***In Vitro* Translation of Oogenetic Messenger RNA of Sea Urchin Eggs and Picornavirus RNA with a Cell-Free System from Sarcoma 180**

(mengo virus/poly(A))

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ABSTRACT A cell-free protein-synthesizing system prepared from mouse sarcoma 180 was characterized by use of RNA from mengo virus and sea urchin egg. In the presence of exogenous mammalian transfer RNA, total sea urchin egg RNA and mengo RNA direct incorporation of [³H]leucine into acid-insoluble material. The system is extremely efficient in that a stimulation of 100-times over background can be obtained. Studies with formylmethionyl-transfer RNA, as well as with inhibitors of initiation, indicate that multiple initiation occurs; further, 85-90% of all chains made *in vitro* are subsequently released from ribosomes. An average translation time of 3.5 min was determined with messenger RNA of sea urchin egg, and product analysis indicates that high-molecular-weight products (greater than 50,000 molecular weight) are being made *in vitro*. Sequences of sea urchin egg RNA containing poly(A) act as messenger RNA.

A cell-free system prepared from Krebs ascites mouse cells has been used for translation of viral RNAs and messenger RNAs from various sources (1-10). This system has been characterized by its high efficiency and its wide range of translation of different messages, such as rabbit globin mRNA (11) and HeLa cell and sea urchin embryo histone mRNA (12). Aviv *et al.* (7) and Metafora *et al.* (13) have reported that the system was dependent upon added tRNA, although Jacobs-Lorena and Baglioni (11) have found no such dependency.

In this paper we report a new cell-free protein-synthesizing system prepared from mouse sarcoma 180 cells. This system has a high efficiency of translation of both viral and nonviral messages, and has a different spectrum of translation from that of the Krebs ascites system. We report high stimulation, multiple initiations, and 90% efficiency of termination. This system is capable of translating mRNA from sea urchin eggs, as well as those of viral origin.

MATERIALS AND METHODS

Nucleoside triphosphates, phosphocreatine, and creatine kinase were purchased from Sigma Biochemicals. Puromycin dihydrochloride was purchased from Calbiochem. Pactamycin (Upjohn) and aurin tricarboxylic acid (Matheson) were the kind gifts of Dr. H. R. Mahler. [³H]Leucine (35.5 Ci/mol) and [³H]phenylalanine (6.15 Ci/mol) were purchased from New England Nuclear Corp. [³H]-Formylmethionine tRNA was prepared from chicken liver (Wang, S., Ph. D. Thesis, Indiana University, 1973). Rat-liver tRNA was purchased from General Biochemicals, Chagrin Falls, Ohio. *Strongylocentrotus purpuratus* was obtained from Controlled Environments, Bellingham, Wash.

Growth of Tumors. Sarcoma 180 cells were propagated intraperitoneally as described (14). Cells were routinely harvested 10 days after injection.

Sarcoma 180 Cell Extract Was Prepared by a modification of the procedure described by Aviv *et al.* (7). Harvested cells were quickly chilled to 0-4°, washed at least three times in two volumes of 35 mM Tris·HCl (pH 7.5)-140 mM NaCl, and centrifuged at 60 × *g* to remove blood. Tumor cells were further packed by centrifugation at 250 × *g* for 10 min. The cells were then suspended in slightly less than twice the packed volume in a buffer containing 10 mM Tris·HCl (pH 7.5)-10 mM KCl-1.5 mM Mg(CH₃COO)₂. Nonidet P-40 (Shell Chemical Co.) was added such that the final concentration was 0.5%. After 10 min at 0-4°, 95% of all cells were lysed.

The suspension was then brought to a final concentration of 30 mM Tris·HCl (pH 7.5)-125 mM KCl-5 mM Mg·(CH₃COO)₂-7 mM 2-mercaptoethanol and centrifuged at 30,000 × *g* for 10 min. The supernatant was then incubated at 37° for 60-120 min in the presence of 1 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, 0.16 mg/ml of creatine kinase, and 40 μM (each) of 20 amino acids. Incubation was terminated by a 4° centrifugation at 30,000 × *g* for 10 min. The supernatant was passed through a Sephadex G-25 column equilibrated with buffer containing 30 mM Tris·HCl (pH 7.5)-120 mM KCl-5 mM Mg(CH₃COO)₂-7 mM 2-mercaptoethanol. The eluted lysate was stored in small aliquots at -70°; frozen lysate retains activity for several weeks. Ribosomal salt washes (0.5 M KCl) were prepared as described by Shafritz and Anderson (15). Salt washes were made to 0.12 M in KCl by dialysis, and preparations were stored at -70°.

