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The ribosomal fraction mediates the translational enhancement associated with the 5'-leader of tobacco mosaic virus

Daniel R.Gallie*, Virginia Walbot and John W.B.Hershey[1]

Department of Biological Sciences, Stanford University, Stanford, CA 94305 and [1]Department of Biological Chemistry, University of California, Davis, CA 95616, USA

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ABSTRACT

The Ω sequence at the 5'-terminus of tobacco mosaic virus (TMV) RNA acts as a translational enhancer. The differential in Ω-associated translational enhancement between the in vitro translation system derived from wheat germ (WG) and that from rabbit reticulocytes (MDL) was exploited to identify that lysate component which was responsible for a lysate's characteristic reponse to Ω. Using fractionated MDL and WG lysates, which were reconstituted in various combinations, the high salt-washed ribosomal fraction was determined to be the responsive element in a lysate. Analysis of Ω's ability to enhance translation was greatest at low mRNA and high ribosomal concentrations and to occur in the early phase of an in vitro translation assay. Translation of Ω-containing CAT mRNA was more sensitive to the presence of micrococcal nuclease than CAT mRNA without an Ω. In substitution experiments, WG ribosomes functioned at much reduced efficiency in MDL as did MDL ribosomes in WG lysate. The initiation factor-containing fraction of one system could not, as a whole, functionally replace that of the other and actually acted to inhibit translation in the heterologous system.

INTRODUCTION

Although for many years control of gene expression at the translational level has been known in prokaryotes, only recently has translational control been found to exist in eukaryotes. There are now several examples of such control: the mRNAs from the cpa1 gene (1) and GCN4 genes (2, 3) of yeast, the hsp22 and hsp70 mRNAs of Drosophila (4,5), the B class of mRNAs from cytomegalovirus (6), and the ferritin mRNAs of human (7) and rat (8). These mRNAs have been shown to be under specific regulatory control from either cis or trans-acting factors; RNA sequence 5' to the coding region is required for regulation to take place.

Besides those mRNAs under specific control, there is little known about the role that 5' untranslated leader sequences play in the translational efficiency of a particular mRNA. The leader sequences from several plant and animal viruses have been

analyzed for their effect on translation of chimeric mRNAs (9,10). These viral leaders do not seem to be under specific regulatory control, rather they influence the rate of translation. One of these leaders, the 68 base untranslated leader of tobacco mosaic virus (referred to as Ω) has been analyzed in some detail for its ability to enhance translation (9,11,12,13). Translation of reporter mRNAs both *in vivo* and *in vitro* was greatly enhanced when Ω was present at the 5' terminus of the mRNA. This enhancement was observed in plant, animal, and even prokaryotic translational systems. Ω possesses the ability to bind two ribosomes simultaneously in the presence of sparsomycin (14), an inhibitor of elongation. It was hypothesized that disome formation might account for Ω's ability to enhance translation (11). A survey of viral leader sequences which could form disomic structures, however, indicated no correlation between disome formation and the ability to enhance translation (9). Deletion of those sequences within Ω which were implicated in the binding of the second ribosome lead to a significant reduction in the ability of this leader to enhance translation in microinjected *Xenopus* oocytes, but not in electroporated tobacco protoplasts, its native host (9). Although a consistent correlation between disome formation and translation enhancement cannot be made, it seems likely that Ω operates at the level of translational initiation rather than translocation or mRNA stability (9,11).

In addition to determining those sequences necessary for enhancement, we are interested in elucidating the mechanism by which Ω stimulates translation. Using a chloramphenicol acetyltransferase (CAT) mRNA as the reporter mRNA, the enhancement observed in rabbit reticulocyte lysate was markedly less than that observed in wheat germ lysate (11). By isolating the ribosomal and the initiation factor-containing fractions from these two systems and reconstituting them in various homologous or heterologous combinations, we have identified the components of each lysate responsible for the characteristic response to the presence of Ω in a mRNA.

MATERIALS AND METHODS

Plasmids and enzymes

The pSP64 derivative containing Ω has been described (11). The Ω derived sequence which is included within the 5'-end of the chloramphenicol acetyltransferase (CAT) mRNA is defined as: 5'-UAUUUUUACAACAAUUACCAACAACAACAAACAACAAACAACAUUACAAUUACUAUUUACA-AUUACA-3'

The CAT gene from Tn9 was obtained from T.J. Close. SP6 RNA-polymerase, human placental RNase inhibitor, micrococcal (Staphylococcal) nuclease, and BglII were purchased from Pharmacia Ltd. or Boehringer-Mannheim.

