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Differential Effect of Ca^{+2} on the Translation of Yeast Mitochondrial and Some Viral RNA's in an E.coli Cell-Free System

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SUMMARY: Addition of 6mM $CaCl_2$ to an E.coli cell-free system resulted in a several-fold enhancement of yeast mt RNA translation and in a severe inhibition of protein synthesis directed by MS2, Qβ and T_5 RNA's. $CaCl_2$ did not alter the Mg^{+2} optimum or the time-course of protein synthesis and had no apparent effect on RNA degradation. Formaldehyde treatment of MS2 RNA markedly diminished the $CaCl_2$-mediated inhibition of its translation. Addition of equimolar amounts of EGTA, together with $CaCl_2$, abolished the effect of the latter on cell-free protein synthesis. FMet tRNA binding to ribosomes was enhanced by $CaCl_2$ in the presence of mt RNA, inhibited in the presence of MS2 RNA, and unaffected in the presence of formaldehyde-treated MS2 RNA. Maximal effect on initiation complex formation was observed with 0.1 mM $CaCl_2$.

INTRODUCTION

Translation of RNA preparations in cell-free systems and characterization of the product is frequently the method of choice for assessing the qualitative, and even quantitative, composition of the mRNA population. In this approach it is assumed that all mRNA species are equally well translated when the overall incorporation is optimized. This assumption may, however, not be realized in view of reports that widely different conditions are required for the translation of different mRNA's in prokaryotic (e.g. 1,2) and eukaryotic systems (e.g. 3-5). These different conditions may actually resemble intracellular mechanisms of translational control (cf. 6).

It was noted in the course of translation of yeast mitochondrial RNA (mt RNA) in an E.coli cell-free system, that $CaCl_2$, added *in vitro*, specifically enhanced this translation and inhibited the translation of several viral RNA's. These experiments suggest that the "efficiencies" of mRNA's may be altered by ions in a manner specific to the various mRNA's and that the effect of Ca^{+2} depends on the secondary structure of the RNA.

MATERIALS AND METHODS: Yeast mitochondrial (mt) total RNA was prepared as previously described (7). MS2 RNA was extracted with phenol from CsCl-banded phage and precipitated with ethanol (8). Qβ RNA was a generous gift from Dr. W.T.Hsu and T_5 RNA from Dr. S.B.Weiss. Formaldehyde treatment of MS2 RNA was done according to the procedure of Lodish (9).
E.coli cell-free protein synthesis: S-30 was prepared from quarter log E.coli Q13 cells according to Nathans (10) and stored in aliquots at -70°. Incubation mixtures contained the following components in a final volume of 0.05 ml: 20mM Tris-HCl, pH 7.8, 65mM NH_4Cl, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 25 μg/ml

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Table 1. Effect of $CaCl_2$ on the translation of several RNA's in an E.coli cell-free system

	[^{3}H]leucine incorporation into protein, cpm/5 µl reaction mixture					
Additions	None	mt mRNA	Total mt RNA	MS2 RNA	T_5 mRNA	Qβ RNA
None	1,133	4,572	3,876	13,207	37,916	13,731
6 mM $CaCl_2$	1,371	12,159	14,206	3,958	24,162	2,305

Amounts of RNA added to 0.05 ml reaction mixtures: 5 µg mt mRNA, 2.5 µg MS2 RNA, 4 µg T_5 mRNA, 1.9 µg Qβ RNA and 25.6 µg total mt RNA.

creatine phosphokinase, 6µM [^{3}H] leucine (4Ci/mmole), 30µM of each of the other amino acids, 5 µg leucovorin, 10 µl S-30 and varying amounts of RNA. At the end of 30 min incubation at 37°, aliquots were spotted on Whatman 3MM paper discs and processed as previously described (11).

