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DIFFERENTIAL EFFECTS ON RNA TRANSLATION BY A KCl EXTRACT OF RETICULOCYTE RIBOSOMES: CHARACTERISTICS OF AN INHIBITORY FRACTION*

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Summary — A KCl extract of rabbit reticulocyte ribosomes has been demonstrated to markedly stimulate the translation of various messenger RNAs in a cell-free system from Krebs II ascites tumor cells. In contrast, the translation of encephalomyocarditis viral RNA is strongly inhibited by the same extract. Fractionation of the KCl extract allows the separation of these inhibitory and stimulatory activities. The inhibitory activity has been shown to be the consequence of an unusual endonuclease, associated with ribosomes, that produces approximately 4 S products from the degradation of globin mRNA and viral RNA.

An unfractionated KCl extract of reticulocyte ribosomes can stimulate the translation of messenger and viral RNAs by a cell-free system from Krebs II ascites tumor cells (1,2). However, the crude extract also has a significant inhibitory effect which has not been previously described beyond brief mention (2). While attempting to improve in vitro translation of various RNAs by the cell-free ascites system, we observed that the addition of a crude extract from reticulocyte ribosomes produced differential effects (3). The translation of EMC RNA was markedly inhibited, whereas translation of all other mRNAs was significantly stimulated. In this paper we describe the fractionation of the extract, whereby the stimulatory and inhibitory activities have been separated. Furthermore, the unusual nucleolytic activity of the inhibitory fraction is reported.

MATERIALS AND METHODS

In Vitro Protein Synthesis — The cell-free system from Krebs II ascites tumor cells was prepared and utilized as described by Boime and Aviv (4) except that the preincubation step was extended to 100 min at 37°C. Each cell-free preparation was optimized for Mg^{2+}, KCl, mRNA, and tRNA concentrations for the translation of each mRNA. Except where noted, incubations were for 90 min at 37°C, and results are normalized to 50-µl reaction volumes.

tRNA Preparation — Ascites cells were extracted with phenol, and the RNA was extracted with 1 M NaCl followed by isopropanol fractionation according to the procedure of Von Ehrenstein (5).

Abbreviations: EMC RNA, encephalomyocarditis viral RNA; MOPC, mineral oil-induced plasmacytoma.

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mRNA Preparations — Rabbit globin mRNA either was a generous gift of Dr. W. C. Merrick or was purchased from Amersham/Searle or prepared by us from rabbit reticulocyte polysomes by the preliminary method of Brawerman (6). The latter method was followed by purification of the mRNA by affinity chromatography on poly U-Sepharose-4B and sucrose gradient centrifugation, with the 9 to 10 S fraction collected. Poly A mRNA from ascites cells was isolated and purified in the same manner, and the 10 to 16 S fraction was collected after sucrose gradient centrifugation. MOPC mRNA was a generous gift of Dr. D. Roop. EMC RNA was prepared from virus propagated in Krebs II ascites tumor cells (4).

Preparation and Fractionation of KCl Extracts of Ribosomes — An initial 0.5 M KCl extract of rabbit reticulocyte ribosomes was donated by Dr. W. C. Merrick. Subsequently, we made similar preparations from rabbit and sheep reticulocytes by dialysis of the "KCl wash fraction" followed by DEAE-cellulose batch absorption and 70% ammonium sulfate precipitation as described by Crystal et al. (7). These extracts were fractionated by stepwise elution from DE-23 cellulose by use of 0.1, 0.2, 0.3, and 0.4 M KCl contained in 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA (Buffer A) for elution. Each elution step was terminated when the monitored A_{280} returned to the base line. Peak protein pools collected from the stepwise elution were designated fractions 1, 2, 3, and 4 respectively. They were reduced in volume by ultrafiltration, adjusted to contain 0.1 M KCl and 20% glycerol in buffer A, and stored at -85°C.

Sucrose Density Gradient Analysis —EMC RNA (5 μg) and globin mRNA (5.4 μg) per 50-μl reaction volume were incubated with DEAE-cellulose fraction 1 (42 μg of protein) in buffer A at 37°C for the indicated times. The incubations were terminated by addition of 10 μg of proteinase K for each 50-μl sample; then the samples were incubated for 10 min at 37°C and iced. The entire samples (100 μl) were then layered on appropriate sucrose gradients and centrifuged for various times and speeds at 5°C. Sedimentation patterns were determined by gradient fractionation, monitored and recorded at 254 nm. Sedimentation markers were tRNA from ascites cells ("4 S") and rRNA from reticulocyte ribosomes ("18 S" and "28 S").