Virus Production. L-cells, grown in suspension, were used to propagate mengo virus. The suspension media consisted of 50% minimal essential medium (Eagle), 50% nutrient mixture F-10 with 15% fetal-calf serum (GIBCO), and 0.1% methyl cellulose. An inoculum of 5 × 10⁶ of nontrypsinized L-cells was necessary to induced exponential growth. Monolayer L-cells were completely adapted to suspension growth in 48 hr, at which point only 10% fetal-calf serum was needed to sustain growth. Cells were maintained at 37° on a rotary shaker at a setting of 100 rpm.

L-cells were grown to a final concentration of 1 × 10⁶ cells per ml. Mengo and bovine enterovirus production was as described (16, 17) except that the incubation period was

FIG. 1. Effect of added RNA on incorporation of $[^3\text{H}]$ leucine. Stimulation of polypeptide synthesis by various amounts of RNA. Sea urchin egg RNA ($\circ$). Stimulation of poly(U)-Sephadex-bound fraction of sea-urchin egg RNA ($\bullet$). Ribosomal salt wash was present. Mengo virus RNA without ribosomal salt wash ($\blacktriangle$), with ribosomal salt wash ($\triangle$). Background ($-$ RNA) was 2050 cpm in 0.1 ml total volume. Incubation was for 45 min, and each point represents duplicate samples. One A_{260} unit equals about 40 μg of RNA (7).

extended to 24 hr to insure two rounds of replication. Virus titer was about 3×10^9 plaque-forming units per ml.

Preparation of RNA. Unlabeled RNA was extracted from the viral lysate as described by Eggen and Shatkin (18). Sea urchin egg RNA was extracted by the pH 9 phenol-chloroform method (19, 20). Poly(U)-Sephadex affinity chromatography was used to detect the presence of poly(A) sequences in sea urchin egg RNA, and to purify poly(A)-containing RNA from total egg RNA (21, 22).

Assay of Amino-Acid Incorporation. Amino-acid incorpora-

TABLE 1. Translation of RNA by sarcoma 180 and Krebs ascites *in vitro* lysates

mRNA	Sarcoma 180		Krebs ascites	
	+RNA (cpm)	-RNA (cpm)	+RNA (cpm)	-RNA (cpm)
BEV-1	20,628	1,488	5,828	2,889
Reovirus	25,741	1,488	5,816	2,889
Mengo	34,798	4,815	33,493	3,096
Sea urchin egg	46,493	1,984	49,685	2,841

All samples contained 0.2 A_{260} units of RNA. Figures are average of duplicate samples. All incubations were for 45 min. BEV-1, bovine enterovirus-1.

FIG. 2. Incorporation of $[^3\text{H}]$ formyl- $[^{14}\text{C}]$ methionine into trichloroacetic acid-precipitable products by sarcoma 180 *in vitro* system. To each reaction mixture containing 0.2 A_{260} unit of total sea-urchin egg RNA, 0.015 ml of $[^3\text{H}]$ formyl- $[^{14}\text{C}]$ methionine tRNA (semi-purified chicken liver tRNA^{Met}, charged with chicken-liver synthetase and formylated with *E. coli* transformylase) was added. At the conclusion of the designated incubation time the *in vitro* incubation mixtures were layered onto 6-ml cushions of 20% sucrose solution and centrifuged for 4 hr at $150,000 \times g$. The upper half of each was precipitated with trichloroacetic acid. The ribosomal pellet was suspended, mixed with the lower half of the cushion, and precipitated with trichloroacetic acid. All samples were done in duplicate. Total incorporation of $[^3\text{H}]$ formyl groups ($\square$); incorporation of $[^3\text{H}]$ formyl groups into released polypeptide chains ($\blacktriangle$); incorporation into bound polypeptides ($\bullet$). $[^{14}\text{C}]$ Methionine counts (not shown) were also incorporated into products.

