A COMPARISON OF THE TRANSLATION OF MENGO VIRUS RNA AND GLOBIN mRNA IN KREBS ASCITES CELL-FREE EXTRACTS

B. LEBLEU, U. NUDEL, E. FALCOFF*, C. PRIVES and M. REVEL

Department of Biochemistry, The Weizmann Institute of Science, Rehovot, Israel

1. Introduction

The control of protein synthesis by the action of mRNA or cistron specific initiation factors is now well established in *E. coli* [1]. In mammalian cells, however, where the existence of tissue-specific translation factors has been suggested [2], the situation is less clear.

Globin mRNA has been translated in such different heterologous cell-free systems as reticulocytes from other animal species [3], *Xenopus laevis* eggs [4], Krebs or Landschutz ascites cells [5], chick embryo muscle cells [2] and rabbit liver [6]. Some other mRNA species which have been successfully translated in heterologous systems are lens mRNA in ascites and reticulocyte extracts [7], and myosin mRNA in a reticulocyte cell free system [2]. Some of these reports have shown a requirement for cell specific fractions in the initiation of translation of heterologous mRNA [2, 6], while others have demonstrated no such requirement.

That mRNA can be translated in a heterologous system has been generally interpreted as a lack of specificity in recognition of the mRNA by the ribosome machinery. However, in most cases no attempts have been made to establish the optimum conditions for translation by addition of cell components homologous to the message used.

The results presented here indicate that reticulocyte factors stimulate selectively globin mRNA translation in Krebs cell-free extracts. Endogenous

protein synthesis and mengo virus RNA translation in these extracts are not or much less stimulated. Addition of similar Krebs cell fractions produces an opposite effect to that of reticulocytes factors.

2. Methods

2.1. *Preparation of Mengo virus*

Mengo virus was grown in L cells (strain L929) and purified as described previously [8]. A further purification step by CsCl equilibrium centrifugation was performed. CsCl was added to a final density of 1.32 g/cm^3 to a solution of Mengo virus (1.15×10^{12} pfu/ml) in 35 mM Tris-HCl (pH 7.6), 146 mM NaCl. Centrifugation was carried out for 24 hr at 40,000 rpm at $4°$ in the Spinco SW 50 rotor. The virus band ($\rho = 1.33$ g/cm^3) was isolated and dialyzed overnight against 35 mM Tris-HCl (pH 7.6) 146 mM NaCl.

2.2. *Isolation of Mengo viral RNA*

Mengo viral RNA was extracted from the purified virus by the procedure of Kerr et al. [9]. It sedimented as a single 37 S peak upon sucrose gradient centrifugation. Viral RNA was stored in aqueous solution (1 mg/ml) in liquid air.

2.3. *Isolation of globin mRNA*

Globin mRNA was extracted by the phenol—chloroform—isoamylalcohol procedure [10] from the 15 S messenger ribonucleoprotein released by EDTA from reticulocyte polyribosomes [11].

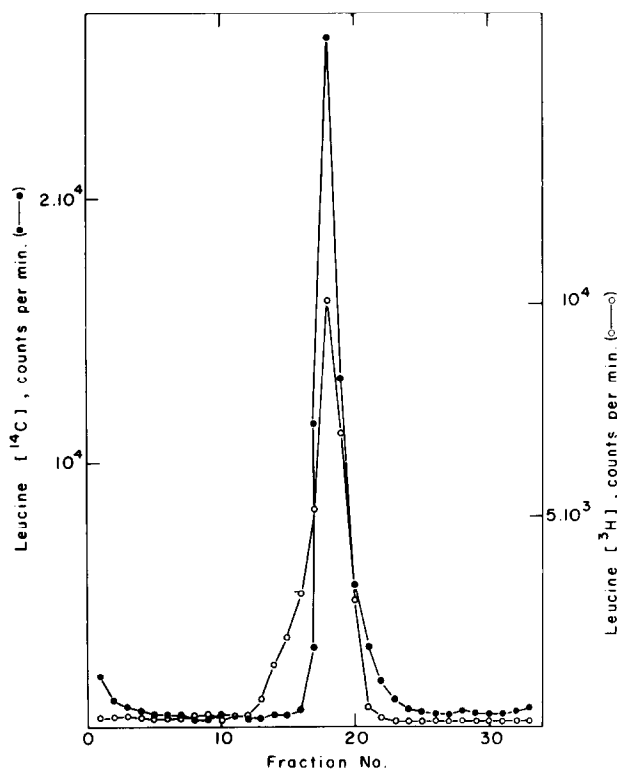* Laboratoire Pasteur de l'Institut du Radium, Paris, France.