RNA synthesis

SP6 derivatives containing CAT or Ω-CAT gene constructs were linearized with BglII which cuts the DNA approximately 450 base pairs downstream of the CAT gene. In vitro transcription of linearized plasmid DNA was carried out using bacteriophage SP6 RNA polymerase as described (15). mRNAs were synthesized in the absence of cap (GpppG) and were quantitated by formaldehyde-agarose gel electrophoresis as described (15).

Fractionation of the lysates

Message-dependent lysate (MDL) was prepared from rabbit reticulocyte lysate as described (18). One ml of the lysate was centrifuged at 85,000 rpm for 90 min at 4°C. The top two-thirds of the supernatant (S-100) was removed and saved, the remaining supernatant was discarded. The ribosomal pellet (P-100) was resuspended in one-tenth the original lysate volume in high salt buffer (20 mM HEPES (KOH) pH 7.2, 10 mM NaCl, 6.1 mM $Mg(OAc)_2$, 0.1 mM EDTA, 0.5 M KCl, 0.1 mM DTT), and the ribosomes pelleted as described above. The high salt-washed ribosomal pellet (HSW Rib) was resuspended in one-tenth the original lysate volume of low salt buffer (20 mM HEPES (KOH) pH7.2, 10 mM NaCl, 1.1 mM $Mg(OAc)_2$, 0.1 mM EDTA, 25 mM KCl, 10%(v/v) glycerol, 0.5 mM DTT). The high salt wash supernatant (HSW) was dialyzed for 4 hr against low salt buffer.

One ml of wheat germ lysate was fractionated into S-100, P-100, and the HSW ribosomes obtained from the P-100 as described

above for the rabbit reticulocyte lysate. As the WG lysate was prepared in 120 mM KCl, the initiation factors (eIFs) are present in the S-100 fraction of the lysate. The WG eIFs from the S-100 were concentrated into two ammonium sulfate fractions: the A fraction (0-40% saturation with ammonium sulfate) and the B fraction (40-60% saturation with ammonium sulfate) as described (16). The two resulting precipitates were collected by centrifugation at 15,000 rpm for 15 min, the pellets resuspended in one-tenth the original lysate volume in 20 mM HEPES (KOH) pH7.6, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 10 % glycerol, and then dialyzed against 100 volumes of the same buffer for 12 hr.

In vitro translation

Wheat germ was generously supplied by Dr. Joanne Ravel. Wheat germ lysate was prepared and used according to Davies *et al*. (17). Messenger-dependent rabbit reticulocyte lysate (MDL) was used as described by Pelham and Jackson (18). Incubations (25 µl) were carried out at 30°C for 60 min unless otherwise indicated. Four µl of each reaction mixture were then removed to determine the level of incorporation of ^{35}S-methionine into trichloroacetic acid-insoluble material. Each reaction was sampled in duplicate, and the resulting counts per minute (cpm) were averaged.

RESULTS

Jobling and Gehrke (10) demonstrated that the enhancing effect of the untranslated leader of alfalfa mosaic virus RNA 4 on chimeric mRNAs containing this sequence was optimized at high input RNA concentration in wheat germ lysate (WG) and rabbit reticulocyte lysate (MDL) *in vitro* translation systems. Although the untranslated leader from TMV (Ω) was shown to enhance translation *in vitro* as well as *in vivo*, its stimulatory effect was determined for only a single input RNA concentration (11). In order to determine whether Ω's ability to enhance translation was insensitive to RNA concentrations, a range of RNA concentrations was tested in WG and MDL using CAT mRNA with or without Ω at the 5' end. Fig. 1 demonstrates the Ω-associated enhancement observed in three independent experiments. In the WG system, Ω was most stimulatory (4 to 7 fold) at low RNA