tion was measured by a modification of the procedure described by Aviv *et al.* (7). Each 0.05-ml reaction mixture contained 60 mM Tris-HCl (pH 7.5), 2.5 mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 14 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, 0.032 mg/ml of creatine kinase, 4 μM (each) 19 nonradioactive amino acids, 5 μM $[^3\text{H}]$ leucine, 120 mM KCl (or salt wash as indicated), and 0.01 mg of commercial rat-liver tRNA. To this reaction mixture 50 μl of preincubated sarcoma 180 cell-free lysate was added, which contained 10 mg/ml of protein as determined by the method of Lowry *et al.* (23). Mengo virus RNA or crude sea urchin egg RNA was added as indicated and the mixture was incubated for 45 min at 37° . The reaction was stopped by addition of 2 ml of cold 10% trichloroacetic acid. Samples were heated at $90-95^\circ$ for 20 min. After cooling, precipitates were collected on 0.45- μm Millipore filters, washed five times with 5% trichloroacetic acid, dried, and counted in a Beckman liquid scintillation counter.

Gel Electrophoresis. Polyacrylamide gel electrophoresis on gels containing Na dodecyl sulfate and urea were done as described (24, 25). In preparation for counting, the gels were frozen and sliced into 1-mm sections and placed in vials. To each 0.2 ml of 15% H_2O_2 was added, and vials were capped and incubated at 60° overnight. After cooling, 2 ml of a Scin-

TABLE 2. Effects of ribosomal salt wash from various sources on translation of RNA by sarcoma 180 cell-free system

Source of ribosomal wash	Sea urchin egg RNA		Mengo virus RNA	
	addition of salt wash			
	+	-	+	-
Sea urchin embryos	42,697	15,975	66,489	32,186
Mengo virus infected-L-cells	1,069	19,675	68,924	36,875
Sarcoma 180 cells	63,497	14,825	72,682	31,682

Samples contained 0.2 A₂₆₀ units of RNA. Incubation was for 45 min. Incorporation figures are averages of duplicate samples. Background (-RNA) incorporation was 1070 cpm.

tisol (Isolab, Inc.) solubilizer-toluene (1:1) mixture and 10 ml of scintillation fluid were added to each vial.

RESULTS

Stimulation of Polypeptide Synthesis with Exogenous mRNAs. Sarcoma 180 extracts direct incorporation of [³H]-leucine into acid-insoluble material in response to both sea urchin egg RNA and picornavirus mRNA, and of [³H]-phenylalanine in response to poly(U). The specificity of the reaction is strictly dependent on the Mg²⁺ concentration. Natural mRNAs are optimally translated between 2 and 3 mM Mg²⁺, and poly(U) between 5 and 7 mM. We have found that by varying the preincubation period the sarcoma 180 system can be made tRNA dependent. A minimum of 0.01 mg of mammalian tRNA is used per reaction mixture.

The extent of polypeptide synthesis increases linearly with increasing amounts of both sea urchin egg and virus RNA (Fig. 1). We were unable to saturate the system with total sea urchin RNA or with virus RNA. However, we were able to reach saturation by the addition of about 30 µg of purified sea urchin mRNA prepared by poly(U)-Sephadex chromatography. The stimulation of [³H]leucine incorporation is 300-fold that of background at saturation with purified mRNA in the sarcoma 180 system, whereas, at saturation we could only obtain 15- to 20-fold stimulation with the Krebs ascites system.

About 3-5% of the total sea urchin egg RNA is bound to the poly(U)-Sephadex column. This fraction stimulates incorporation *in vitro* 10-fold more effectively than does total egg RNA (Fig. 1).

Besides mengo virus, bovine enterovirus-1 and reovirus RNA have been translated in the sarcoma 180 system. These messengers all yield a 10- to 15-fold stimulation over endogenous incorporation. We found minimal translation of bovine enterovirus-1 and reovirus RNAs in a Krebs ascites extract (Table 1). Mengo virus and sea urchin egg RNAs stimulated both systems to about the same extent with amounts of RNA below that saturating the system (Table 1).