Fig. 1. Polyacrylamide gel analysis of the products synthe-
sized under direction of hemoglobin mRNA (●—●—●). *In
vivo* ^{3}H-labeled globin was added as internal marker
(○—○—○).

Fig. 2. Polyacrylamide gel analysis of the products synthe-
sized under direction of residual endogenous mRNA
(○—○—○) and Mengo viral RNA (●—●—●). The arrows indi-
cate the position where bovine serum albumin (BSA), chyr
trypsin (C), α-amylase (αA) and globin (G) migrate.

2.4. Preparation of cell free system from Krebs ascites cells

Extracts derived from Krebs II ascites cells were
prepared and pre-incubated according to Mathews
and Korner [12].

For the amino acid incorporation assay, 0.05 ml
incubation mixtures were generally used, containing
0.02 ml of pre-incubated S-30 (30 A_{260} units per
ml), 30 mM Tris-HCl (pH 7.6), 3.5 mM $MgCl_2$,
80 mM KCl (unless otherwise indicated), 7 mM β-
mercaptoethanol, 1 mM ATP, 0.5 mM GTP, 5 mM
creatine phosphate, 7.5 μg creatine kinase, 0.1 mM
of each amino acid except the radioactive one(s) and
0.15 μCi of [14C] leucine (351 μCi/μmole) or 0.15
μCi ^{14}C protein hydrolysate (50 μCi/μmole) for the
mengo product analysis.

After 30 min of incubation at 37°, 40 μl aliquots
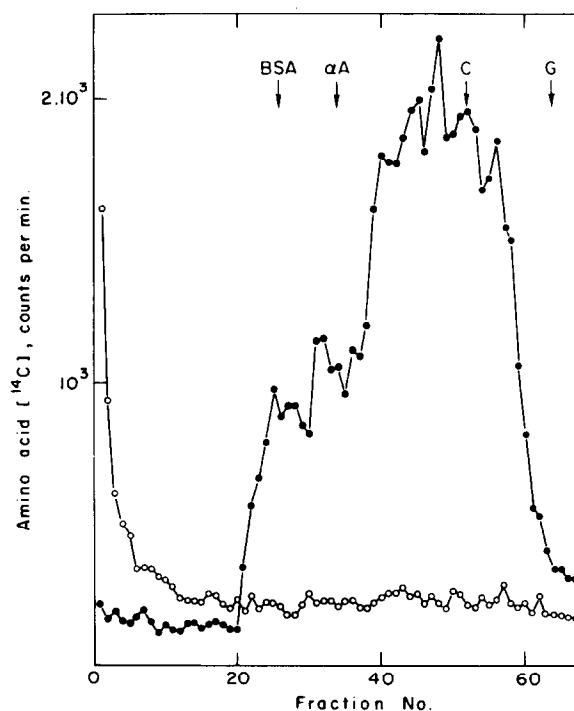were spotted on Whatman No. 3 filter paper discs

and processed for measurement of amino acid inco:
poration into protein [13].

Krebs sap and ribosomes were prepared accord-
ing to Mathews and Korner [12].

2.5. Preparation of a 0.5 M wash fluid from reticuk cyte ribosomes (reticulocyte RWF)

0.5 M KCl ribosomal wash fluid and pH 5.0 en-
zyme fraction were prepared from rabbit reticulo-
cyte polyribosomes as described by Shafritz and
Anderson [14].