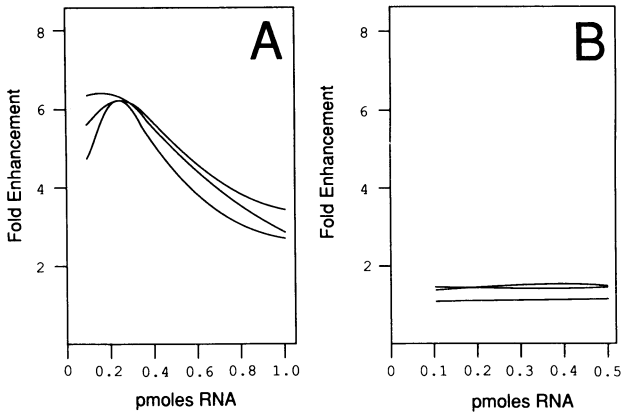

Fig. 1. Comparison of the translational efficiencies of CAT, Ω-CAT, and TMV RNAs in WG lysate (A) and MDL (B). The fold-enhancement afforded by the presence of Ω on CAT mRNA from three separate experiments was plotted as a function of mRNA concentration.

concentrations (0.05 to 0.35 pmole input RNA) and less so (2.7 to 3.5 fold) at higher RNA concentrations (0.7 to 1.05 pmole input RNA). The lower enhancement at the higher RNA concentrations was not the result of ^{35}S-met exhaustion as ^{35}S-met incorporation remained linear throughout the range of RNA levels tested. In contrast to WG lysate, the enhancement observed in MDL remained constant (1.4 to 1.6 fold) throughout the range of RNAs tested.

To determine whether Ω-associated enhancement was constant over the time interval of a WG translation assay, aliquots from translations of CAT or Ω-CAT mRNAs were analyzed for ^{35}S-met incorporation at several times. Ω was found to be most enhancing during the early phase of the assay (Fig. 2). After 40 min, translation of Ω-CAT mRNA had ceased, whereas translation of CAT mRNA continued throughout the entire 75 min assay. This observation suggests that the half-life of Ω-CAT mRNA was less than that of CAT mRNA or that translation of Ω-CAT consumed a limiting factor more rapidly than did CAT mRNA.

The role of Ca++ in translation is not well understood but it is considered to have some importance for efficient translational initiation (19). Increasing amounts of EGTA were

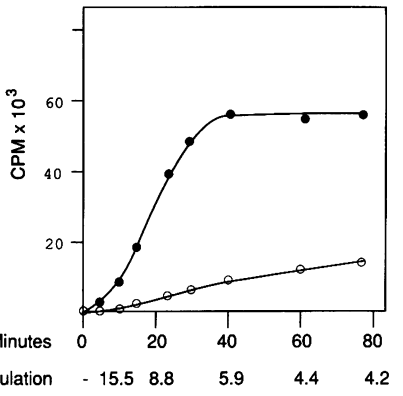

Fig. 2. Ω-associated enhancement in WG lysate as a function of time. O, CAT mRNA; ●, Ω-CAT mRNA. The Ω-associated stimulation was calculated for each time point and is shown beneath the X-axis. Similar displays of the fold stimulation are included in all subsequent figures. CPM, counts per minute.

added to the assay systems to ascertain whether Ω may confer an advantage to CAT mRNA translation under low Ca++ conditions. At EGTA concentrations in excess of 1 mM, translation of CAT mRNA dropped until it ceased at 9 mM (data not shown). In contrast, translation of Ω-CAT mRNA remained virtually unaffected between 1 mM and 9 mM EGTA; at 20 mM this mRNA retained 50% of the efficiency it possessed at 1 mM. In MDL, EGTA had little effect on overall translation, confirming a recent report (19). In the presence of increasing EDTA concentrations, translation of both CAT and Ω-CAT mRNAs was completely inhibited at 2mM EDTA in both in vitro systems (data not shown), a concentration at which almost all the Mg++ present in either lysate (approximately 2.25 mM) would have been chelated. Increasing Ca++ concentrations had a generally negative effect on translation; Ω-CAT was more resistant than CAT mRNA. This ion effect resulted in increasingly greater Ω-associated enhancement (data not shown).