FMet tRNA binding was measured by a modification of the procedure of Blumberg et al.(12). Ribosomes were pelleted from S-30 by a 2-hour centrifugation at 165,000x g and extracted for 1 hour in 0.4M NH_4Cl in 0.02M Tris-HCl, pH 7.5, 0.02M MgOAc, 5mM EDTA, and 5mM DTT. The final ribosomal pellet was suspended in the above buffer containing 50% glycerol, and aliquots were stored at -70°. [^{3}H] FMet $tRNA_{fMet}$ was prepared according to Kelmers et al.(13). FMet tRNA binding to ribosomes was followed in reaction mixtures which contained the following components in a final volume of 0.05 ml: 0.04 M Tris-HCl, pH 7.5, 0.08M NH_4Cl, 0.02mM GTP, 6mM MgOAc, $3x10^3$ cpm [^{3}H]FMet $tRNA_{fMet}$, 10 OD_{260} units low-salt washed ribosomes and varying amounts of RNA. Following incubation at 37° or 25°, the reaction was terminated by addition of 2.5 ml cold buffer (0.04M Tris pH 7.5, 0.08M NH_4Cl, 6mM MgOAc) containing 10^{-5}M aurintricarboxylic acid. Reaction mixtures were filtered through HAWP Millipore filters, washed with 30 ml buffer, dried and counted in a toluene-based scintillation fluid.

Materials: E.coli B $tRNA_{fMet}$ was purchased from Boehringer; radioactive amino acids were obtained from Amersham; ATP,GTP, CTP, creatine phosphate and creatine phosphokinase were supplied by Sigma. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Yeast mitochondrial RNA exhibited a much lower efficiency than MS2 RNA in directing protein synthesis in an E.coli cell-free system (Table 1). This lower coding efficiency was not the result of a grossly different amino acid composition of the products of these RNA's and was not overcome by altering Mg^{+2} concentrations in the reaction mixtures (results not shown). This could be due to incomplete purification or partial degradation of mt RNA. However, the addition of 6 mM $CaCl_2$ to the cell-free system resulted in an enhancement of mt RNA translation by several

Table 2: Effect of Ca^{+2} on the immunoprecipitation of mt RNA translation products with antibodies against cytochrome oxidase

$[CaCl_2]$	RNA added	Input cpm	Radioactivity precipitated with antibodies: anti cytochrome oxidase cpm	%	from preimmune serum cpm	%
0	None	50880	782	1.5	605	1.2
	mt RNA	75880	2642	3.5	1394	1.8
6 mM	None	46500	652	1.4	469	1.0
	mt RNA	146960	4348	3.0	1759	1.2

62 µg mt RNA were incubated in 0.2 ml reaction mixtures which contained 0.02 ml post-ribosomal supernatant, 3 OD_{260} of low salt washed ribosomes, 0.14 M NH_4OAc pH 6.7, instead of Tris , and the other components described in Methods. Following incubation, reaction mixtures were diluted 5-fold to 0.01 M NaP_i pH 7.5, 0.1 M NaCl, 1% Triton X-100 and 0.2% SDS and incubated 5 hrs at 4° with antibodies raised in rabbits ag-inst holo-cytochrome oxidase or with IgG from preimmune serum,followed by an overnight incubation withgoat anti-rabbit serum. Precipitates were washed by 3 centrifugations through 0.5 M over 1 M sucrose step gradients, dissolved and counted in Instagel.

fold, whereas the translation of MS2, Qβ, and T_5 viral RNA's was severely curtailed under these conditions (Table 1).

Mitochondrial RNA from petite yeast strains can be translated in an E.coli cell-free system (14), and its translation was also enhanced by Ca^{+2} (15). In addition, mt RNA-directed product can be precipitated by antibodies directed against cytochrome oxidase (15,16). The proportion of immunoprecipitated product was similar with and without Ca^{+2} (Table 2), and the immunoprecipitated peptides exhibited the same size distribution (not shown). This indicated that roughly the same mRNA species were translated in both cases, albeit at a higher rate in the presence of Ca^{+2}. It is, however, not certain that the effect is specific to mt RNA or to all T_5 mRNA species since amino acid polymerization directed by poly (GU) was also enhanced in the presence of Ca^{+2} (results not shown). Furthermore, experiments, now in progress, indicate that the enhancement of mRNA translation by ions is not due to the fact mt RNA is heterologous in this system.

