transcription assay (22). The mobility of the band detected for each clone using early-labeled RNA (lanes E) is the same as the one of the RNA selected from the late-labeled RNA (lanes L) and is very similar to the mobility of the RNA detected by blot analysis of total poly(A)⁺ RNA from late in development (Fig. 1, lanes 5). This indicates that the poly(A)⁺ RNA labeled at the beginning of development represents a discrete species of RNA, with the same size as the RNA that is synthesized and accumulates late in development. This suggests that they are the same gene products.

Hybridization Competition Between Late Accumulated mRNA and Early Synthesized mRNA. The data presented in Fig. 2 suggest that the RNA species synthesized early in

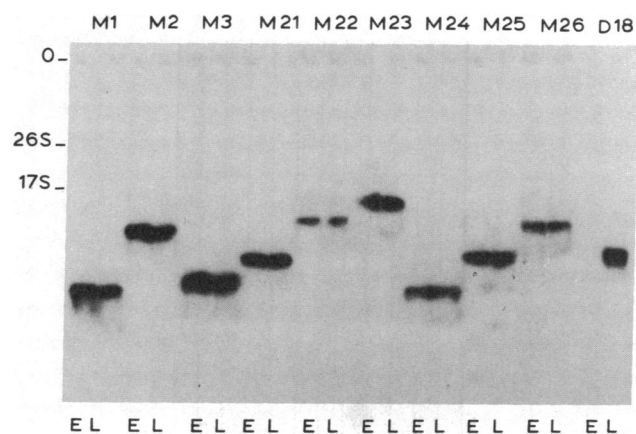


FIG. 2. RNA synthesis during early and late development. Cells (7×10^7) placed on filters in MES-PDF (phosphate-deficient Mes-buffered medium) (20) were incubated for 2 hr with 10 mCi of [³²P]P_i (specific activity 40 mCi/ μ g; 1 Ci = 37 GBq) added immediately after starvation or 10 hr later. The two preparations of cytoplasmic poly(A)⁺ RNA (1.2×10^7 and 1.4×10^7 cpm/ μ g, respectively) were hybridized separately for 2 days to 20 μ g of cloned DNAs immobilized on nitrocellulose paper, as described (20). The hybridized RNA was released from the paper by the procedure described in ref. 9. Aliquots of the early-labeled RNA (lanes E) and the late-labeled RNA (lanes L) released from each clone were electrophoresed in two adjoining lanes of a formaldehyde-containing gel under the same conditions used for the analysis shown in Fig. 1. Clone M3 corresponds to a constitutive RNA. D18 is a developmentally specific clone (22). The exposure time for autoradiography was 3 days.

development are the same sequences that accumulate in the cytoplasm after cellular aggregation. This interpretation is supported by hybridization competition analysis, an example of which is shown in Fig. 3. Here poly(A)⁺ cytoplasmic RNA was prepared from cells labeled with [³²P]P_i at the beginning of development (from 0 to 2 hr, rows a–c) or later (from 10 to 12 hr, rows d–f). RNA species of different size were separated by subjecting the two RNA preparations to electrophoresis and cutting each lane into 12 slices. The RNA recovered from each slice was hybridized to cloned DNA M3 (Fig. 3A), whose corresponding RNA is constitutively expressed (see Fig. 1), and to cloned DNA M5 (Fig. 3B), whose RNA is developmentally regulated. It appears that the early-labeled RNA that hybridized to a given DNA has roughly the same size as the corresponding late-labeled RNA (compare rows a and rows d), a result in agreement with the data shown in Fig. 2.

The hybridization of early- and late-labeled RNA was repeated in the presence of a 100-fold excess of nonradioactive poly(A)⁺ RNA derived from 2-hr or 12-hr developing cells. The mRNA from late developing cells competes with all labeled RNAs (Fig. 3A and B, rows c and f), but RNA from early developing cells competes only with the labeled RNA corresponding to the constitutive clone M3 (Fig. 3A, rows b and e) and does not prevent the hybridization of the early- or of the late-labeled RNA to the developmentally specific clone M5 (Fig. 3B, rows b and e). A similar result was obtained with the other constitutive clones and developmentally regulated clones listed in Fig. 1 (data not shown). Thus the early-synthesized RNA and the late-synthesized RNA compete for hybridization to the cognate gene, which constitutes definitive evidence that they are indeed the same sequences.