The effect of micrococcal nuclease on Ω-associated enhancement

As reported previously (11), the presence of additional sequence at the 5' end of a mRNA does not mimic the enhancement seen for Ω. This would suggest that the mechanism of Ω-associated enhancement is not increased protection from 5'->3'

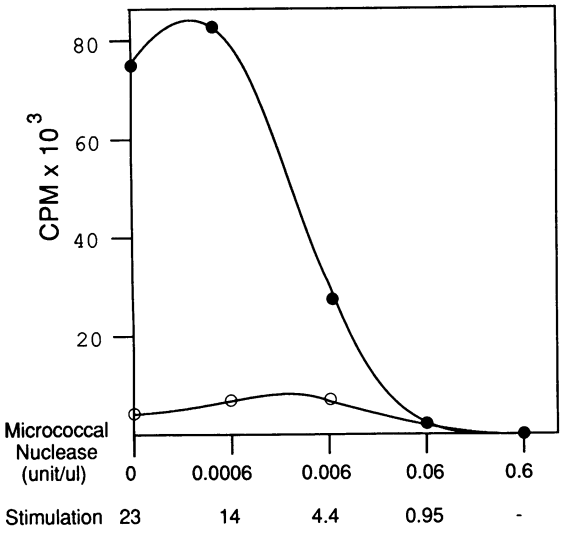

Fig. 3. Translational efficiency of CAT and Ω-CAT mRNAs in WG in the presence of micrococcal nuclease. O, CAT mRNA; ●, Ω-CAT mRNA.

exonuclease activity; furthermore, such an activity has not been described in either eukaryotic or bacterial systems. It is possible that Ω does provide some protection against endonuclease attack, at least within the untranslated leader. To test this possibility, increasing amounts of micrococcal nuclease were added to WG translations reactions of CAT and Ω-CAT mRNA. Because of the Ca++ requirement by the nuclease, the translations were conducted in the presence of 1.25 mM $CaCl_2$ which resulted in a greater differential between CAT and Ω-CAT mRNA translational efficiency. Translation of Ω-CAT mRNA began to exhibit a marked reduction at micrococcal nuclease concentrations above $6x10^{-4}$ units/ml whereas translation of CAT mRNA was not affected until the concentration had increased an order of magnitude, i.e., greater than $6x10^{-3}$ units/ml (Fig. 3). At a nuclease concentration of $6x10^{-2}$ units/ml, the efficiency of Ω-CAT mRNA translation had fallen to that of CAT mRNA translation and for concentrations much greater than $6x10^{-2}$ units/ml, there was no detectable translation for either form of the mRNA. We conclude that Ω is providing no additional protection to a mRNA against a

general endonuclease such as micrococcal nuclease; and because the Ω-containing form of the CAT mRNA was more sensitive to the nuclease, it may, in fact, provide less protection.

The effect of initiation factors on Ω-associated enhancement

As was demonstrated recently (13), Ω's ability to bind two ribosomes simultaneously is not the mechanism by which it brings about translational enhancement, at least in tobacco protoplasts. It is possible that Ω interacts with the eIFs more efficiently, thus raising the local concentration of the eIFs around the Ω-containing mRNA. At least for the cap binding complex, competition for these particular eIFs may be one of the components which produces the different efficiencies seen for various mRNAs in in vitro translation (20). An equally likely hypothesis is that the presence of Ω lowers or obviates the need for a particular eIF. As the WG system is more responsive to the presence of Ω on a mRNA than is MDL, it may be that the responsive component is a WG eIF which is not present (or present at lower amounts) in MDL. To study this possibility, the effect of additional eIFs in a homologous or heterologous assay was tested. Rabbit reticulocyte eIFs were isolated as a high salt-