2.6. Preparation of a 0.5 M KCl wash fluid from Krebs ribosomes (Krebs RWF)

Ribosomes were prepared from non pre-incubat
Krebs S-30. They were salt-washed either for 4 hr c
overnight at 0° in a buffer containing 30 mM Tris
Cl (pH 7.6), 5 mM Mg chloride, 7 mM β-mercapto-

Table 1
Effect of inhibitors of initiation on the translation of hemoglobin mRNA and Mengo RNA.

| mRNA | [^{14}C]leucine incorporation (pmoles) | | | | | |
| | Endogenous | | Hemoglobin mRNA | | Mengo virus RNA | |
Additions	None	Reticulocyte RWF*	None	Reticulocyte RWF	None	Reticulocyte RWF
	2.94	5.8	8.1	20.5	7.9	8.6
+ ATA	2.25	2.6	1.7	1.3	1.6	1.1
+ NaF	1.9	2.3	1.6	1.1	2.6	2.6

Hemoglobin mRNA (0.2 μg) or Mengo virus RNA (1 μg) were incubated in 0.05 ml protein synthesis reaction mixture as described in Methods. 0.015 ml of reticulocyte RWF (60 μg protein) were added where indicated. NaF and ATA were added at final concentrations of 10 mM and 0.1 mM, respectively. Results are expressed in pmoles leucine incorporated per 0.02 ml of Krebs S-30. Endogenous not subtracted.

* Stimulation of the endogenous activity by reticulocyte RWF is mainly due to the presence of some hemoglobin mRNA in this fraction, since analysis of the product shows the synthesis of globin chains. This contribution to the endogenous activity was subtracted from the results in tables 2 and 3.

Table 2
Relative translation of Mengo virus RNA and hemoglobin mRNA in Krebs ascites extracts.

| | mRNA | [^{14}C]leucine incorporation (pmoles) | | | | | |
| | | Hemoglobin mRNA | | Mengo virus RNA | | Ratio hemoglobin/Mengo | |
	Additions	None	Reticulocyte RWF	None	Reticulocyte RWF	None	Reticulocyte RWF
Exp. 1	80 mM KCl	2.9	24.7	8.5	9.2	0.34	2.68
Exp. 2	80 mM KCl	1.6	15.5	2.6	2.65	0.61	5.84
	100 mM KCl	1.35	10.1	3.2	7.5	0.42	1.34
	120 mM KCl	0.2	4.2	1.4	4.6	0.14	0.81

Hemoglobin mRNA (0.2 μg) or Mengo virus RNA (1 μg) were incubated in 0.05 ml protein synthesis reaction mixtures as described in Methods. 0.015 ml of reticulocyte RWF (60 μg of proteins) were added where indicated. Results are expressed in pmoles leucine incorporated per 0.02 ml Krebs S-30. The background incorporation due to residual endogenous activity of the cell free system was subtracted.

ethanol and 30% glycerol (v/v) to which KCl had been added with stirring to a final concentration of 0.5 M. After spinning down the salt-washed ribosomes at 50,000 rpm at 4° for 3 hr, the supernatant was dialyzed for 6 hr against the same buffer with a KCl concentration reduced to 125 mM, and stored in small aliquots in liquid air.