Mt RNA-directed protein synthesis was proportional to Ca^{+2} concentration up to 6 mM and remained constant at higher concentrations (Fig. 1). This contrasts the inhibition of protein synthesis by higher than optimal concentrations of Mg^{+2} and suggested that Ca^{+2} did not act to replace Mg^{+2}. Indeed, mt RNA-directed protein

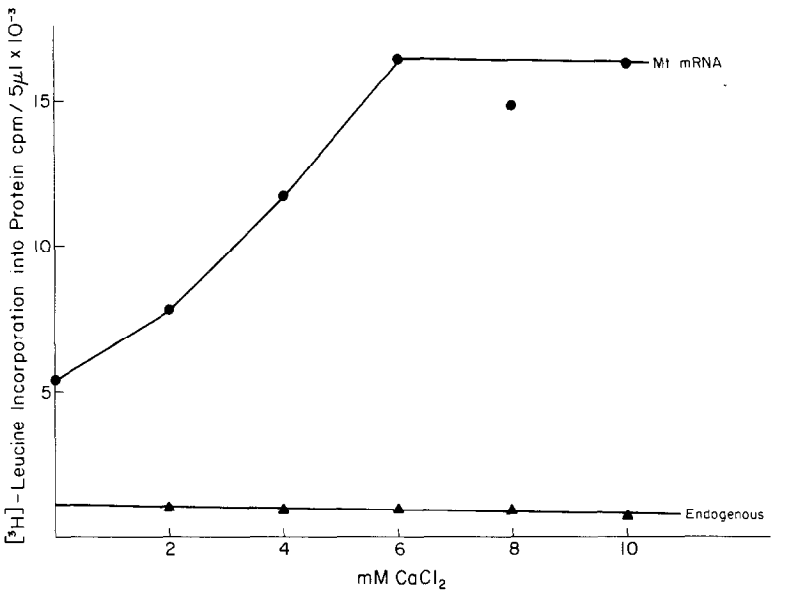

Fig. 1. Effect of $CaCl_2$ concentration on mt RNA translation. ▲—▲, endogenous incorporation; ●—●, synthesis directed by 1.6 µg mt mRNA.

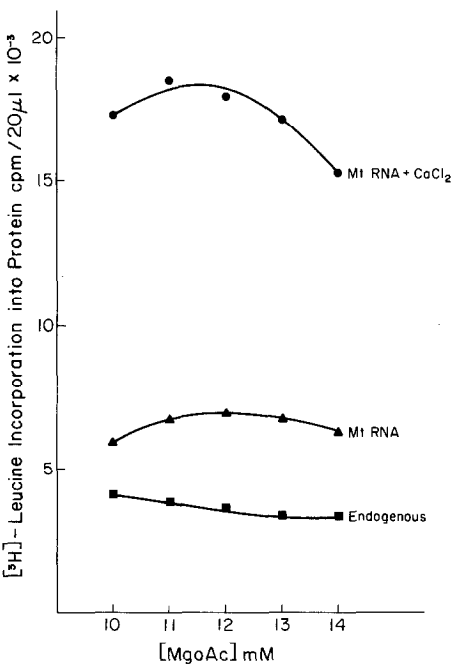

Fig. 2. Effect of Mg^{+2} concentration on mtRNA-directed protein synthesis. Additions: ■—■, none; ▲—▲, 19.5 µg mtRNA; ●—●, 19.5 µg mtRNA + 5 mM $CaCl_2$. Other conditions as described in Materials and Methods.

