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Utilization of cytoplasmic poly(A)$^+$RNA for protein synthesis in preimplantation mouse embryos

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SUMMARY

The distribution of cytoplasmic poly(A)$^+$RNA between subribosomal (<80S) and ribosomal/polysomal (≥80S) ribonucleoprotein particles has been investigated in mouse morulae and blastocysts. After labelling for 24 h with [5,6-^{3}H]uridine, late morulae (96 h post-hCG), early blastocysts (100 h post-hCG), or late blastocysts (120 h post-hCG) were homogenized in detergent buffer, and 11 000 *g* supernatants were prepared and centrifuged through 15–40 % sucrose gradients. Poly(A)$^+$RNA was isolated from the appropriate gradient fractions by affinity chromatography. In late morulae beginning to undergo cavitation, poly(A)$^+$RNA was evenly distributed between the two types of RNP particles whereas it was almost entirely in the ribosomal/polysomal fraction in early and late blastocysts. The sedimentation profile (in 5–20 % sucrose gradients) of poly(A)$^+$RNA from the ribosomal/polysomal fraction of late morulae and blastocysts was the same, with a range of 4S to greater than 28S and a modal peak slightly smaller than 18S. Roughly 75 % of this RNA was judged to be functional mRNA based on the EDTA sensitivity of the RNP particles containing it. Poly(A)$^+$RNA from the subribosomal fraction of late morulae displayed a similar range of sedimentation values, but was enriched for a component sedimenting at 6–7S. These results demonstrate that the subcellular distribution of poly(A)$^+$RNA shifts as cavitation begins, with the decline of the non-translating, subribosomal fraction. Although most of this fraction in late morulae is probably too small to constitute translatable mRNA, the remainder may represent a reserve available for recruitment into polyribosomes to support blastocyst expansion. Since little non-translating mRNA exists in blastocysts, the increasing rate of protein synthesis during blastocyst expansion must be driven by mRNA accumulation and/or stabilization.

INTRODUCTION

Preimplantation development in the mouse comprises a series of morphogenetic events several of which have been shown to require the transcription of embryonic genes. Inhibitors such as actinomycin D and α-amanitin have been employed to demonstrate the transcriptional dependency of the cleavage divisions as well as cavitation, hatching, and attachment to the substratum (reviewed by Sherman, 1979; Magnuson & Epstein, 1981). One of the most interesting findings to have come from such experiments is that hatching and attachment can still occur on schedule in late blastocysts treated from the early blastocyst stage with α-amanitin

Key words: mRNA utilization, mouse embryos, subribosomal RNP.

(Schindler & Sherman, 1981; Kidder & McLachlin, 1985), implying that the mRNAs needed for these processes are synthesized long in advance and that either those mRNAs or their translation products (or both) are stable. The half-lives of a variety of individual polypeptides in the early blastocyst have been measured, and were found to vary widely: some polypeptides have half-lives on the order of one or a few hours, others more than 30 h (Brinster, Brunner, Joseph & Levey, 1979). There are also long-lived mRNAs in early blastocysts, the overall or mean cytoplasmic poly(A)$^+$RNA half-life increasing from about 10 h in morulae to almost 20 h (Kidder & Pedersen, 1982). Thus it is possible that post-transcriptional regulatory mechanisms such as mRNA stabilization govern the production of short-lived proteins required for late blastocyst functions utilizing mRNAs synthesized in the early blastocyst stage.

In the present study, we have extended our examination of post-transcriptional regulation in blastocysts by focussing on mRNA utilization. We have examined the distribution of poly(A)-containing RNA between non-translating (<80S) and putative translating (≥80S) ribonucleoprotein (RNP) particles in embryos undergoing cavitation and blastocyst expansion. We find that such regulation does occur, since the subribosomal fraction of newly synthesized poly(A)$^+$RNA, which constitutes about 50 % of total cytoplasmic poly(A)$^+$RNA in late morulae, is severely reduced in blastocysts. However, most of the subribosomal poly(A)$^+$RNA molecules in late morulae are too short to represent the precursors of mRNAs being translated in blastocysts.