RESULTS AND DISCUSSION

Effect of KCl Extract on RNA Translation — Initial translation of various mRNAs by the cell-free ascites system was unsuccessful except with EMC RNA. This lack of success was attributed to loss of labile initiation factors due to the extended preincubation period of 100 min at 37°C. This period had been optimized previously only to provide maximal depletion of endogenous message without affecting subsequent EMC RNA translation. Conceivably, the surviving concentration of initiation factors was sufficient only for EMC RNA translation. To correct this situation, we prepared 0.5 M KCL extracts of reticulocyte ribosomes, treated them to deplete nucleic acids, and tested for possible stimulation of mRNA translation. In Table I, the results of these trials are shown. As shown in Experiments 1 to 3, the translation of globin, MOPC, and poly A-containing ascites cell mRNAs was markedly stimulated by the addition of the 0.5 M KCl extract from reticulocyte ribosomes. Surprisingly, the opposite effect was noted with EMC RNA (Experiment 4); the translation was strongly inhibited by the 0.5 M KCl extract.

Fractionation of the KCl Extract — Whether the differential effects of the KCl extract were due to one or more factors was resolved by fractionation on DEAE-cellulose. The

TABLE I: Effect of 0.5 M KCl extract on translation of RNAs by cell-free ascites protein synthesis system

Experiment[a]	Addition[b,c] (μg)	pmol amino acid incorporated
1	—	1.0
	globin mRNA (4.0)	6.9
	KCl extract (180)	11.1
	globin mRNA + KCl extract	150.9
2	—	3.7
	MOPC mRNA (3.2)	4.0
	KCl extract (180)	19.4
	MOPC mRNA + KCl extract	64.9
3	—	1.9
	ascites poly A mRNA (14.3)	7.5
	KCl extract (180)	13.2
	ascites poly A mRNA + KCl extract	46.7
4	—	0.5
	EMC RNA (8.1)	29.6
	KCl extract (340)	2.0
	EMC RNA + KCl extract	5.1

[a]Experiments 1 and 4 contained 4.3 mM Mg^{2+}, 97 mM KCl; Experiments 2 and 3 contained 3.5 mM Mg^{2+} and 78 mM KCl.

[b]Experiments 1 and 2 contained the same KCl extract preparation; Experiments 3 and 4 contained different preparations.

[c]Experiments 1 to 3 used [^{14}C]leucine, 330 μCi/μmol; Experiment 4 used [^{14}C]phenylalanine, 477 μCi/μmol.

protein fractions produced by an increasing stepwise KCl elution were tested for stimulation of globin mRNA or inhibition of EMC RNA translation. As shown in Figure 1, the stimulatory activity resided mainly in fraction 3, and the inhibitory activity was greatest in fraction 1 and trailed off to zero in fraction 4. It is clear that the differential effects of the KCl wash on RNA translation reside in separate factors of inhibition and stimulation. These factors have a molecular weight greater than 10,000, since they are not removed by dialysis nor by Amicon UM-10 ultrafiltration.

Characterization of the Inhibitory Fraction — Since the stimulatory activity residing in fraction 3 presumably was due to known initiation factors that are limiting in our cell-free ascites system, we decided to concentrate further efforts on fraction 1, which markedly inhibits EMC RNA translation. Earlier results (not shown) on the hydrolysis of ^{3}H-labeled EMC RNA by the 0.5 M KCl extract indicated that the inhibitory effect might be due to nuclease activity. Therefore, EMC RNA and globin mRNA were incubated with fraction 1, the reactions were terminated by the addition of proteinase K to destroy the presumed