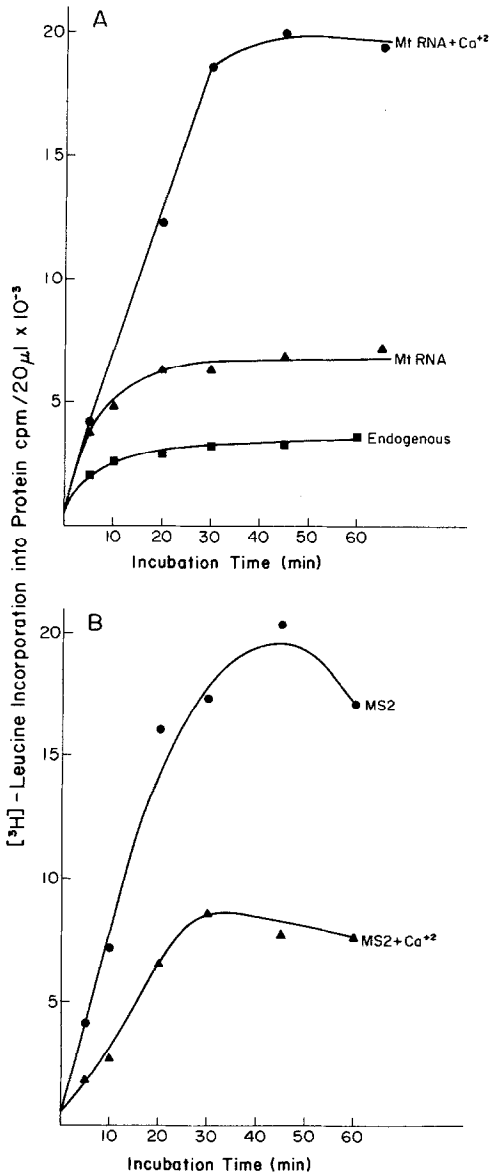

Fig. 3. Time course of protein synthesis in the presence of Ca^{+2}. Where indicated 19.5 µg mtRNA or 2.3 µg MS2 RNA with or without 5 mM $CaCl_2$ were added. Other details are as in Materials and Methods.

A. MtRNA directed incorporation. Symbols: ■——■, no additions; ▲——▲, mtRNA; ●——●, mtRNA + Ca^{+2}.

B. MS2 RNA directed incorporation. Symbols: ●——●, MS2 RNA; ▲——▲, MS2 RNA + Ca^{+2}.

synthesis was optimal at the same Mg^{+2} concentration with and without Ca^{+2} (Fig.2). As may be seen in Fig. 3, protein synthesis followed essentially the same time course with and without Ca^{+2}. Protein synthesis was strongly inhibited by the addi-

Table 3. EGTA abolishes the effect of $CaCl_2$ on the translation of RNA

	[^{3}H]leucine incorporation into protein, cpm/20 µl			
Addition	None	$CaCl_2$	EGTA	EGTA+$CaCl_2$
None	2,539	2,038	2,232	2,477
Mt RNA, 19.2 µg	5,279	10,268	3,941	4,209
MS2 RNA, 1.2 µg	8,813	4,474	9,671	8,083

Specific radioactivity of [^{3}H]leucine was 2.3 Ci/mmole. Where indicated, 5 mM $CaCl_2$ and/or EGTA were added. Other details are described in Methods.

tion of 6mM $CuSO_4$ or $ZnCl_2$, while $MnCl_2$ had no significant effect. On the other hand, addition of an equivalent amount of EGTA, a known Ca^{+2} chelating agent (17), abolished the effect of Ca^{+2} on the translation of mt and MS2 RNA's (Table 3).

Cremer and Schlesinger reported (18) that a nucleolytic activity in an E.coli K12 cell-free system was inhibited by 7mM $CaCl_2$. The addition of $CaCl_2$ to the cell-free system had no apparent effect on the rate of degradation of [^{3}H]adenine labeled mt RNA (not shown). This may be due either to the different E.coli strain employed in these experiments or to the fact that total mt RNA, and not mRNA, was utilized. Also, it is not certain whether the same RNA species are degraded in the presence and absence of Ca^{+2}.

The ratio of the three gene products of MS2 varies in the couse of its translation both in vivo and in vitro (19,20) and in both cases the coat protein comprises most of the product. This can be grossly altered by a mild treatment of the RNA with formaldehyde (9). Such treatment of MS2 RNA resulted in a considerably diminished effect of Ca^{+2} on its translation (Table 4), suggesting that the effect of Ca^{+2} on the coding efficiency of mRNA is dependent on the secondary structure of this RNA.

In isolated experiments, it was noted that a diminished activity of aged MS2 RNA preparations, probably due to degradation, was accompanied by a much smaller effect of Ca^{+2} on their translation, indicating that this effect is related to the structural features of the intact polycistronic RNA. Indeed, as shown by Jacobson (21) an increase in $MgCl_2$ concentration resulted in an increasing number of loops and more structural complexity of MS2 RNA. Such loops can be expected to hinder initiation complex formation and they are not as readily formed in partially degraded MS2 RNA.

