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Different mRNAs Have Different Nuclear Transit Times in *Dictyostelium discoideum* Aggregates

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Nuclear processing of mRNA precursors in differentiating multicellular *Dictyostelium discoideum* aggregates is markedly slower than in growing amoebae. Thus, we have been able to determine the time of nuclear processing of individual mRNA species in postaggregating cells by following the incorporation of $^{32}PO_4$ into nuclear and cytoplasmic RNA complementary to cloned cDNAs. Precursors of mRNAs synthesized during both growth and differentiation remain in the nucleus for about 25 to 60 min. By contrast, typical mRNAs which are synthesized only by postaggregative cells have nuclear processing times between 50 and 100 min. Depending on the particular mRNA, between 20 and 60% of nuclear transcripts are converted into cytoplasmic mRNA. A third class of mRNAs are transcribed from a set of repetitive DNA segments and are expressed predominantly during differentiation. Nuclear precursors of these mRNAs are extensively degraded within the nucleus or very rapidly after transport to the cytoplasm. Those sequences that are stable in the cytoplasm exit from the nucleus only after a lag of over 2 h. Thus, mRNAs encoded by different genes that are subject to different types of developmental controls display different times of transit to the cytoplasm and different efficiencies of nuclear processing. Differential nuclear processing may contribute to the regulation of the level of individual cytoplasmic mRNAs.

Synthesis of a protein can be regulated at many levels (9). During differentiation of *Dictyostelium discoideum* protein synthesis has been shown to be regulated at the levels of transcription, translation, and stability of the mRNA (22). We show here that the level of cytoplasmic mRNA in developing cells can also be regulated at the level of nuclear processing and transport to the cytoplasm.

Differentiation of growing *Dictyostelium* amoebae is induced by amino acid starvation; an initial response to starvation is a preferential inhibition of translation of certain mRNAs (2). During and just after formation of multicellular aggregates, about 2,000 to 3,000 new species of mRNA appear on the polyribosomes (5). Induction of most of these mRNAs is at the transcriptional level (20, 33). Many of these developmentally regulated RNAs are specifically labilized when the multicellular aggregates are disaggregated; the stability of many members of this class of mRNAs is specifically increased by high levels of extracellular cyclic AMP and possibly also by cell-cell contact (7, 24, 26).

In growing cells processing of mRNA and rRNA is rapid; only a few minutes are required for a newly synthesized mRNA or rRNA to be processed within the nucleus and exit into the cytoplasm (13, 23). There is no accumulation of nuclear precursor to rRNA, or of nuclear polyadenylated [poly(A)$^+$] RNA. One of the predominant alterations in nucleic acid metabolism after induction of differentiation is a markedly reduced rate of nuclear RNA processing (17). Several nuclear pre-rRNAs are accumulated (4), and nuclear poly(A)$^+$ RNA does not exit into the cytoplasm until 1 or 2 h after synthesis (14). Although we do not understand why the rate of processing, transport, or both, of nuclear pre-mRNAs in multicellular aggregates is so slow, this system is an appropriate one in which to determine the limiting step in nuclear processing for individual mRNAs. We show here that in multicellular aggregates 50 to 100 min is required for most species of developmentally regulated mRNAs to be transported to the cytoplasm, whereas only 25 to 60 min is required for processing of most species of "common mRNAs," i.e., those synthesized during both growth and differentiation.

One particular class of developmentally regulated mRNAs share sequences homologous to a set of repetitive DNA sequences (34). We show here that many of the poly(A)$^+$ nuclear RNAs

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which contain these transcripts are degraded within the nucleus or immediately after transport to the cytoplasm. Only a small fraction of these RNAs accumulate in the cytoplasm, and these exit the nucleus only after a lag of over 2 h.

The existence of multiple classes of mRNA with different nuclear processing times in the same cells suggests that the rate of nuclear processing is not simply a reflection of the energetic or metabolic state of the cell during differentiation. It is at least partially linked to some structural features of individual mRNA precursors which in turn may play a role in post-transcriptional control of gene expression.