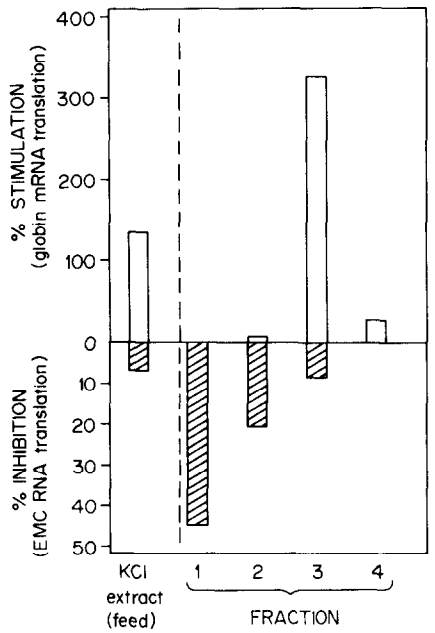

FIG. 1. Effect of fractions from DEAE-cellulose chromatography of the KCl extract on the stimulation (globin mRNA) or inhibition (EMC RNA) of translation. Input was 0.7 μg EMC RNA and 11.0 μg globin mRNA. Each assay contained 4.3 mM Mg^{2+} and 97 mM KCl. Results are normalized to an input of 10 μg of protein for each fraction tested. Remainder of assay and fractionation conditions are given in "Materials and Methods."

nuclease, and the reaction mixtures were analyzed by sucrose density gradient centrifugation. The effect on EMC RNA is shown in Fig. 2A. Even at zero time, which is after a 10-min incubation with proteinase K, the rapid nucleolytic activity almost completely degraded the RNA (thus a zero time without fraction 1 is illustrated). After a 30-min incubation with fraction 1, practically all RNA was degraded to products sedimenting at the same rate as a 4 S RNA marker. The EMC RNA preparation used in this experiment evidently was already somewhat degraded (zero-time curve), indicating that it contained endogenous nuclease activity that caused degradation slowly with time even without added fraction 1 (results not shown). The nuclease activity in fraction 1 also degraded globin mRNA, as shown in Fig. 2B). The globin mRNA is evidently not as labile as EMC RNA, since the zero-time incubation with fraction 1 was not completely degraded. Furthermore, some 9 to 10 S RNA still remained after a 30-min incubation. In contrast to EMC RNA, globin mRNA minus fraction 1 showed identical patterns with zero-time and 30-min incubations (results not shown). We have performed sedimentation experiments with each individual component of the incubation mixture and with the four ribonucleotides to ensure against misinterpretation of the profiles in Fig. 2 (results not shown).

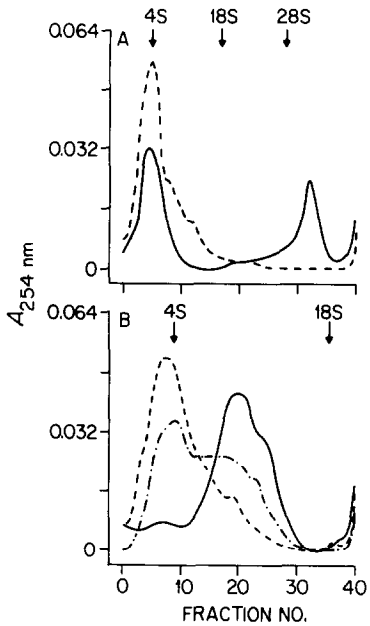

FIG. 2. Effect of DEAE-cellulose fraction 1 on mRNA. RNA was incubated with fraction 1 as detailed in "Materials and Methods," then subjected to sucrose density gradient centrifugation. A, EMC RNA at zero time (——) without fraction 1 and after 30 min incubation (- - -) with fraction 1; 50,000 rpm for 4.8 hr in a 10 to 30% gradient. B, globin mRNA at zero time (——) without fraction 1, and at zero time (- · -) and after 30 min incubation (- - -) with fraction 1; 56,000 rpm for 6.5 hr in a 5 to 20% gradient. Absorbance due to fraction 1 has been subtracted from appropriate profiles. Sedimentation is from left to right.

To further explore the enzymic nature of fraction 1, we tested the heat lability of the preparation. Incubation of fraction 1 at 55°C prior to protein synthesis assays with EMC RNA indicated that the inhibitory activity declined from 86% to approximately 50% after 5 min of heat treatment and then remained constant for at least 25 min of further heat treatment. The same phenomenon was observed after heating at 48°C, except that approximately 30 min of heat treatment was required for a constant value of inhibitory activity to be reached. These observations indicate that possibly two species of nuclease exist in fraction 1.

Since the experiment illustrated in Fig. 2B indicated that fraction 1 degraded globin mRNA, albeit more slowly than EMC RNA, we next tested the effect of preincubation of globin mRNA and fraction 1 prior to the protein synthesis assay. The results clearly indicated that fraction 1 also inhibited globin mRNA translation even though the addition of fraction 3 was necessary for significant translation. Thus, in previous experiments with unfractionated KCl extract the nucleolytic activity towards globin mRNA was masked or protected against.