Table 4. Alteration of the effect of $CaCl_2$ on MS2 RNA translation by formaldehyde treatment of the RNA

Addition	[^{14}C]amino acid incorporation into protein, cpm/20 µl		
	None (A)	5 mM $CaCl_2$ (B)	Effect of $CaCl_2$ B/A
None	7,975	6,826	
MS2 RNA, 1.2 µg	14,070	9,095	
	(6,097)	(2,269)	0.37
CH_3O-MS2 RNA, 1.2 µg	15,060	12,365	
	(7,087)	(5,539)	0.78
Mt RNA, 16 µg	10,670	13,134	
	(2,697)	(6,308)	2.34

1.2 microcuries [^{14}C]protein hydrolysate were added with 3×10^{-5} M each of methionine, aspartate, asparagine, glutamine and cysteine. Figures in parentheses represent net RNA-directed cpm calculated by substracting endogenous activity.

Usually amino acid incorporation into protein is complete after several minutes of incubation in cell-free systems, such as rat liver microsomes, which display a poor ability to initiate protein synthesis with added natural mRNA's. In these experiments, added mRNA's were translated over a 30-40 min period. The products of MS2 RNA translation in these experiments were immunoprecipitable with anti MS2 serum and exhibited the characteristic distribution upon SDS-polyacrylamide gel electrophoresis. Based on the ratio of the various MS2 gene products (19,20), seen also in these experiments, it was calculated that 8-10 coat molecules were produced per molecule of MS2 RNA. Obviously, such calculations are not yet possible for mt RNA translation since most of the mt gene products and their relative rates of synthesis either *in vivo* or *in vitro* are not known. Nevertheless, the similar fraction of mt RNA-directed product precipitated as cytochrome oxidase peptides (Table 2) suggested several rounds of initiation with this RNA as well. It was, therefore, reasoned that the results in Fig. 3 were explicable as an effect of Ca^{+2} ions on the initiation complex formation with the various RNA's.

This inference was borne out by following the binding of FMet tNRA to ribosomes. The effect of Ca^{+2} on initiation complex formation mediated by mt RNA could be demonstrated either by use of limiting amounts of RNA or by lowering the incub-

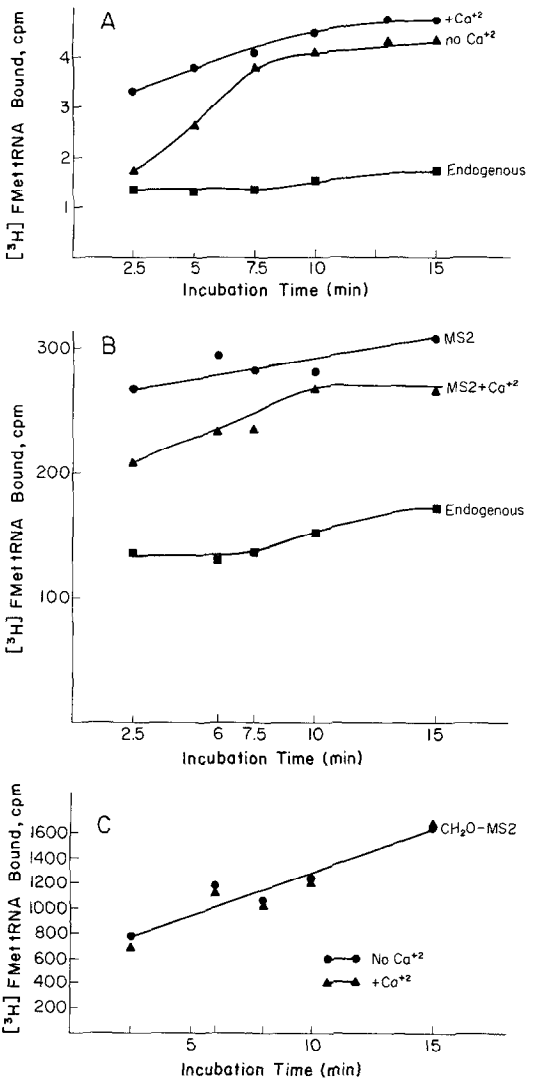

Fig. 4. Effect of Ca^{+2} on FMet tRNA binding. Where indicated, 4 µg Mt RNA, 4 µg MS2 or 1.2 µg CH_2O MS2 RNA were added with and without 0.1 mM $CaCl_2$. Incubation temperatures were 25° in A and C and 37° in B. Other details are as in Materials and Methods.