MATERIALS AND METHODS

Collection and labelling of embryos

Ha(ICR) female mice obtained from West Seneca Laboratories, Inc., Roswell Park Memorial Institute (Buffalo, New York) were superovulated with 5 i.u. pregnant mare's serum gonadotropin followed 48 h later by 5 i.u. human chorionic gonadotropin (both hormone preparations were supplied by Sigma Chemical Co., St Louis, Missouri). The females were mated with $CB6F_1/J$ males from the Jackson Laboratories (Bar Harbor, Maine). 8-cell embryos were flushed from oviducts and uteri on the morning of day 3, and early blastocysts from uteri on the morning of day 4, using flushing medium one (Spindle, 1980). Day-3 embryos were pooled in modified standard egg culture medium (Spindle, 1980) whereas day-4 embryos were pooled in modified Eagle's basal medium (BME+AA of Spindle, 1980) supplemented with 5 % foetal bovine serum and 5 % newborn bovine serum. The same two media were used for labelling the embryos with [5,6-^{3}H]uridine (38–40 Ci mmol^{-1}; New England Nuclear, Boston, Massachusetts): day-3 embryos were cultured from 72 to 96 h or 76 to 100 h post-hCG in a precursor concentration of 5–15 μM and day-4 embryos from 96–120 h post-hCG in a concentration of 2–3 μM. By the end of the labelling periods, the day-3 embryos had become cavitating morulae or early blastocysts and the day-4 embryos had become expanded, hatching blastocysts (arrested or retarded embryos were discarded). For labelling of embryo DNA, day-3 embryos were cultured overnight to the early blastocyst stage, then labelled for 2 h with 1 μM-[6-^{3}H]thymidine (16 Ci mmol^{-1}; New England Nuclear). The embryos were cultured in an incubator which maintained a temperature of 36·5–37·5 °C and a humidifed atmosphere of 5 % CO_2 in air. After the labelling period, some of the embryo batches were treated briefly with a pronase solution (1 %) to remove zonae (this step was eventually found to be unnecessary), then washed through five drops of cold phosphate-buffered saline containing 0·3 % polyvinylpyrrolidone and 10 mM-phenylmethylsulfonylfluoride.

Embryo lysis and preparation of 11 000 g supernatants

Embryo lysis was accomplished using a combination of gentle homogenization and detergent treatment. Washed embryos were homogenized at 0° in 400 μl lysis solution using the 'A' pestle of a Dounce homogenizer. Two different lysis solutions were used in the experiments reported here: one consisted of TAM buffer [20 mM-Tris-HCl pH 7·5, 100 mM-NH_4Cl, 5 mM-$Mg(CH_3COO)_2$, 5 mM-dithiothreitol (DTT)] containing 1 % Nonidet P-40; the other consisted of TSM/EGTA buffer [40 mM-Tris-HCl pH 7·5, 150 mM-NaCl, 20 mM-$Mg(CH_3COO)_2$, 10 mM-[ethylenebis-(oxyethylenenitrilo)] tetracetic acid (EGTA), 5 mM-DTT] containing 1 % NP-40 and 0·5 % deoxycholic acid (DOC, sodium salt). Both lysis solutions also contained 10 μg ml^{-1} cycloheximide and either placental ribonuclease inhibitor (Sigma) or RNasin (Biotec, Madison, Wisconsin) at a concentration of 100 units/ml. The lysates were transferred to Eppendorf micro test tubes (1·5 ml) and centrifuged for 10 min in a Sorvall HB-4 rotor at 4 °C and 12 000 r.p.m. (11 000 *g*). The supernatants were carefully removed and either loaded immediately onto sucrose gradients or stored at −70 °C for analysis at a later time.