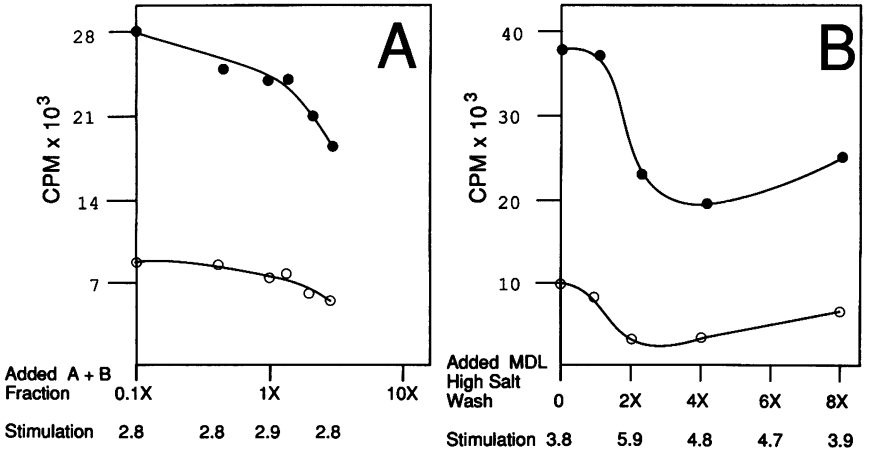

Fig. 4. The effect on enhancement in WG lysate by supplementation with initiation factors isolated from either WG lysate (A) or MDL (B). O, CAT mRNA; ●, Ω-CAT mRNA. X-axis scale refers to the fraction of the physiological level normally present in a lysate which is added back to the translation assay.

wash of the ribosomes (see Material and Methods). The eIFs of WG were isolated as two fractions (referred to as A and B) from the WG lysate S-100. When the WG A and B fractions were added to WG total lysate they had a moderately negative effect on the overall level of translation at high concentrations, but the fold-enhancement conferred by Ω was virtually unaffected (Fig. 4A). When the rabbit reticulocyte eIFs were added to the heterologous WG system, there was a sudden decrease in the overall level of translation but again little change in the Ω-associated enhancement (Fig. 4B). When rabbit reticulocyte eIFs were added to the homologous MDL system, the overall level of translation increased, and there was a small, but measurable increase in the Ω-associated enhancement (a 30% real increase over a 9 fold increase in the concentration of eIFs)(Fig. 5A).

The presence of the WG A and B fractions in MDL had a marked negative effect on translation but no effect on the enhancement by Ω (Fig. 5B). This dramatic negative effect on translation in rabbit reticulocyte lysate by WG eIF-containing fractions can be attributed primarily to the B fraction of WG (data not shown). Even the presence of low concentrations of the B fraction in MDL had a substantial negative effect on translation. But neither the presence of A fraction added alone nor B fraction added alone altered the Ω-associated enhancement typically observed in MDL.

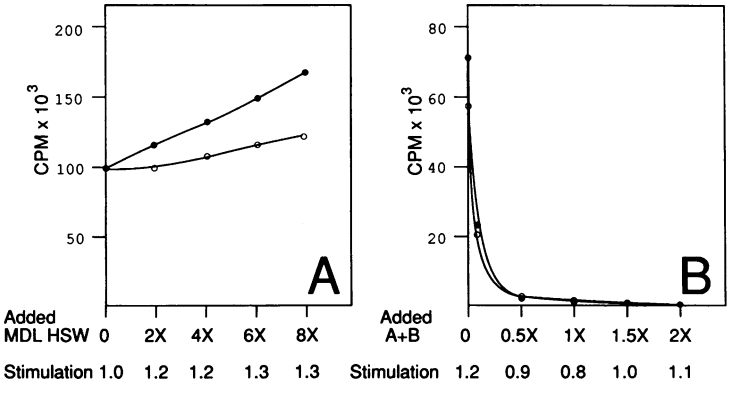

Fig. 5. The effect on enhancement in MDL lysate by supplementation with initiation factors isolated from either MDL (A) or WG lysate (B). O, CAT mRNA; ●, Ω-CAT mRNA. X-axis scale as defined in Fig. 6.