A. Mt RNA mediated. Symbols: ■——■, no additions; ▲——▲, Mt RNA; ●——●, MtRNA + Ca^{+2}.

B. MS2 RNA mediated symbols: ■——■, no additions; ●——●, MS2 RNA; ▲——▲, MS2 RNA + Ca^{+2}.

C. CH_2O-MS2 RNA mediated. Symbols: ●——●, no Ca^{+} ; ▲——▲, with Ca^{+2}.

ation temperature to 25° (cf. 12). Binding of FMet tRNA, mediated by mt RNA, was enhanced by Ca^{+2} (Fig. 4A), binding mediated by MS2 RNA was inhibited (Fig. 4B), whereas binding mediated by formaldehyde-treated MS2 RNA was unaffected (Fig. 4C). As seen further in Fig. 4, FMet tRNA binding in the presence of mt RNA was enhanced during the early periods of incubation and reached the same plateau earlier when

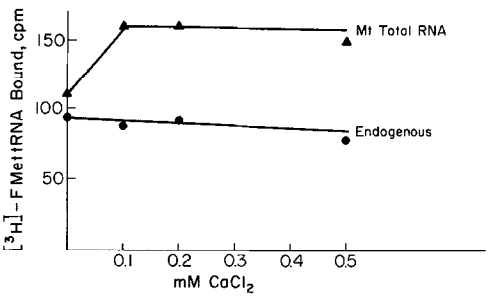

Fig. 5. Effect of Ca^{+2} concentration on mtRNA mediated FMet tRNA binding. Reaction mixtures were incubated for 90 sec at 37°. Symbols: ●——●, no additions; ▲——▲, 4 µg mtRNA

Ca^{+2} was present. Similarly, the plateau obtained with MS2 RNA is also the same with and without Ca^{+2} (Fig. 4B). The fact that the same plateau levels were obtained with and without Ca^{+2}, due to the single cycle of binding measured in this assay, is consistent with the lack of a Ca^{+2} effect on mt RNA degradation mentioned earlier.

The concentrations of $CaCl_2$ employed in these experiments were several orders of magnitude higher than the concentrations of ionic Ca^{+2} involved in several cellular processes (22) and raised a doubt as to the physiological significance of this effect. The discrepancy might be due to the disruption of cellular organization during the preparation of the cell-free extract. Also, the concentrations of Ca^{+2} mentioned in this work are those of total $CaCl_2$ added, and not those of free ionic Ca^{+2}, which may be much lower, as are those measured *in vivo*. It is, therefore, noteworthy that maximal enhancement of FMet tRNA binding, with only washed ribosomes and mt RNA present, was observed already with 0.1 mM $CaCl_2$ (Fig. 5). This is compatible with a possible removal of Ca-binding proteins and lipids in the course of preparing ribosomes for this assay.

Ionic conditions were shown to exert a translational control *in vitro* (3-5) in a manner explicable by the model Lodish (23) since the effects on the translation of one mRNA species were examined in each case. The model assumes permanent differences in the efficiencies of various mRNA species in order to show that during a limitting protein synthetic capacity only the more efficient mRNA's will be translated. The results of this work demonstrate opposing effects of Ca^{+2} on the translation of mitochondrial and several viral RNA's even in the absence of other competing RNA's. They would, thus, be more representative of events in developing systems where the sequential synthesis of proteins is accomplished by utilizing an mRNA population already present prior to the particular stage (24-26). A

changing ionic environment, either through a sequential synthesis of ion carriers (27), during muscle contraction (28), or following fertilization (29), may, thus, result in altered efficiencies of the various mRNA's and yield a changing pattern of proteins synthesized.

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