mRNA Stability in Early and Late Development. The data in Fig. 3 confirm that the mRNA sequences that are synthesized early in development and that hybridize to developmentally specific clones do not accumulate at a significant extent in the cytoplasm until cellular aggregation occurs. This finding

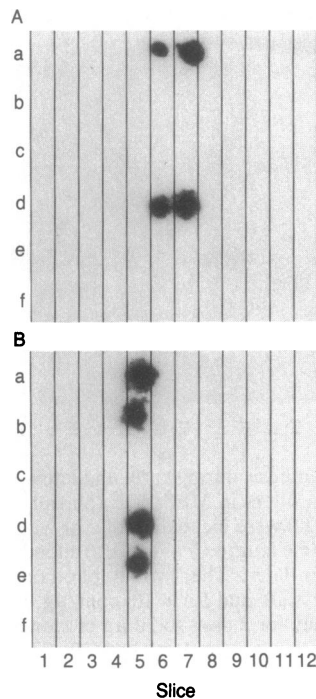


FIG. 3. Competition-hybridization analysis of poly(A)⁺ RNA synthesized early and late during development. Cytoplasmic poly(A)⁺ RNA was prepared from 7×10^7 cells incubated with 10 mCi of [³²P]P_i from 0 to 2 hr (rows a–c) and from 10 to 12 hr (rows d–f) of development. The two RNA preparations (1.3×10^7 and 1.5×10^7 cpm/ μ g, respectively) were electrophoresed in parallel lanes of a formaldehyde-containing gel. Each gel lane was cut into 10 0.6-cm slices. Each slice was squeezed into a bag containing 3 ml of hybridization buffer and a strip of nitrocellulose paper, corresponding to each vertical lane, with cloned DNA M3 (A) or M5 (B) spotted in 3 aliquots of 2 μ g each. The hybridization conditions were the same as in Fig. 2, but in rows b and e, 10 μ g of cytoplasmic poly(A)⁺ RNA extracted from cells at 2 hr of development were added to the hybridization mixture. In rows c and f, 10 μ g of poly(A)⁺ RNA derived from 12-hr developing cells were used as competitor. Exposure time for autoradiography was 48 hr. Most of 26S RNA was in slice 3 and most of 17S RNA was in slice 4.

suggests that the early-synthesized mRNAs might be highly unstable. The stability of the early- and late-labeled RNA was compared by following the incorporation of ³²P during a 4-hr continuous labeling, a procedure previously used to study the effect of cell disaggregation and of cAMP on mRNA stability (5, 6).

When RNA labeled between 10 and 14 hr of development was used (Figs. 4B and 5B), the labeled RNA hybridizing to each clone—including a DNA clone complementary to ribosomal RNA (pR1.5)—increased linearly for 4 hr after a lag of about 1.5 hr. This lag was presumably the time required for the phosphate pool to equilibrate with exogenous ³²P and for the processed RNAs to exit from the nuclei (23). Similar kinetics of uptake were observed for total poly(A)⁺ RNA (Fig. 5B). With RNA labeled from 0 to 4 hr (Figs. 4A and 5A), the amount of labeled RNA hybridizing to the ribosomal DNA clone and to the three constitutive clones M1, M2, and M3 increased linearly. However, the label hybridized to the developmental specific clones reached a plateau almost immediately after the lag period. The clear interpretation of this result is that late in development, all the mRNAs that were analyzed have a half-life of several hours or more (see ref. 5 for discussion), but that at the beginning of development, only the constitutive mRNAs are stable. The developmentally specific mRNAs have a half-life that cannot be measured precisely but that is almost certainly <30 min.