Fractionation of 11 000 g supernatants on sucrose gradients

Supernatants from 100 to 1000 embryos were layered on 15–40 % gradients of sucrose in TAM buffer; each gradient tube contained a 0·5 ml cushion of 2 M-sucrose at the bottom. The gradients were spun at 40 000 r.p.m., 4 °C for 120 min in a Beckman SW-40 rotor. Polyribosome preparations from mouse liver or from cultured *Xenopus* kidney cells were included in the same gradients to provide optical density markers. The marker preparations also provided an internal control for monitoring the stability of RNP: neither long-term storage at −70 °C nor co-homogenization and sedimentation with embryo lysates had any effect on the size distribution of marker polyribosomes.

After centrifugation, gradients were fractionated with continuous monitoring of optical density using an ISCO density gradient fractionator. A sample, consisting of 1/20 of each gradient fraction, was taken for precipitation with trichloroacetic acid (TCA) and measurement of radioactivity by scintillation counting. The fractions corresponding to ribonucleoprotein (RNP) sedimenting at less than 80S were then pooled, as were those corresponding to RNP sedimenting at or greater than 80S (see Fig. 1), using the position of the 80S marker ribosome peak as a guide. The pooled fractions were made 0·3 M with NaCl and precipitated with two volumes of 95 % ethanol together with 200 μg yeast RNA (Sigma) as carrier.

Assay of ribonucleoprotein preparations for poly(A)$^+$RNA

The amount of radiolabelled poly(A)$^+$RNA in subribosomal and ribosomal/polysomal preparations was determined by affinity chromatography on oligo(dT)cellulose (Type 3, Collaborative Research Inc., Lexington, Massachusetts). Ethanol precipitates were dissolved in 0·5 ml of binding buffer [10 mM-Tris-HCl, pH 7·5, 500 mM-NaCl, 2 mM-disodium ethylenediamine tetraacetate (EDTA)] containing 2 % sodium dodecyl sulphate (SDS). Affinity chromatography was then carried out as described previously (Kidder & Pedersen, 1982). The RNA that bound to and was eluted from the column was precipitated with TCA (100–200 μg yeast RNA was added as carrier) and the precipitates were collected on glass fibre filters (Whatman GF/F). After thorough drying, the filters were either counted directly in Omnifluor (New England Nuclear) or eluted with 0·01 N-KOH and counted in Ready-Solv HP/b (Beckman). In the former case, the c.p.m. data were corrected for counting efficiency (57 %) and for β-absorption by the filter (30 %, based on a test series of TCA precipitates prepared according to the same protocol) to arrive at an estimate of d.p.m. Data from TCA precipitates eluted from the filters were corrected for a counting efficiency of 46–48 %. The two methods of estimating d.p.m. were in excellent agreement.

Analysis of size distributions of poly(A)$^+$RNA

Material bound to and eluted from the oligo(dT)-cellulose column was precipitated with ethanol, then dissolved in gradient buffer (10 mM-sodium acetate, pH 5·1, 1 mM-EDTA, 0·1 % SDS). The samples were heated briefly to 65 °C, cooled, then separated, along with *Xenopus*