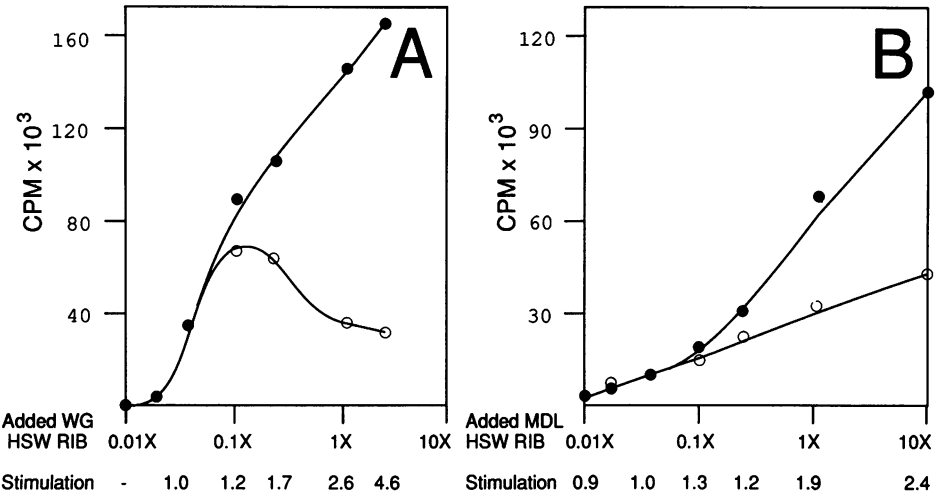

Fig. 6. The effect of ribosome concentration on Ω-associated enhancement in a homologous assay system. High salt-washed ribosomes isolated from WG lysate were added to WG S-100 (A). High salt washed ribosomes isolated from rabbit reticulocyte lysate were added to MDL S-100 (B). O, CAT mRNA; ●, Ω-CAT mRNA. X-axis scale refers to the fraction of the physiological level normally present in a lysate which is added back to the S-100-based assay.

The effect of increasing ribosome concentration on Ω-associated enhancement

As added eIFs from WG or rabbit reticulocyte had little or no effect on the differential translation of Ω-containing mRNAs and those without the Ω enhancer, it was of interest to determine the effect that a range of ribosome concentrations would have on Ω's ability to stimulate translation. Each translation system was fractionated into S-100 and high salt washed ribosomes (HSW Rib), and then the HSW Rib fraction was added back to the S-100 components in a homologous or heterologous reaction mixture. When the HSW Rib of WG were present in the homologous S-100 at only 0.01X to 0.05X their normal concentration, the presence of Ω at the 5' end of the CAT mRNA afforded no translational advantage to the mRNA (Fig. 6A). Above 0.05X normal ribosomal concentration, the translational efficiencies of the two forms of the mRNA began to diverge with the CAT mRNA reaching a maximum

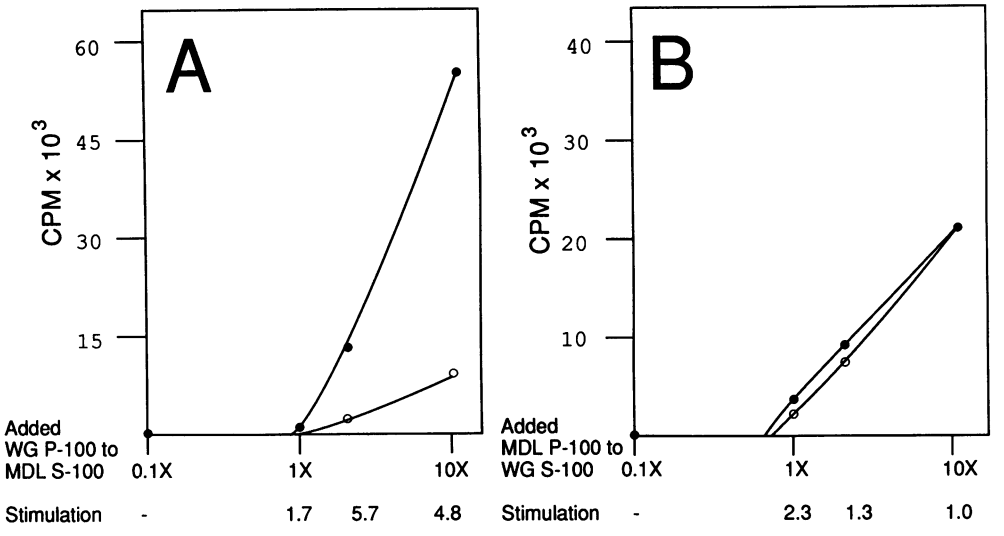

Fig. 7. The effect of ribosome concentration on Ω-associated enhancement in a heterologous assay system. The WG P-100 fraction isolated from WG lysate was added to MDL S-100 (A). The MDL P-100 fraction isolated from MDL was added to WG S-100 (B). O , CAT mRNA; ●, Ω-CAT mRNA. X-axis is as defined in Fig. 6.

efficiency between 0.1X and 0.2X and the Ω-CAT mRNA continuing to increase in efficiency up to 2X the physiological ribosomal concentration. As a result, the fold-enhancement observed for Ω also increased with the ribosomal concentration.