MATERIALS AND METHODS

Isolation of ^{32}P-labeled RNA from nuclei and cytoplasm. *D. discoideum* AX3 cells were grown and harvested as previously described (5, 6). A sample of 10^8 cells was plated on each Millipore filter (4.2-cm diameter) resting on two filter pads saturated with morpholineethanesulfonic acid-PDF buffer (21). At the time of labeling, each filter was removed from the pads and deposited upon a 20-μl drop of water containing 3 mCi of carrier-free $^{32}P_i$. At the end of the labeling period, cells were removed from the filter by shaking in a 50-ml centrifuge tube containing 30 ml of PDF buffer and small pieces of ice. Cells were then collected by centrifugation and lysed by vigorous mixing in 0.5 ml of lysis buffer (6) on a Vortex mixer for 2 min. The lysate was then centrifuged in an Eppendorf model 5141 centrifuge for 3 s to remove unbroken cells. The supernatant was transferred to a second tube and centrifuged for 2 min to pellet nuclei. Nuclei were then lysed in 0.5 ml of HMK buffer (6) containing 0.3% sodium dodecyl sulfate.

Total RNA was phenol extracted from both nuclei and cytoplasm and separated into poly(A)$^+$ and non-polyadenylated [poly(A)$^-$] species by oligodeoxythymidylate chromatography as described previously (25).

Hybridization of ^{32}P-labeled RNA to cloned DNA. Isolation and characterization of cloned common and developmentally regulated DNAs was detailed previously (7, 20, 25, 26). Cloned DNAs were immobilized to a set of filters and hybridized to [^{32}P]RNA under conditions in which the ^{32}P radioactivity in the RNA-DNA hybrid is proportional to the amount of [^{32}P]mRNA species in the preparation (26). Different exposures of the radioautogram were scanned with a Joyce-Loebel microdensitometer with a peak exposure of less than 1 optical density unit, conditions within the linear range of the film and the instrument. The same film was used to scan all of the "spots" corresponding to a single clone; thus, the average area under the peaks (see Fig. 3) is a valid measure of the relative amount of [^{32}P]RNA corresponding to that DNA sequence.

Controls for separation of nucleus and cytoplasm. Because we employed a rapid procedure to separate nuclei from cytoplasm, it was essential to assess the degree of contamination of each fraction. Contamination of cytoplasm by nuclei was tested by labeling plated cells with $^{32}PO_4$ for 5 min. Cells were lysed and nuclei and cytoplasm were separated as described above. Poly(A)$^+$ RNA was isolated from each cellular fraction. In one trial, 98% of the labeled cellular poly(A)$^+$ RNA was found in nuclei and only 2% was found in cytoplasm. In another trial, 94% of labeled poly(A)$^+$ RNA was found in nuclei and 6% was found in cytoplasm. Since it is not known whether all labeled poly(A)$^+$ RNA after a 5-min pulse should be in the nuclei, 6% can be taken as an upper limit of contamination of cytoplasm by nuclear material.

To determine the degree of contamination of nuclei by cytoplasm, growing cells were labeled for 15 h with [^{3}H]uracil. Total RNA was then extracted from both nuclei and cytoplasm and hybridized to filter-bound DNA derived from clone SC79, which specifies a very abundant mRNA in growing cells (7). Twenty-fold more labeled cytoplasmic RNA hybridized than nuclear RNA. Thus 5% can be considered an upper limit of contamination of nuclei by cytoplasmic material.

RESULTS

Incorporation of ^{32}P into nuclear and cytoplasmic RNA species. To follow the accumulation of ^{32}P radioactivity in individual species of poly(A)$^+$ and poly(A)$^-$ RNA in both nuclei and cytoplasm, cells were labeled with $^{32}PO_4$ from 14 to 18 h of development, a time when both common and aggregation-stage mRNAs are synthesized (20). Poly(A)$^+$ and poly(A)$^-$ nuclear and cytoplasmic RNAs were isolated from cells labeled for different times and were hybridized to filter-bound cloned DNAs representing either common or developmentally regulated transcripts. Hybridization was performed under conditions in which the amount of label bound to the filter is proportional to the amount of the labeled RNA species in the preparation (26).

No labeled poly(A)$^-$ RNA, either nuclear or cytoplasmic, hybridized to any of the clones tested, regardless of the time of ^{32}P labeling (Fig. 1, 2). This indicates that nuclear pre-mRNAs are polyadenylated within 45 min of synthesis, since this was the earliest time point that was studied.