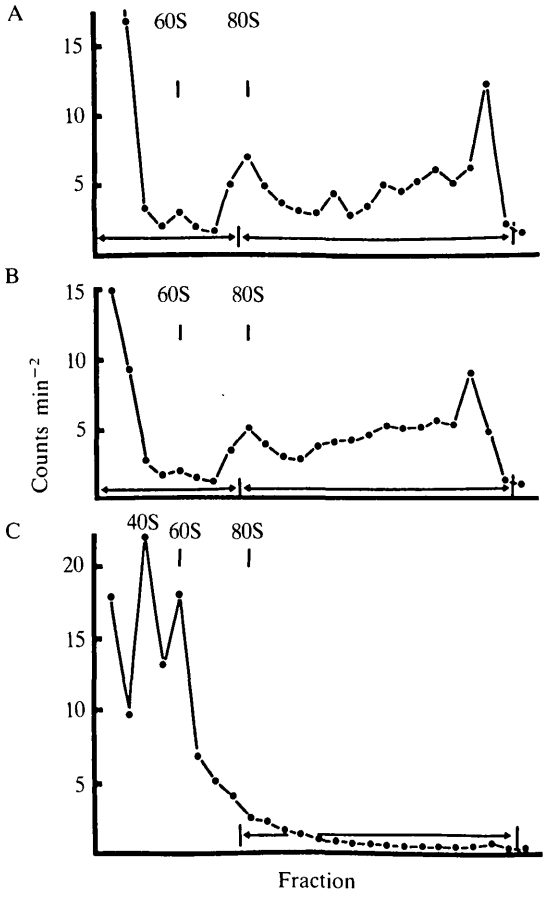

Fig. 1. Distribution of ribonucleoprotein after sedimentation through 15–40 % sucrose gradients. Arrows indicate fractions pooled for isolation of poly(A)$^+$RNA. Mouse liver ribosome preparations provided optical density markers.

(A) Approximately 900 late morulae (92 h post-hCG) had been labelled for 24 h with [5,6-^{3}H]uridine.

(B) Approximately 500 hatching blastocysts (122 h post-hCG) had been labelled for 26 h; one-half of the supernatant was layered on the gradient.

(C) Distribution after treatment of the other half of the supernatant (part B) with EDTA.

kidney cell or mouse liver RNA markers, on 5–20 % sucrose gradients in the same buffer (centrifugation was carried out at 4°C, 40000 r.p.m. for 8·5 h in a Beckman SW-41 rotor). Fractions were precipitated with TCA and collected on GF/F filters.

RESULTS

Fractionation of cytoplasmic ribonucleoprotein

A previous study of the time course of [^{3}H]uridine incorporation into mRNA of early blastocysts demonstrated that label accumulates for 20 h or more (Kidder & Pedersen, 1982). In order to maximize the amount of label in the poly(A)$^+$RNA

fraction and allow the distribution of label among RNP classes to approach the steady-state condition, embryos were cultured in the presence of radioactive precursor for 24 h. Virtually all (99 %) of the incorporated uridine in the 11 000 *g* supernatants was solubilized by a 90 min treatment with 0·3 N-KOH at 37 °C.

Our identification of RNP as containing either non-translating (subribosomal) or potentially translating (ribosomal/polysomal) mRNA is based solely on the sedimentation rate of cytoplasmic poly(A)$^+$RNA in sucrose gradients (Fig. 1). We verified that the 11 000 *g* supernatants were not likely to be contaminated with nuclear RNP by labelling blastocysts for 2 h with [^{3}H]thymidine; the embryos were then homogenized in one of the two lysis solutions. Only 3–6 % of the total incorporated label was released into the supernatant. As a test of the authenticity of poly(A)$^+$RNA in the ribosomal/polysomal region of the gradients as functional mRNA, its sensitivity to EDTA treatment was determined (Penman, Vesco & Penman, 1968). Before layering on the gradient, the 11 000 *g* supernatant was divided into two portions: one was diluted with an equal volume of TSM/EGTA buffer, the other with the same buffer containing 100 mM-EDTA. The supenatants were allowed to stand at room temperature for 15 min before centrifugation, and the EDTA-treated supernatant was separated on a gradient containing 10 mM-EDTA. This treatment caused extensive disruption of ribosomes and polyribosomes (Fig. 1C), and reduced the amount of poly(A)$^+$RNA in the ribosomal/polysomal region to a fraction (mean = 0·25 ± 0·13, n = 6) of that in the same region of control gradients. Thus most (average 75 %) of the labelled poly(A)$^+$RNA in RNP sedimenting at or greater than 80S can be considered to be mRNA in translation.

Distribution of poly(A)$^+$RNA in late morulae and blastocysts

Measurements of the label in poly(A)$^+$RNA from subribosomal and ribosomal/polysomal regions of sucrose gradients are given in Table 1; the gradients were divided as illustrated in Fig. 1. Although the two lysis solutions differed in their efficiency of solubilization of RNP into the 11 000 *g* supernatant, both procedures provided data indicating an even distribution of poly(A)$^+$RNA between the ribosomal and ribosomal/polyribosomal RNP components from late morulae. More rigorous homogenization methods, designed to solubilize a greater proportion of incorporated label into the supernatant, resulted in the leakage of nuclear contents into the supernatant or partial disruption of polyribosomes. In Table 1, the proportion of incorporated label in the supernatants is slightly underestimated, since the supernatants were not drawn off quantitatively.