2.7. Analysis of the cell-free products

After the 30 min incubation of the cell-free system at 37°, a mixture of the 20 essential amino acids (0.25 mM final concentration) was added and the incubation continued for an additional 30 min. The samples (or purified globin [15]) were processed for SDS polyacrylamide gel analysis as described by Boime et al. [16] (7.5% polyacrylamide gels [17] were used except for the experiment illustrated in fig. 1 where 15% polyacrylamide gels were used). The gels were frozen and sliced; 0.2 ml of NCS solubilizer was added to each 1 mm thick slice (in some cases two slices were combined). After 6 hr, 3.5 ml of toluene scintillation fluid was added and the vials
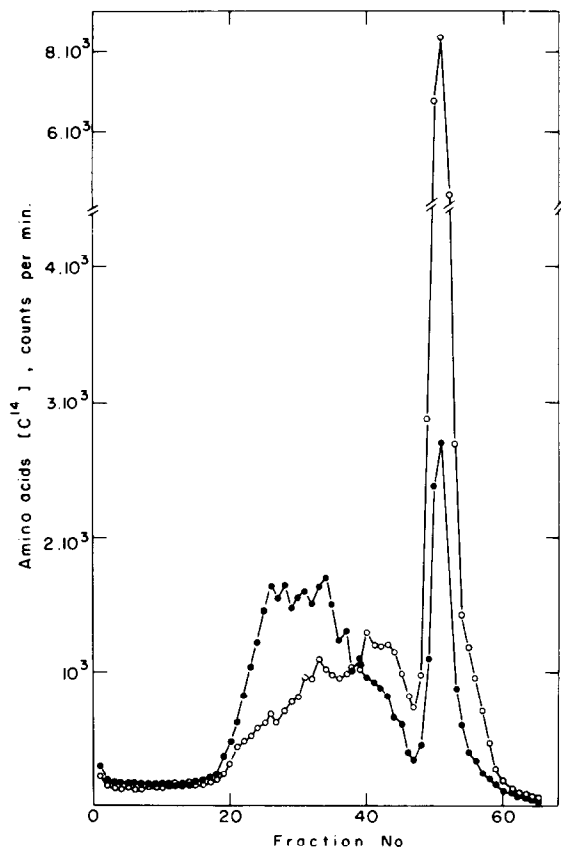
Fig. 3. Polyacrylamide gel analysis of the products synthe-
sized after simultaneous addition of hemoglobin mRNA and
Mengo viral RNA. (●—●—●) without and (○--○--○) with added
reticulocyte RWF (180 μg of protein). Hemoglobin mRNA
(0.8 μg) and Mengo viral RNA (5 μg) were incubated in
0.15 ml protein synthesis reaction mixture as described in
Methods.

were shaken gently at 37° overnight. The gels were
standardized with the following purified proteins:
globin (16,000), chymotrypsin (25,000), α-amylase
(45,000) and bovine serum albumin (68,000).

3. Results

3.1. Translation of Mengo and globin mRNA in a Krebs ascites cell free system

Purified rabbit globin mRNA can be translated in
a cell free system derived from Krebs ascites cells
[5]. Analysis of the products obtained by polyacryl-
amide gel electrophoresis shows that the 9 S RNA

directs globin chain synthesis exclusively (fig. 1). In
this particular experiment, in vivo-labelled ^{3}H-globin
was coelectrophorized with the ^{14}C-labelled cell-free
product but the same pattern was also obtained in
the absence of added carrier.

Addition of purified 37 S Mengo virus RNA to a
Krebs ascites cell-free system promotes the synthesis
of polypeptide chains which are clearly different
from those produced by the endogenous messenger
or by globin mRNA (fig. 2). It is known that in vivo
the RNA is first translated into giant polypeptide
chains which are then cleaved into the final viral
proteins [18]. The products obtained in vitro will
differ depending on whether or not the cleavage
mechanism is operating. In our conditions (80 mM
KCl a fairly reproducible pattern of products varying
in size from 20,000 to 60,000 daltons were observed,
with few higher molecular weight polypeptide
chains. This would suggest that the cleavage process
is, in fact, in operation. At a higher concentration of
KCl (120 mM) however, we observed the accumula-
tion of higher molecular weight products similar to
those obtained by Eggen and Shatkin [19]. A similar
dependence of the size of the product on the con-
centration of KCl has been seen with encephalomyo-
carditis (EMC) RNA (Mathews, personal communica-
tion.)

As it is difficult to determine the correct physio-
logical ionic conditions, we have compared the
translation of Mengo RNA and hemoglobin mRNA
at different ion concentrations. In both cases the
$MgCl_2$ concentration optimum was found to be 3.5
mM. The optimum KCl concentration (in the pres-
ence of reticulocyte factors (see below) was found
to be 80 mM for globin and 100 mM for Mengo. In
the absence of added factors the optimum for both
was around 80 mM KCl, which was used unless other-
wise stated.