After a 20- to 30-min lag period, the amount of ^{32}P radioactivity in nuclear poly(A)$^+$ RNA complementary to each clone, in most cases, increased at a linear rate (Fig. 1 and 3). The lag period is presumably due, at least in part, to the time required for the pool of precursor nucleotides to become fully labeled. Clones SC79, SC29, CZ5, CZ12, and CZ22 encode single mRNAs which are transcribed and accumulate in growing cells as well as throughout differentiation (7, 20, 25, 26). The amount of ^{32}P radioactivity in nuclear poly(A)$^+$ RNA complementary to these clones reached a maximum value at 90 min after the start of labeling. This amount remained constant or declined slightly during the next 135 min. ^{32}P radioactivity appeared in cytoplasmic RNA complementary to these clones after a lag of 50 to 70 min. Thereafter, the

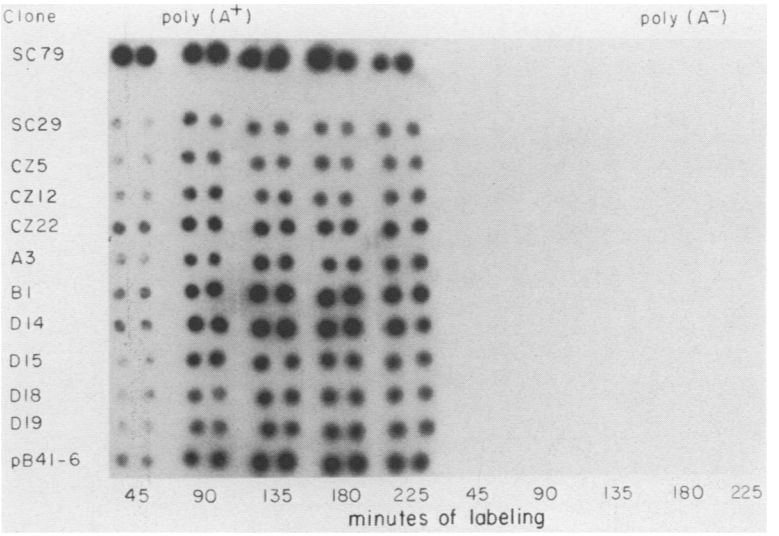

FIG. 1. Uptake of ^{32}P into nuclear poly(A)$^+$ and poly(A)$^-$ RNA species during a continuous labeling between 14 and 18 h of development, Poly(A)$^+$ and poly(A)$^-$ RNA were labeled and isolated, and then each RNA was hybridized to a filter containing duplicate spots of each of the 12 cloned DNAs studied; each spot contained 2 μg of DNA. Each hybridization reaction contained the fractionated RNA from 10^8 cells. For the nuclear poly(A)$^+$ RNA, the amount (cpm) of ^{32}P radioactivity used was as follows for the respective time points: 45 min, 160×10^3; 90 min, 480×10^3; 135 min, 820×10^3; 180 min, $1,060 \times 10^3$; 225 min, $1,100 \times 10^3$. Nuclear poly(A)$^-$ RNAs were 800×10^3, $2,400 \times 10^3$, $4,100 \times 10^3$, $5,100 \times 10^3$ and $4,800 \times 10^3$, respectively. Hybridizations were carried out at 37°C for 96 h. The filters were washed and treated with RNase as described in reference 26. Exposure was for 3 days at −70°C with an intensifying screen.

amount of radioactivity increased at an approximately linear rate for at least 2 h (Fig. 2 and 3). For each of these clones, the rate of accumulation of ^{32}P radioactivity in cytoplasmic RNA achieved linearity at the approximate time when the amount of nuclear radioactivity had reached a steady state. Based on the time elapsing between the incorporation of ^{32}P label into nuclear RNA and its appearance in the cytoplasm, we conclude that, after aggregation, different common mRNA molecules spend between 20 to 30 min (CZ5, CZ22) and 50 min (SC29) within the nucleus before export into the cytoplasm. Nuclear precursors to SC79 and SC29 mRNAs appear to be converted into cytoplasmic mRNAs efficiently, since the curve describing the rate of accumulation of ^{32}P in cytoplasmic mRNA parallels that for the nuclear pre-mRNA. By contrast, only 20 to 30% of the precursors to common mRNAs CZ5 or CZ12 appear to be processed into mRNA.

Nuclear processing of aggregation-stage mRNAs in the same cells is much slower although there is considerable variability in the processing and transport times for different species. Clones A3, B1, D14, D15, D18, and D19 all encode single mRNA species which are not transcribed in growing cells (20, 26). Transcription and mRNA accumulation begins between 4 and 8 h (clones D15, D18) or between 8 and 12 h (all of the others; reference 20). After a lag period of 25 to 30 min, accumulation of ^{32}P radioactivity into nuclear poly(A)$^+$ RNAs complementary to these clones increased at a linear rate until 135 min and remained roughly constant thereafter (Fig. 3). There was a lag of 100 to 120 min for appearance of radioactivity in cytoplasmic RNA complementary to these clones; thereafter radioactivity accumulated at a linear rate for at least 90 min. We conclude that nuclear precursors for these RNAs spend an average of 50 min (D15) to 85 min (D14) within the nucleus before export into the cytoplasm, a processing time significantly longer than for the common RNAs transcribed in the same cells. The graphs in Fig. 3 are consistent with the notion that there is no extensive nuclear degradation of these sequences and that about 50% of the nuclear transcripts are conserved as mRNAs.