In contrast to the data from late morulae, those from early and late blastocysts indicate almost complete utilization of poly(A)$^+$RNA for translation. In several late blastocyst batches, in fact, incorporation into poly(A)$^+$RNA of the subribosomal RNP component was virtually undetectable. This result provides additional assurance that the 11 000 *g* supernatants are not contaminated with nuclear RNP.

Size distribution of poly(A)$^+$RNA

As a first step in characterizing the subribosomal poly(A)$^+$RNA of late morulae, this RNA was subjected to centrifugation through 5–20% sucrose gradients. Poly(A)$^+$RNA from the ribosomal/polysomal RNP component of both late morulae and late blastocysts was analysed similarly for comparison. The size distributions are shown in Fig. 2. The ribosomal/polysomal fraction of poly(A)$^+$RNA from the two stages is similar, spanning the range from 4S to greater than 28S with a modal peak slightly smaller than 18S. The size distribution of the late morula subribosomal fraction is rather different, being enriched in a component sedimenting at 6–7S but displaying the same overall size range. It is

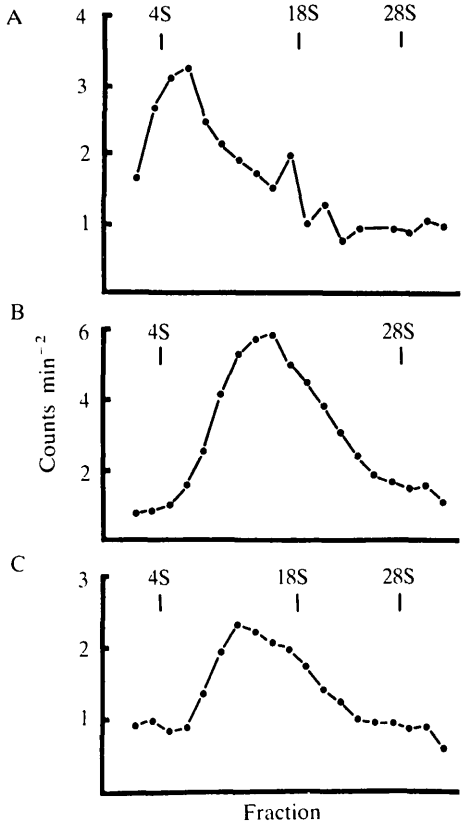

Fig. 2. Distribution of poly(A)$^+$RNA after sedimentation through 5–20% sucrose/SDS gradients. Total RNA from mouse liver provided optical density markers.

(A) Poly(A)$^+$RNA from subribosomal particles (< 80S) of approximately 900 late morulae (92 h post-hCG) labelled for 24 h with [5,6-^{3}H]uridine.

(B) Poly(A)$^+$RNA from ribosomal/polysomal particles (≥ 80S) of the same embryos.

(C) Poly(A)$^+$RNA from ribosomal/polysomal particles (≥ 80S) of approximately 250 hatching blastocysts (122 h post-hCG) labelled for 26 h. One-half of the supernatant was layered on the gradient.

Table 1. *The distribution of newly synthesized poly(A)$^+$RNA between subribosomal and ribosomal/polysomal fractions in late morulae, early and late blastocysts*