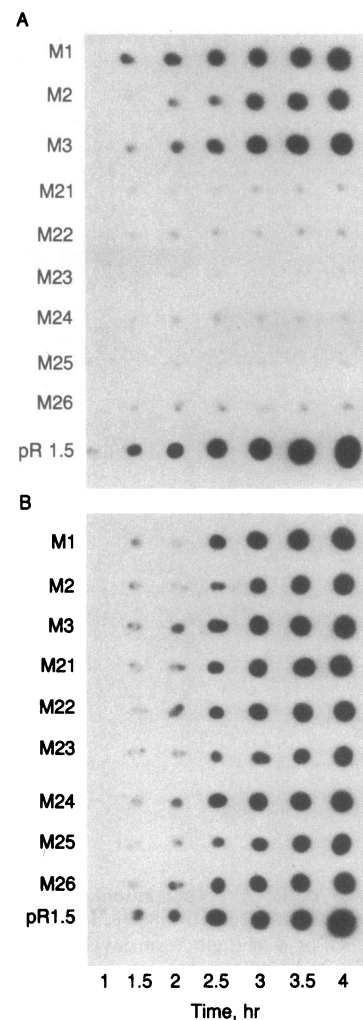


FIG. 4. Incorporation of ³²P into cytoplasmic polyadenylated RNA species during early and late development. Cells (5×10^7) were labeled with 10 mCi of [³²P]P_i, beginning immediately after starvation (A) or 10 hr later (B). At the indicated times, cytoplasmic poly(A)⁺ RNA was isolated and hybridized to the indicated DNAs. For the hybridization reaction, the procedure described in ref. 5 was used to normalize the amount of poly(A)⁺ RNA present in hybridization mixtures containing RNA labeled at different stages of development. Clone pR1.5 is a ribosomal DNA clone (see ref. 16), and it was hybridized to an aliquot (1%) of the poly(A)⁺ RNA preparations in the presence of 20 μ g of unlabeled poly(A)⁺ RNA extracted from vegetative cells, to reduce the intensity of the signal to a level comparable to the signals given by the other clones. Time of exposure for autoradiography was 48 hr.

DISCUSSION

The results reported here indicate that mRNA stabilization is a major mechanism of control of gene expression during development of *D. discoideum*.

We have shown that six mRNA species—corresponding to six randomly chosen clones, the basis of selection being that they each contain a single developmentally regulated gene—accumulate detectably in the cytoplasm only after the formation of tight cellular aggregates at the midpoint of the differentiation cycle. However, they are synthesized from the very beginning of development at a rate comparable to that measured in the postaggregation phase (Figs. 1–3). Because of the inefficient uptake of ³²P label from the growth medium, we were unable to determine whether these genes are also transcribed in growing cells.

Early in development the six mRNA species studied here are highly unstable, whereas in the cellular aggregate they