Ribosomal Salt Wash. Many authors have reported the use of ribosomal salt wash containing initiation factors to enhance *in vitro* protein synthesis (15, 26, 27). In the presence of a salt wash from sea urchin ribosomes, stimulation was increased 2-fold (Table 2). Salt washes were also prepared from ribosomes of L-cells infected with mengo virus and from sarcoma 180 ribosomes. The wash from ribosomes of L-cells infected

with mengo virus stimulated translation of mengo virus RNA, but appeared to inhibit translation of sea urchin egg RNA. However, a salt wash from sea urchin ribosomes stimulated mengo virus translation. Likewise, the salt wash from sarcoma 180 ribosomes stimulated translation of both messengers. All salt washes contained from 3.5 to 5 mg protein per ml.

Initiation and Termination. The observation that the addition of mRNA enhances the incorporation of radioactive amino acids in a cell-free system does not necessarily imply that true initiation of synthesis of polypeptides has occurred. The inhibitors of protein synthesis, pactamycin and aurin tricarboxylic acid, inhibit initiation of protein synthesis in cell-free systems (28-30). When pactamycin or aurin tricarboxylic acid was added to the sarcoma 180 system stimulated by sea urchin egg RNA at time 0, inhibition proportional to the pactamycin or aurin tricarboxylic acid concentration was observed. 1 µM pactamycin inhibits 50% of the product directed by sea urchin RNA. 10 µM aurin tricarboxylic acid inhibits polypeptide synthesis by 80%. These results are compatible with published data (28-30). When pactamycin (1 µM) was added 0, 15, and 30 min after the reaction was initiated, immediate inhibition of incorporation was noted. The inhibition data suggest that, unlike the Krebs ascites system in which initiation of polypeptide synthesis directed by exogenous message appears to be completed between 10 and 20 min and that further incorporation is due to elongation (18), initiation in the sarcoma 180 system is still taking place even after 30 min.

Although formylmethionine-tRNA is not the normal initiating tRNA in eukaryotic systems, fMet-tRNA^{Met} can initiate protein synthesis *in vitro*. Labeled formylated methionine can be traced in the mammalian system since the formylated methionine is not removed after the release of the polypeptide from the polysome (31). We examined the extent of initiation by following the release of trichloroacetic acid-precipitable protein containing labeled formylmethionine for 45 min (Fig. 2). The data presented here also demonstrate

TABLE 3. Release of polypeptide chains from ribosomes

Treatment of 0.1 ml of incubation mixtures after incubation	Supernatant (cpm)	Pellet (cpm)	Released chains (%)
1.6 ml of TS + 0.06 M KCl 4° for 15 min	35,900	4,780	88
0.125 ml of CSB + 0.025 ml of 0.01 M puromycin 4° for 15 min	22,800	2,450	90.5
0.125 ml of CSB + 0.025 ml of 0.01 M puromycin 37° for 10 min	23,500	1,585	94

At the end of the indicated treatment periods all samples were cooled to 0°, made to a final volume of 1.7 ml with TS buffer [0.15 M Tris·HCl (pH 7.5)-25 mM Mg(OAc)₂-35 mM 2-mercaptoethanol], and centrifuged at 150,000 × g for 1.5 hr. Supernatants were collected and precipitated with trichloroacetic acid for counting. Pellets were suspended in 0.14 M NaCl-35 mM Tris·HCl (pH 7.5), precipitated with trichloroacetic acid, and prepared for counting. Values are averages of duplicate samples. CSB refers to the compensating salt buffer of Blobel and Sabatini [0.10 M Tris·HCl (pH 7.5)-0.01 M Mg(OAc)₂-1 M KCl] (35).

FIG. 3. Electrophoretic separation of polypeptides synthesized by sarcoma 180 system directed by sea urchin RNA. An incubation mixture to which total sea urchin RNA was added was incubated for 45 min, then centrifuged for 90 min at $150,000 \times g$. The supernatant was withdrawn and prepared for electrophoresis on a 5% polyacrylamide gel containing Na dodecyl sulfate and urea. Counts per slice (—○—). Bovine-serum albumin (molecular weight = 68,000) and chymotrypsinogen (molecular weight = 25,000) were used as molecular weight markers. Background was 30 cpm.

that about 90% of all polypeptides made *in vitro* are released from polysomes during this period. We have also tested the efficiency of the sarcoma 180 system by forcing termination in the presence of puromycin. In the absence of puromycin 88% of all polypeptides are released, while in the presence of the antibiotic, this number increased to 90–94% (Table 3). Mathews and Korner (3) and Eggen and Shatkin (18) have shown that in the Krebs ascites *in vitro* system only 20–30% of all polypeptides produced are released.