Both Mengo and globin mRNA translation are
blocked by inhibitors of polypeptide chain initiation
such as ATA [20] or NaF [21] (table 1). The effi-
ciency of translation of globin mRNA was compar-
able to that reported by others [5], that is, 3—4
globin chains per mRNA chain in 30 min. This value
did not vary significantly when globin mRNA was
purified by either the standard sucrose centrifugation
[11], or by adsorption and elution from a poly U
cellulose column (Lebleu, Nudel and Caput; unpub-

Table 3
Effect of Krebs RWF and reticulocyte RWF on Mengo RNA and hemoglobin mRNA translation.

mRNA:		[^{14}C] leucine incorporation pmoles		
		Hemoglobin mRNA	Mengo RNA	Ratio hemoglobin / Mengo
	Fraction added			
Expt. 1	None	10.4	5.7	1.8
	Reticulocyte RWF	21.6	4.2	5.1
	Krebs RWF	7.2	4.9	1.4
	Reticulocyte + Krebs RWF	10.8	4.8	2.2
Expt. 2	None	1.9	6.6	0.28
	Reticulocyte RWF	12.9	7.4	1.74
	Krebs RWF*	0.3	5.9	0.05
	Reticulocyte + Krebs RWF	9.1	9.6	0.94
Expt. 3	None	4.9	4.4	1.1
	Reticulocyte RWF	21.7	3.6	6.0
	Krebs sap	2.5	4.3	0.58
	Retic. RWF + Krebs sap	6.3	4.7	1.34

Hemoglobin mRNA (0.2 μg) or Mengo virus RNA (1 μg) were incubated in 0.05 ml protein synthesis reaction mixtures as described in Methods. Reticulocyte RWF (20 μg protein), Krebs RWF from two different preparations (50 μg protein or 270 μg protein where (*) indicated) and Krebs sap (160 μg protein) were added as indicated. Results are expressed as in table 2. Endogenous activity subtracted.

lished results). In all cases the homogeneity of the globin mRNA was found to be greater than 90% by polyacrylamide gel electrophoresis. In the case of Mengo RNA, the efficiency of translation was similar to what has been reported by others for EMC RNA [16]. Both messenger RNA's were added to the cell-free system in non-saturating amounts.

3.2. Effect of addition of reticulocyte subfractions

The efficiency of the Krebs ascites cell-free extract could be markedly increased by the addition of a 0.5 M KCl wash fluid from reticulocyte polyribosomes (RWF) [22, 23] prepared as described by Shafritz and Anderson [14]. An 8 to 10-fold stimulation of globin synthesis is observed (table 2).

A pH 5.0 precipitate of reticulocyte supernatant which is active to complement reticulocyte polyribosomes (results not shown) has practically no stimulatory effect on globin translation in the Krebs cell-free system. It is, therefore, neither tRNA nor elongation factors which stimulate globin synthesis in the Krebs extract. Addition of purified Krebs tRNA had likewise no effect.

The stimulatory effect of reticulocyte RWF probably results essentially from the addition of initiation factors contained in this fraction [14, 23], which is required for globin chain initiation by 0.5 M KCl washed reticulocyte polyribosomes [23. 24]. In the Krebs ascites cell free extract, reticulocyte RWF stimulates the transfer of ^{32}P-labelled globin mRNA from the 40 S ribosomal subunit to polysomes (Nudel et al., to be published). Moreover, the stimulation of globin mRNA translation by reticulocyte RWF is entirely abolished by known inhibitors of initiation such as ATA or NaF, (table 1).

A comparison of Mengo and globin mRNA translation shows that the reticulocyte RWF has a pronounced differential effect on the efficiency of the Krebs ascites cell-free extract in translating the two mRNA's.

The reticulocyte RWF stimulated globin synthesis much more than Mengo RNA translation (table 2). At 80 mM KCl, there was no stimulation of Mengo RNA translation. At high KCl concentration, Mengo translation in the Krebs extract alone was smaller

and could be stimulated by adding reticulocyte RWF. Table 2 demonstrates, however, that in all ionic conditions, the translation ratio of globin/Mengo RNA is increased 3–8-fold by the addition of reticulocyte RWF.

When Mengo and globin RNA were added together to the cell free system, this messenger specific effect is even more clearly illustrated. The translation of each messenger was separately measured by analysis of the products after polyacrylamide gel electrophoresis. As shown in fig. 3, addition of reticulocyte RWF strongly stimulated the translation of globin mRNA, but decreased that of Mengo RNA. When both mRNA's are in competition, the reticulocyte RWF therefore favors synthesis of globin in this heterologous system.