Clone pB41-6 contains DNA sequences which are repeated 100 to 300 times in the *Dictyostelium* genome. It hybridizes to a large number of mRNA species, and the abundance of this class of mRNAs increases 20- to 50-fold during the first 12 h of differentiation (34). In postaggregation cells, incorporation of ^{32}P into nuclear

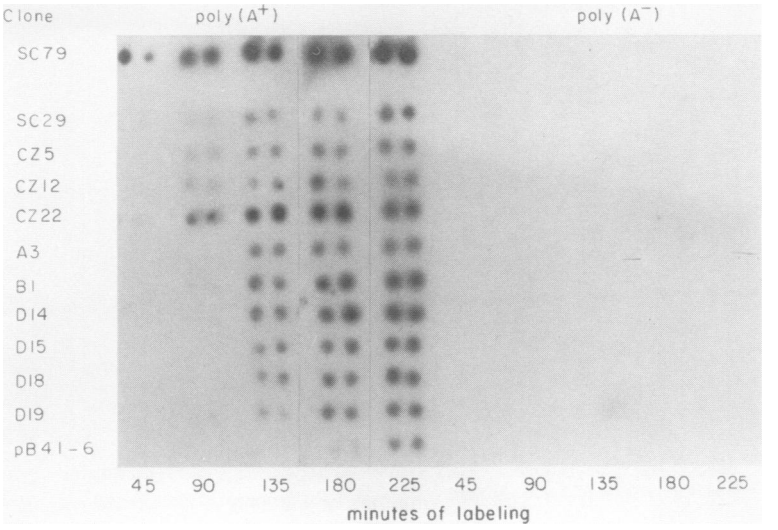

FIG. 2. Uptake of ^{32}P into cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNA species during a continuous labeling between 14 and 18 h of development. Poly(A)$^+$ RNA and poly(A)$^-$ RNA were labeled, isolated, and hybridized as described in the legend to Fig. 1. For the cytoplasmic poly(A)$^+$ RNAs, the following amounts (cpm) of [^{32}P]RNA were used: 45 min, 30×10^3; 90 min, 210×10^3; 135 min, 425×10^3; 180 min, 630×10^3; 225 min, 990×10^3. Cytoplasmic poly(A)$^-$ RNAs were 400×10^3, $1,400 \times 10^3$, $2,460 \times 10^3$, $3,600 \times 10^3$, and $5,200 \times 10^3$, respectively.

poly(A)$^+$ RNA complementary to pB41-6 reached a maximum value by 90 min (Fig. 3). However, accumulation of ^{32}P radioactivity in cytoplasmic RNA complementary to this clone began only after a lag period of at least 135 min. The rate of accumulation of pB41-6 sequences in the cytoplasm was at least fourfold lower than the rate of incorporation into nuclear RNA. These results indicate that a significant fraction, probably greater than 75%, of RNA transcripts complementary to pB41-6 are turned over within the nucleus or immediately after export into the cytoplasm. In addition, export to the cytoplasm of the stable pB41-6–related species is much slower than for other mRNA species in the same cells.

DISCUSSION

Processing of mRNA precursors in *Dictyostelium* and other eucaryotic microorganisms is less complex than in mammalian cells and appears to involve fewer processing reactions. Many *Dictyostelium* genes, such as actin and discoidin, lack introns (19, 27, 29). Only one known *Dictyostelium* gene contains introns; the two introns are small (~100 base pairs) and within the protein-coding region (18). In vertebrates, most protein-coding genes contain multiple introns; the size of each can range up to several thousand bases (1, 9). The average size of nuclear *Dictyostelium* pre-mRNA is only 20% larger than that of cytoplasmic mRNA, and in growing cells over 50% of the sequences in nuclear poly(A)$^+$ RNA are exported to the cytoplasm as mRNA (13, 14). The present studies (Fig. 3) indicate that, in the postaggregation cells, nuclear mRNA precursors are converted to mRNAs with varying efficiencies. Precursors to common mRNAs such as SC29 and SC79 and regulated mRNAs such as B1, D15, D18, and D19 are converted to mRNAs with efficiencies greater than 60 to 70% (Fig. 3). In contrast, only about 20% of the precursors to CZ5 and CZ22, both common mRNAs, are converted to mRNA, and precursors to pB41-6–related mRNAs are processed even less efficiently. Because of the relatively few time points we were able to take, it is not possible to compute the exact fraction of each pre-mRNA sequence which appears in the cytoplasm.