Similarly, when rabbit reticulocyte HSW Rib were present in the homologous S-100 at a concentration 0.01X to 0.1X that which is physiological, the translational efficiencies of the two forms of the mRNA were equivalent; Ω-containing mRNA was progressively more efficiently translated above 0.1X the normal ribosome concentration (Fig. 6B). Neither the CAT nor the Ω-CAT mRNA reached a maximal efficiency but continued to increase in expression up to 10X the physiological ribosomal concentration. Here as in the WG system, the fold-enhancement afforded by Ω continued to increase with the ribosomal concentration.

When the WG P-100 was added to the MDL S-100, overall translational efficiency decreased markedly so that measurable amounts of ^{35}S-met incorporation were not detected until the ribosomal concentration reached the normal (1X) level (Fig. 7A).

The same was observed when the MDL P-100 was added to the WG S-100 (Fig. 7B). Nevertheless, the P-100 fraction of the lysate was shown to be responsible for the characteristic response towards Ω. The presence of the WG P-100 in the MDL S-100 gave rise to Ω-associated enhancement which was typical for that routinely observed in WG lysate (Fig. 7A); conversely, the presence of the MDL P-100 in the WG S-100 resulted in the lower translational response to Ω similar to that seen for MDL (Fig. 7B).

Isolation of the lysate fraction which is responsive to Ω

As established in the previous experiment, the responsive element to Ω was localized to the P-100 of both lysates. Because the P-100 contains eIFs as well as ribosomes, it was of interest to fractionate both P-100 preparations and to substitute each fraction from one lysate system for the fraction of the other. The WG lysate was fractionated as before into S-100, P-100, HSW Rib, A and B fractions. The MDL was fractionated into the MDL S-100, P-100, HSW Rib, and HSW (containing the MDL eIFs). Because the ribosomes of one lysate operate at a much reduced efficiency in the S-100 of the other, it was necessary to use a high input level of mRNA (2.5 pmole or 1 μg). At these high input levels, the Ω-associated enhancement is artificially low in each reconstituted homologous system as the available ^{35}S-met becomes exhausted. As a result, the level of enhancement for the reconstituted WG system, i.e., experiments 2-4 (Table 1), ranged from 2.3 to 3.9 fold, although the overall level of translation was quite high.

When the MDL P-100 was substituted for the WG P-100, the level of translation dropped by over an order of magnitude, and the enhancement afforded by the presence of Ω on the CAT mRNA was only marginally detected or lost (1.03 fold). Once the MDL ribosomes had been subjected to a high salt wash and thereby further depleted of their MDL eIFs, translational efficiency in the WG S-100 dropped another order of magnitude. Nevertheless, the enhancement by Ω was only 1.3 fold, again similar to that seen in the MDL system. If the WG eIFs, in the form of the A and B fractions were added to the MDL HSW Rib, they could not functionally replace the MDL eIFs (and as shown above, the B

TABLE 1
Isolation of the lysate fraction which is reponsive to Ω

Experiment	Components in assay	mRNA	^{35}S-met incorporation into protein (cpm)	Fold Stimulation
1	WG S-100	CAT	0	-
		Ω-CAT	0	-
2	WG S-100 + WG P-100	CAT	705043	1
		Ω-CAT	1598112	2.3
3	WG S-100 + WG HSW Rib	CAT	252564	1
		Ω-CAT	987422	3.9
4	WG S-100 + WG HSW Rib + A + B	CAT	256342	1
		Ω-CAT	968076	3.8
5	WG S-100 + MDL P-100	CAT	28398	1
		Ω-CAT	29379	1.03
6	WG S-100 + MDL HSW Rib	CAT	1945	1
		Ω-CAT	2525	1.3
7	WG S-100 + MDL HSW Rib + A + B	CAT	1859	1
		Ω-CAT	1795	0.97
8	WG S-100 + MDL HSW Rib + MDL HSW	CAT	11155	1
		Ω-CAT	9982	0.89
9	WG S-100 + MDL P-100 + MDL HSW	CAT	33367	1
		Ω-CAT	38659	1.2
10	MDL S-100	CAT	0	-
		Ω-CAT	0	-
11	MDL S-100 + MDL P-100	CAT	313543	1
		Ω-CAT	307190	0.98
12	MDL S-100 + MDL HSW Rib	CAT	185377	1
		Ω-CAT	193858	1.05
13	MDL S-100 + MDL HSW Rib + MDL HSW	CAT	354490	1
		Ω-CAT	327006	0.92
14	MDL S-100 + WG P-100	CAT	203875	1
		Ω-CAT	978833	4.8
15	MDL S-100 + WG HSW Rib	CAT	25737	1
		Ω-CAT	95227	3.7
16	MDL S-100 + WG HSW Rib + A + B	CAT	44178	1
		Ω-CAT	225308	5.1
17	MDL S-100 + WG HSW Rib + MDL HSW	CAT	14651	1
		Ω-CAT	45432	3.1
18	MDL S-100 + WG HSW Rib + MDL HSW + A + B	CAT	25091	1
		Ω-CAT	107891	4.3
19	MDL S-100 + MDL HSW + A + B	CAT	0	-
		Ω-CAT	0	-