Labelling period (hours post-hCG)	Lysis solution	Proportion of incorporated uridine in supernatant	d.p.m. in poly(A) $<80S$	$^+$RNA* $\geqslant 80S$	Proportion of mRNA in ribo/poly. fraction
72–96	TAM, 1 % NP-40	0·37	1149	1646	0·59
(late morulae)		0·36	832	808	0·52
		0·35	818	533	0·39
					$\bar{x} = 0·50$
72–96	TSM/EGTA,	0·43	498	608	0·55
(late morulae)	1 % NP-40, 0·5 % DOC	0·43	631	457	0·42
		0·48	270	292	0·52
					$\bar{x} = 0·50$
76–100	TSM/EGTA	0·36	15	479	0·97
(early blastocysts)	1 % NP-40, 0·5 % DOC	0·54	60	226	0·79
		0·58	132	453	0·77
		0·59	55	1216	0·96
		0·70	79	948	0·92
					$\bar{x} = 0·88$
96–120	TSM/EGTA,	0·45	430	3475	0·89
(late blastocysts)	1 % NP-40, 0·5 % DOC	0·40	135	1212	0·90
		0·51	306	2478	0·89
		0·61	54	6720	0·99
		0·59	88	577	0·87
		not determined	168	6204	0·97
		not determined	91	2038	0·96
		not determined	96	3606	0·97
					$\bar{x} = 0·93$

* Corrected for recovery after ethanol precipitation.

clear that only a portion of the subribosomal poly(A)$^+$RNA in this stage could be considered a potential precursor to polysomal mRNA.

DISCUSSION

The overall rate of protein synthesis in early mouse embryos increases approximately nine-fold between the 2-cell and early blastocyst stages (Abreu & Brinster, 1978*a*), and most if not all of this synthesis is dependent on embryonic gene transcription (Braude, 1979; Flach *et al.* 1982). The synthesis rates of two of the most prominent groups of proteins in early embryos, actins and tubulins, have also been shown to increase several fold between the 2-cell and blastocyst stages and several fold again by an early outgrowth stage (Abreu & Brinster, 1978*a*,*b*). Greater utilization of cytoplasmic mRNA for protein synthesis is one of several factors which could support an ever-increasing rate of protein synthesis. Our data do, in fact, indicate that a greater proportion of cytoplasmic poly(A)-containing RNA is associated with polyribosomes and utilized for protein synthesis in blastocysts than in late morulae. In late blastocysts, there is little (if any) poly(A)$^+$RNA in the subribosomal fraction of 11 000 *g* supernatants, whereas about half of the cytoplasmic poly(A)$^+$RNA is located in that fraction in late morulae. Likewise, about half of the cytoplasmic poly(A)-containing RNA in cleavage and morula stages is subribosomal (K. Clegg, personal communication), suggesting that the situation in blastocysts represents a departure from conditions obtaining throughout the previous three days of preimplantation development.

Our finding that not all of the poly(A)-containing RNA in the ribosomal/polysomal fraction is EDTA-sensitive is not unique: similar results have been obtained with two-cell to morula-stage mouse embryos (K. Clegg, personal communication) and cultured mammalian somatic cells (Penman *et al.* 1968). Taking this into account, the data indicate that only about 38 % of cytoplasmic poly(A)-containing RNA in late morulae is being translated (50 % of it sediments in the ribosomal/polysomal region, but only about 75 % of this is likely to be associated with polyribosomes). The function of the remaining 62 % which is not being translated remains to be clarified. Judging from the overall size distribution (Fig. 2A) of the subribosomal component, most of it is not typical mRNA. We do not believe that the preponderance of short molecules in this preparation is the result of artifactual degradation during preparation of the embryo lysate, since the lysis solution contained a potent ribonuclease inhibitor which protected both the polyribosome-associated poly(A)$^+$RNA (Fig. 2B,C) and the marker polyribosomes from noticeable cleavage. Furthermore, the size distribution of the subribosomal poly(A)$^+$RNA was reproducible from one batch of late morulae to another. It is possible, however, that the short lengths of most of the subribosomal fraction result from degradation *in vivo*. The molecules sedimenting at 6–7S might represent mRNAs which were being translated earlier, but which by the end of the labelling period were undergoing turnover.