Taking into consideration all of the mRNAs we have studied, it appears that processing of *Dictyostelium* nuclear pre-mRNA is a more conservative process than that in vertebrate cells, where typically less than 10% of the sequences transcribed in heterogeneous nuclear RNA appear as mRNA (8). This difference could be explained, at least in part, by the differences in numbers and sizes of intervening sequences in the two types of cells.

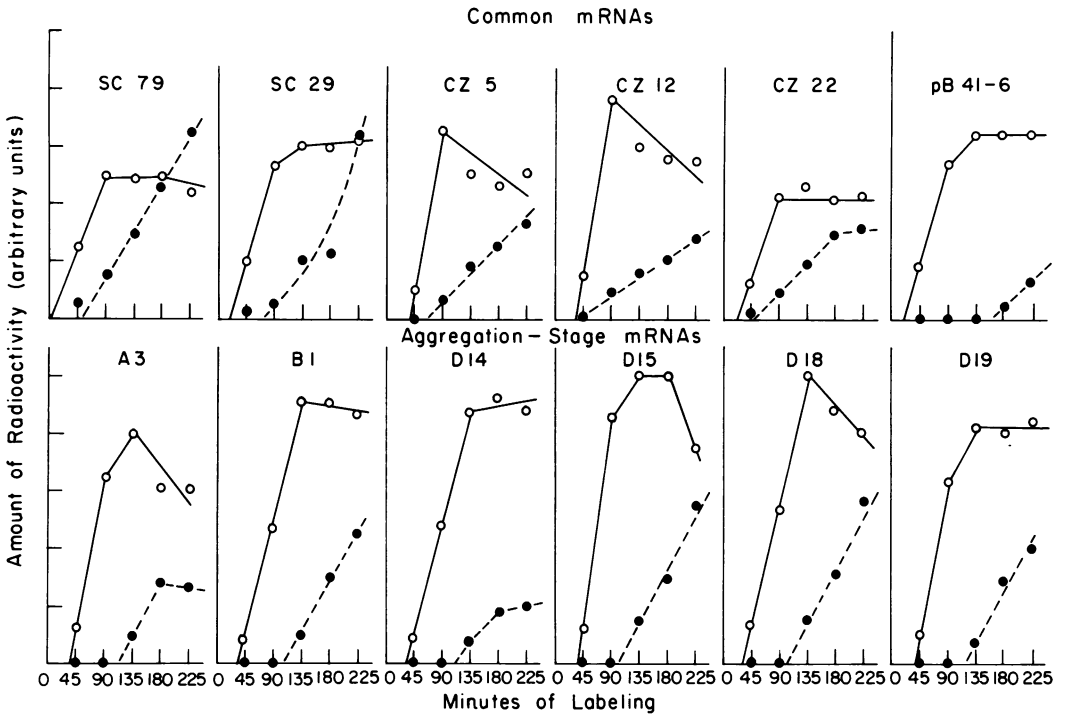

FIG. 3. Quantitation of the autoradiographs shown in Fig. 1 and 2. Different exposures of the radiograms were scanned in a Joyce-Loebel microdensitometer. All scanned intensities had absorbances in the linear range of detection (<1.16 optical density units). Duplicate spots of each cloned DNA were scanned, and the peak heights were averaged. The height of each peak is reported in arbitrary units. Open and closed circles represent nuclear and cytoplasmic poly(A)$^+$ RNAs, respectively.

Dictyostelium mRNA contains many of the post-translational modifications found in vertebrate mRNA. Essentially all *Dictyostelium* mRNAs contain 100 to 150 adenylic acid residues at the 3′ end, as do mammalian mRNAs (12). In mammalian cellular mRNA the 5′-terminal sequences have the formula $m^7G(5')ppp(5')XmpY(m)p\ldots$, where X and Y can be any of the four nucleotides, and Y, as well as X, can contain a 2′-*O*-methylated ribose (8). Only four 5′ sequences are found in *Dictyostelium* mRNA: $m^7GpppAp$ (65%), $m^7GpppGp$ (10%), $m^7GpppAmpAp$ (10%), and $m^7GpppAmpUp$ (10%). Thus, most *Dictyostelium* mRNAs lack 2′-*O*-methylated nucleotides. Additionally, *Dictyostelium* mRNAs lack internal 6-methyladenosine residues found in mammalian cellular mRNA (10).