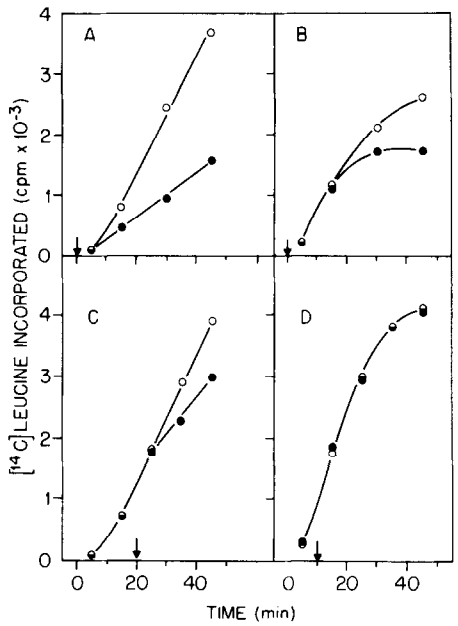

FIG. 3. Time courses of RNA translation and effects of addition of fraction 1. Arrows indicate times of fraction 1 additions (equivalent to 21 µg protein per 50-µl reaction volume). A and C, EMC RNA translations containing 0.68 µg RNA per 50-µl volume. B and D, globin mRNA translations containing 2.7 µg RNA per 50-µl volume. ○, without fraction 1; ●, with fraction 1. Results (cpm) are normalized to 50-µl volumes.

Because the experiments indicated that the nucleolytic activity of fraction 1 affected EMC RNA somewhat differently than globin mRNA, we further examined this phenomenon. When fraction 1 was added to the protein synthesis assay at zero time, the inhibitory effect on the translation of the two RNAs was qualitatively different. As shown in Fig. 3A, the inhibitory action was almost immediate against EMC RNA, but the rate of translation remained linear. Doubling the EMC RNA concentration does not change the qualitative aspects of this inhibition. Conversely, it is shown in Fig. 3B, that the inhibition against globin mRNA translation appeared only after 15 min of incubation time, and the rates were hyperbolic. An interpretation of these results could involve protection of RNA by polysome formation. Thus, fraction 1 was added at various times after the initiation of the reaction. Addition of fraction 1 after 20 min of EMC RNA translation did not change the inhibitory effect qualitatively (Fig. 3C). In contrast, the time course of globin mRNA translation was absolutely unaffected by addition of fraction 1 after 10 min of incubation. This latter result suggests that by 10 min all of the globin mRNA that would be translated in this system was complexed to ribosomes and not affected by added nucleolytic activity once complexed. However, EMC RNA is not protected against an addition of fraction 1 at 20 min, and

perhaps it is degraded while attached to one or more ribosomes. Addition of fraction 1 after 40 min of incubation (results not shown) gave a similar pattern to that shown in Fig. 3C.

The stimulation of translation of all messengers except EMC RNA by the 0.5 M KCl extract or fraction 3 can be accommodated by differences in mRNA association rates with initiation complexes or factors (8–12); depletion of labile initiation factors by extended preincubation of cell-free preparations; and greater susceptibility of EMC RNA to the endonuclease(s).

The inhibitory activity of the KCl extract and fraction 1 is most likely due to endonuclease(s) residing in the reticulocyte ribosome. Hulea and Arnstein (13) have reported small but significant RNase activity in the cytosol and ribosomes of blood reticulocytes, analyzed by production of acid-soluble 260-nm absorbance. Our sucrose gradient analysis (Fig. 2) indicated the degraded RNA products to be approximately 4 S, far too large to be acid soluble. Farkas and Marks (14) partially purified an RNase from the postribosomal supernate of rabbit reticulocytes. This enzyme degraded RNA to oligonucleotides of an average chain length of six, which clearly differentiates it, too, from the activity reported here. We have tested the postribosomal supernate for an effect on EMC RNA translation and have not detected inhibition at protein concentrations even 10-fold higher than those utilized with fraction 1. Conceivably, the characteristics of the nucleases of fraction 1 are comparable to those observed by Brown et al. (15) in extracts of interferon-treated cells. Also, the differential susceptibility of EMC and globin mRNA to degradation might be correlated with the observations of Meyuhas and Perry (16) that small mRNAs are more stable than large mRNAs in vivo. However, our results are too premature for any further comparisons.

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