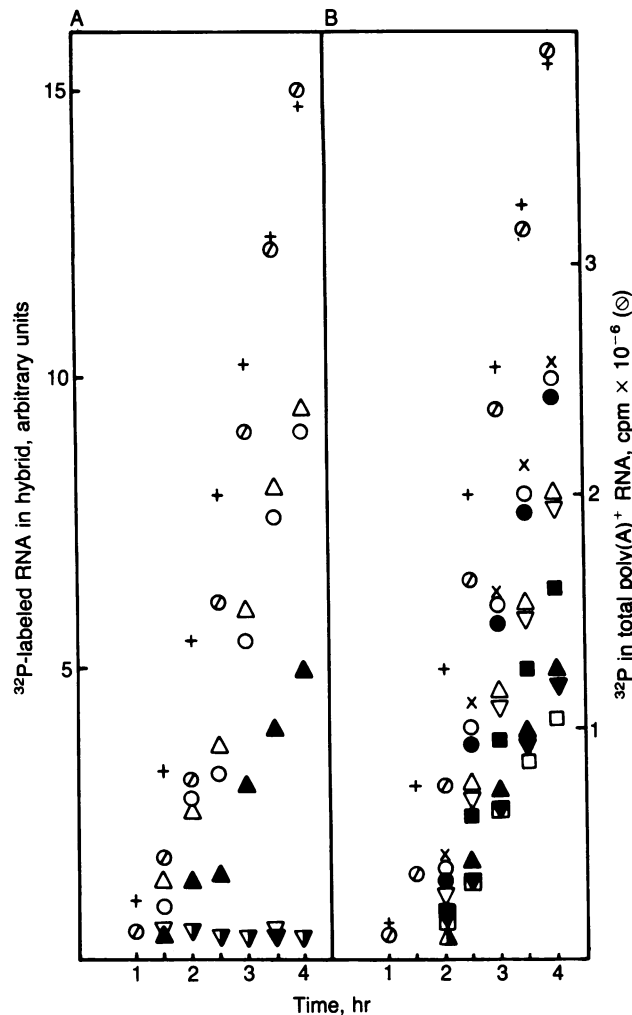


FIG. 5. Quantitation of the autoradiogram shown in Fig. 4. Different exposures of the radiograms were scanned in a Joyce-Loebl microdensitometer using a full-scale pen deflection of 1.16 optical density units, a value within the range of the film. The height of each peak is reported in arbitrary units. The symbols used for each clone are as follows: Δ , M1; \blacktriangle , M2; \circ , M3; \bullet , M21; ∇ , M22; \blacktriangledown , M23; \blacksquare , M24; \square , M25; \times , M26; $+$, pR1.5. *B* corresponds to Fig. 4*B*. In *A*, corresponding to Fig. 4*A*, it has not been possible to plot the data corresponding to all developmentally specific clones, because too many points were almost coincident. The data for clones M22 and M23 are shown as representative of the entire group. Also shown is the incorporation of ^{32}P into total cytoplasmic poly(A)⁺ RNA (\emptyset), measured by scintillation counting of an aliquot of the RNA preparations used in the experiment of Fig. 4, after precipitation with trichloroacetic acid.

seem to be as stable as the constitutive mRNAs (Figs. 4 and 5; refs. 5 and 6). Based on our data, the half-life of mRNA cannot be measured precisely; however, the minimal change in mRNA stability during development can be estimated. In the experiment described in Figs. 4 and 5, in early development no increase could be detected in ^{32}P incorporation into the six mRNAs after their appearance in the cytoplasm. Thus, in early development the half-life of the six mRNAs studied here is probably <30 min (i.e., less than the sampling interval time). In late development the amount of ^{32}P incorporated into the same mRNAs increased linearly for several hours; their half-life is therefore at least several hours.

We have not measured the rate of transcription of our clones by the *in vitro* "run-off" test applied to clone D18 (22). However, from the relative amounts of labeled RNA hybridized to the various clones in several experiments like the one reported in Fig. 4, we estimate that late in development the

rate of synthesis of all the developmentally specific mRNAs studied here (with the exception of clone D18) is at most 2-fold greater than in aggregated cells. Therefore, the increase in stability of these mRNAs must play the major role in controlling their accumulation during development.

Results qualitatively similar to those reported in Figs. 2 and 4 were obtained when total cytoplasmic RNA rather than poly(A)⁺ RNA was used. Thus, the observation that shortening of the poly(A)⁺ tail during development may affect the stability of mRNA molecules (24) does not affect the interpretation of the results reported here. This also holds true, for the same reason, for previous reports on the change in mRNA stability following dispersion of cellular aggregates (5, 6).

In preliminary experiments, we have extended our analysis to 15 other randomly chosen clones. All of them behave as the developmentally regulated clones described in Figs. 1, 2, and 4 of this paper. So far the only clone for which we could not detect early transcription is clone D18. This was not chosen randomly but was picked as a control to validate our experimental approach, as *in vitro* studies had already shown its expression to be regulated at the level of transcription (22). These data suggest that mRNA stabilization may determine the extent of accumulation of most developmentally regulated mRNAs.

The stability of mRNA is generally accepted as an important facet of the regulation of protein synthesis in eukaryotes (25). In mammalian cells most mRNAs have long half-lives (5–15 hr), but a substantial fraction are much less stable (26, 27). There are a few instances in which the stability of a particular mRNA or class of mRNAs is specifically affected by environmental or developmental stimuli. Certain hormones cause specific stabilization of mRNA species (28), and preferential stability is important in the accumulation of globin and myosin mRNAs during the terminal stages of erythropoiesis and myogenesis, respectively (29–32). The example reported here is perhaps the most extensive example of regulation of gene expression at the level of mRNA stability during development so far described.

However, our data do not establish whether the observed changes in mRNA stability represent a primary control mechanism or whether they are the secondary effect of control at the translational level in which mRNAs selectively excluded from polyribosomes are preferentially degraded. Evidence for translational control during development of *D. discoideum* has been reported by several authors (8, 24, 33). Further work is needed to discriminate between these two possibilities.

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