In mammalian cells, capping and polyadenylation of pre-mRNAs occurs during or just after RNA transcription (3, 15, 16, 28, 30, 32). Splicing appears to be a slower process. The order and timing of these post-transcriptional modifications in *Dictyostelium* is not known, although the present study (Fig. 1 and 2) shows that in postaggregating cells nuclear pre-mRNAs are polyadenylated during or within a few minutes after synthesis.

In growing *Dictyostelium*, a typical mRNA exits the nucleus within 4 min after synthesis, a nuclear processing time much shorter than in mammalian cells (23, 26). We do not know why the overall rate of processing and transport of nuclear pre-mRNA is dramatically slowed during differentiation. Nor do we know what step(s) becomes rate limiting. This work indicates, however, that polyadenylation is not rate limiting; furthermore, polyadenylation per se is not sufficient for export of RNA into the cytoplasm.

In postaggregation cells different developmentally regulated mRNAs exit the nucleus after lags of 50 to 85 min. Processing of all common (or nonregulated) mRNAs by the same cells is somewhat quicker; different common mRNAs exit the nucleus 20 to 60 min after synthesis (Fig. 3). However, it should be noted that some of the ^{32}P radioactivity in common RNAs could be incorporated by the few single cells that have not entered multicellular aggregates. The different processing times and efficiencies of different mRNAs may be an important factor in regulating the level of individual cytoplasmic mRNAs. In

particular, our data suggest that the longer processing times of most species of regulated mRNAs might be due to different sets of processing enzymes that modify different classes of coregulated mRNAs.

One mechanism we can envision involves the binding of one species of protein to precursors of certain regulated mRNAs while a different species is bound to many precursors of nonregulated mRNAs. Such a protein could regulate or modulate the rate of any nuclear processing step, be it splicing, capping, or export into the cytoplasm. The existence of such a set of proteins would imply a common recognition signal on all members of each class of mRNAs. In principle this signal could be a common primary, secondary, or tertiary structure in the RNAs; however, we note that none of the common or regulated cDNAs studied here cross-hybridized with each other.

Transcripts complementary to clone pB41-6 are processed into stable mRNA species more slowly and less completely than those of other genes we have studied (Fig. 3). pB41-6 is a 2.6-kilobase segment of genomic DNA which contains sequences repeated 100 to 300 times in the genome (34). A 4.5-kilobase sequence, repeated about 30 times in the genome, contains copies of pB41-6 sequences; this 4.5-kilobase fragment has many of the properties of a transposable element (S. Chung, C. Zuker, and H. F. Lodish, Nucleic Acids Res., in press). Only one strand of pB41-6 is expressed in RNA. All cytoplasmic RNAs which contain pB41-6 sequences appear to be mRNAs, as they are specifically associated with polysomes. The coding strand of pB41-6 contains three open reading frames, of 621, 705, and 789 bases (C. Zuker, J. Cappello, R. L. Chisholm, and H. F. Lodish, submitted for publication). We do not know, however, whether the multiplicity of transcripts that contain pB41-6–related sequences is due to multiple transcripts from one genomic sequence or to transcripts from different genomic segments that contain these repetitive sequences. It is clear, however, that most cytoplasmic RNAs complementary to pB41-6 carry only pB41-6–related sequences and are not associated with single-copy sequences. The nature of the nuclear transcripts which contain pB41-6 sequences, most of which are degraded within the nucleus, is not known. However, accumulation of nuclear and cytoplasmic pB41-6 transcripts increases in parallel during differentiation (C. Zuker, R. L. Chisholm, G. Mangiarotti, and H. F. Lodish, manuscript in preparation).

The *Drosophila* reiterated *copia* sequences have many properties in common with *Dictyostelium* pB41-6. A large fraction of *copia* transcripts accumulate within the nucleus (11, 31). No more than 10% of pulse-labeled *copia* RNA can be chased into the cytoplasm; the remainder is degraded within the nucleus with a half-life of less than 30 min (11). By contrast, cytoplasmic poly(A)$^+$ *copia* RNAs are stable, with a half-life of 7 to 10 h. The reasons why *copia* nuclear poly(A)$^+$ transcripts are processed or exported with an efficiency of 6 to 13% (11) are not known.

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