On the other hand, the subribosomal fraction in late morulae does contain some poly(A)$^+$RNA, albeit a minor fraction, the size range of which is appropriate for mRNA. As mentioned in the Introduction, experiments with transcription inhibitors have provided evidence that some late blastocyst proteins, such as those involved in blastocyst expansion and hatching, are produced from translation of long-lived mRNAs present in the early (day 4) blastocyst stage (Schindler & Sherman, 1981; Kidder & McLachlin, 1985). It is possible that the subribosomal mRNP in late morulae includes a number of stable mRNAs destined for recruitment into polyribosomes as blastocyst expansion begins. The sequence-specific recruitment of mRNA from the non-translating fraction has been documented in surf clam zygotes (Rosenthal, Hunt & Ruderman, 1980) and may occur in mouse zygotes as well since cell-free translation analysis has demonstrated that the enhanced synthesis of certain polypeptides after fertilization is due to the stimulation of translation of their oogenetic mRNAs (Braude, Pelham, Flach & Lobotto, 1979; Cascio & Wassarman, 1982). Pulse-chase experiments, designed to test the hypothesis that poly(A)$^+$RNA shifts from the subribosomal to the ribosomal/polysomal compartment as blastocyst expansion begins, have been inconclusive: since both compartments undergo turnover of poly(A)$^+$RNA (the overall rate being fairly slow in early blastocysts; Kidder & Pedersen, 1982), it is impossible to assess to what extent the ribosomal/polysomal compartment is being augmented during the chase. The role of subribosomal mRNA in late morulae could presumably be clarified if its translation products could be compared with those of the translating mRNA fraction in early blastocysts.

Our finding that about half of the cytoplasmic poly(A)$^+$RNA in early blastocysts is in the subribosomal RNP fraction is consistent with results from a variety of other embryonic and somatic cell systems. In *Drosophila*, 51 % of total poly(A)$^+$RNA is subribosomal in preblastoderm embryos, with 30 % remaining subribosomal during organogenesis (Lovett & Goldstein, 1977). In cleaving sea urchin embryos, 50 % of newly synthesized poly(A)$^+$RNA enters subribosomal particles; this proportion declines steadily to less than 20 % in mesenchyme blastulae (Dworkin & Infante, 1976). In *Xenopus* embryos, more than 90 % of newly synthesized, poly(A)$^+$RNA is in subribosomal particles during cleavage but this proportion declines to about 78 % by the neurula stage (Shiokawa, Misumi & Yamana, 1981). In somatic cells the proportion of mRNA not in polysomes has been estimated to be as little as 12 % to over 70 %, depending on the cell or tissue type, with most falling within the 20–40 % range (Bag & Sells, 1979; Bergmann, Cereghini, Geoghegan & Brawerman, 1982; Gold & Hecht, 1981; Imaizumi-Scherrer, Maundrell, Civelli & Scherrer, 1982; Lee & Engelhardt, 1978; MacLeod, 1975; Ouellette, Ordahl, Van Ness & Malt, 1982; Pratt & Johnson, 1980). Several of these groups of investigators have compared either the coding potential or the sequence diversity of subribosomal mRNA with that of translating mRNA from the same cells, and the most common finding has been that the two RNA populations have overlapping but not identical specificities. In particular, the relative abundances of individual mRNA sequences may differ between the two

mRNA components (Baker & Infante, 1982; Croall & Morrison, 1980; Dworkin & Hershey, 1981; Gold & Hecht, 1981; Imaizumi-Scherrer *et al.* 1982; Infante & Heilmann, 1981; Ouellette *et al.* 1982; Rudensey & Infante, 1979; Tansey & Ruderman, 1983; Walters, Yandell & Enger, 1979; Yenofsky, Bergmann & Brawerman, 1982). Results such as these have led several investigators to postulate that the subribosomal 'free' RNP fraction is enriched in mRNAs which undergo initiation of translation less frequently, and a kinetic model has been constructed to explain the discriminatory translation of individual mRNAs based on initiation efficiency (Godefroy-Colburn & Thach, 1981). Applied to the preimplantation mouse embryo, such a model would predict that during the transition from late morula to blastocyst, the availability of initiation factors increases, allowing efficient translation of messages which had previously initiated translation less frequently. Testing of this hypothesis would require an analysis of the translatability of subribosomal RNP from late morulae in the presence of cytoplasmic components from blastocysts.

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