Products of Sea Urchin Egg RNA. Electrophoretic analysis of the products of a sarcoma 180 system directed by sea urchin egg RNA was done (Fig. 3). Most of the [³H]leucine incorporation was into high-molecular-weight products with an average molecular weight of about 50,000. Similar patterns were obtained with the product of a sarcoma 180 incubation directed by mRNA purified by poly(U)–Sephadex chromatography and with *in vivo* labeled proteins. These results indicate that the released chains are not short, prematurely terminated peptides.

DISCUSSION

In this paper we report an *in vitro* system developed from mouse sarcoma 180 cells which translates efficiently both viral and nonviral message from heterologous sources. Incorporation of [³H]formylmethionine in the sarcoma 180 system is linear during 45 min, and there appears to be no decrease in translation rate during this period of time. Without data on the synthesis of a discrete molecular species we cannot ac-

curately assess the rate of translation. However, the data of Fig. 3 indicate that most of the released chains have molecular weights greater than 25,000. Thus, we estimate from Fig. 2 an approximate time of 3.5 min for synthesis of an average chain (27). Using the same label we have been able to measure the amount of polysomal and free formylmethionine polypeptide. The efficiency of release is about 90%.

We have not observed any specificity of translation of mRNAs in this system, since all mRNAs we have tested have been translated. These include mengo virus, bovine enterovirus-1, and reovirus messages, as well as sea-urchin egg mRNA. The ability to translate all these messages may signify the availability of a large number of specific initiation factors, or a loss in specificity of a few initiation factors.

The observation that the system could be stimulated by addition of ribosomal salt washes from sea urchin, and that inhibition of sea urchin translation occurred on addition of ribosomal wash from L cells infected with mengo virus, would agree with the concept of specificity of initiation factors and perhaps modification of initiation factors during virus infection.

Sea urchin eggs contain a supply of mRNA molecules synthesized during oogenesis and stored in an inactive form until recruited for translation after fertilization. The regulatory mechanisms underlying the turn-on of protein synthesis and mobilization of stored mRNA are poorly understood. On the basis of the amount of translation in a cell-free system from *Escherichia coli* that had been calibrated with MS-2 and *E. coli* RNAs, Slater and Spiegelman (32) have estimated the amount of stored mRNA in sea urchin eggs to be about 4% of the total RNA. Slater *et al.* (20) have demonstrated that poly(A) sequence-containing RNA exists in eggs and that this RNA becomes more adenylated shortly after fertilization.

Using poly(U)–Sephadex columns, we were able to obtain enriched RNA for poly(A)-containing sequences. The bound RNA comprised 3–5% of the total applied to the column and was translated 10-fold more efficiently by the sarcoma 180 system than was total egg RNA. These results confirm that the poly(A)-containing sequences studied by Slater *et al.* (20) are in fact mRNA, and that about 3–5% of the RNA of sea urchin eggs is mRNA. This figure is still uncertain because we have not analyzed our poly(U)–Sephadex-bound fractions for possible contamination by other RNA species. Further, there is a significant, but unquantitated, amount of template activity remaining in the fraction of the RNA that does not bind to poly(U)–Sephadex. This may be accounted for in part or completely by histone mRNA which is present in sea urchin eggs and is known in mammalian systems to lack poly(A) sequences (33).

Polyacrylamide-gel electrophoretic analysis of the products of total protein synthesis directed by sea urchin egg RNA in the sarcoma 180 system indicates that the same range of high-molecular-weight proteins are being synthesized as are observed in *in vivo* synthesis by embryos (Raff *et al.*, unpublished). This system is being exploited for the *in vitro* synthesis of a specific oogenetic mRNA product, microtubule protein (24, 34).

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