3.3. *Effect of addition of Krebs ascites cells subfractions*

Addition to the cell-free system of a 0.5 M KCl wash fluid prepared from Krebs ascites ribosomes gave very different results (table 3). Globin mRNA translation is markedly inhibited, while Mengo RNA translation is not significantly affected. Moreover, addition of Krebs RWF to a system containing reticulocyte RWF indicates a competition between the two groups of factors, and a decrease in the globin/Mengo translation ratio is obtained.

Similar results were obtained with Krebs ascites cell supernatant fraction (sap) (table 3) suggesting that in these cells, the message-specific factors may be in the supernatant as well. These Krebs fractions stimulate endogenous polypeptide synthesis by Krebs ascites cell polyribosomes indicating thereby that they are active and do not contain a general inhibitor of protein synthesis.

When both Mengo and hemoglobin mRNA are added together in a competition experiment and the products analyzed by polyacrylamide gel electrophoresis, the Krebs RWF selectively decreases globin synthesis as compared to Mengo. Synthesis of some Mengo products is even increased. The messenger discriminating properties of Krebs factor therefore contrast with those of reticulocyte RWF (fig. 3).

4. Discussion

While this article was in preparation, Metafora et al. [27] reported very similar findings, but suggested that the stimulation by reticulocyte RWF was unspecific. Our results, however, show that the relative efficiency of Krebs cell-free extracts to translate hemoglobin mRNA as compared to Mengo virus RNA is markedly increased by the addition of reticulocyte factors. When both Mengo and globin mRNA's are in competition, the reticulocyte factors direct the ribosomes to select globin mRNA and under these conditions even produce an inhibition of Mengo RNA translation. Reticulocyte factors, therefore, appear to have messenger discriminating properties.

Reticulocyte RWF contains at least three initiation factors, one of which has been suggested by Anderson [6, 24] and by Heywood [2] to be specific for globin mRNA translation. The stimulation of Mengo RNA observed at high KCl concentration may, in part result from the effect of the non-messenger-specific M1 and M2 factors [14]. This is supported by the fact that addition of reticulocyte RWF shifts the optimal Mg concentration for poly U translation by Krebs extracts from 12 mM to 6 mM with a stimulation of the same order as for Mengo RNA translation (unpublished results). This stimulation is always 3 to 8 times smaller than that of globin synthesis (see table 2).

Endogenous incorporation of leucine, in the absence of added globin mRNA (table 1) is also increased by the reticulocyte factors. In opposition to the interpretation of Metafora et al. [27], however, analysis of the products shows that this is not due to a stimulation of the Krebs mRNA's translation but to the presence of small amounts of globin mRNA in the RWF. Indeed, the only product detected by polyacrylamide gel electrophoresis was globin.

The specific effect of reticulocyte factors on globin synthesis is further illustrated by the fact that adding Krebs RWF or supernatant fractions does not produce the same effect but, on the contrary, decreases the ratio of globin to Mengo RNA translation. We suggest that such specific inhibitions may also result from messenger discriminating factors. This interpretation is based on a similar observation in *E. coli*, in which we have recently found that fac-

tors specific for some mRNA cistrons will inhibit the translation of non-compatible mRNA's [26]. Such inhibitory activities have already been reported by Cohen [5] and Levy et al. [25] in mammalian systems. Mengo RNA translation is not stimulated by the addition of Krebs RWF, possibly because this RNA is already expressed at its maximum capacity in the Krebs extracts used. Another possibility is that Krebs factors are not specific for Mengo RNA translation since Krebs cells are not a good host of this virus. Existence of a factor necessary for Mengo RNA translation could be shown in extracts from L cells grown in suspensions: this factor was practically absent from monolayer cultures of L cells and could not be replaced by reticulocyte RWF (unpublished results).

The demonstration that messenger specific factors, similar to those operating in *E. coli* [1, 26] exist in mammalian cells and differ from one tissue to another will require extensive purification of the elements from several cell-free protein synthesis systems. Preliminary attempts have been reported by Pritchard et al. [24] and by Heywood [2]. Our results would support these conclusions. At least, the present work indicates that although a heterologous system can translate globin mRNA, it does so with an efficiency very much lower than the maximal capacity of the mRNA obtained with homologous factors. Moreover, the ratio at which two different mRNA's can be translated in the same cell-free system is determined by the origin of the factors added.

Acknowledgement

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