fraction is, in fact, inhibitory) as there was no stimulation of translational activity observed (compare experiments 7 to 6 in Table 1). The fold-enhancement remained close to 1.0. The

addition of MDL HSW to MDL HSW Rib substantially restored translational activity but no enhancement was observed for Ω (0.89 fold). Similar results were obtained when MDL HSW was added to MDL P-100 (experiment 9).

When the MDL lysate was fractionated and reconstituted in various fashions (experiments 11-13), no Ω-associated enhancement was observed, i.e., the ratio remained close to 1. If the MDL P-100 was replaced by WG P-100, the enhancement increased to 4.8 fold. If WG HSW Rib were used instead, the level of translation dropped almost an order of magnitude but the level of enhancement remained high at 3.7 fold. Addition of the WG A and B fractions to the components of experiment 15 increased the overall level of translation (experiment 16) and the enhancement observed was 5.1 fold. If MDL HSW was added instead to the components of experiment 15, a reduction in the level of translation was observed but the enhancement was still 3.1 fold. Finally, if the WG A and B fractions were added to the components of experiment 17, there was some restoration of activity, and the enhancement remained high at 4.3 fold. These data suggest that the component which is responsible for a lysate's characteristic response to Ω is contained in the high salt-washed ribosomal fraction.

Exchange translations

If the responsive component of a lysate is confined to the high salt-washed ribosomal fraction, the gradual exchange of the ribosomal fraction of one lysate for the other should result in a steady alteration in the fold-enhancement, independent of the S-100 background. In addition, if the ribosomal fraction is held constant, but the S-100 background is gradually exchanged from one lysate type to the other, the fold-enhancement should remain constant.

Initially, such "exchange translations" of CAT and Ω-CAT mRNAs were carried out using unfractionated MDL and WG lysates (Fig. 8). A series of translations were designed so that the WG lysate was gradually replaced with MDL. The fold-enhancement in the translation containing WG lysate was 8.4. As MDL began to replace the WG lysate, overall translational efficiency dropped as observed previously. The fold-enhancement also dropped from 8.4 to 1.9 by the point at which 50% of the WG lysate had been

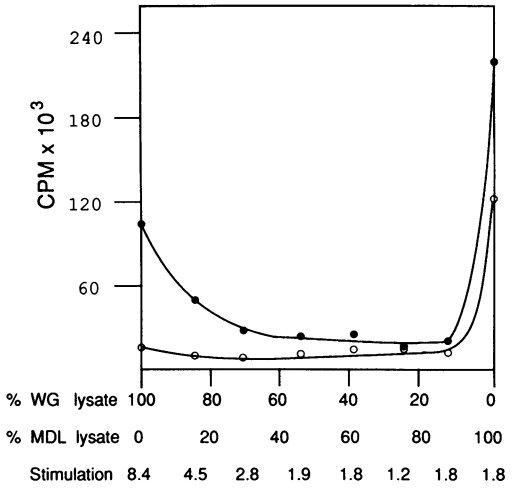

Fig. 8. The effect of gradually exchanging the WG lysate system with MDL on Ω's ability to enhance translation. O , CAT mRNA; ● , Ω-CAT mRNA. X-axis scale represents the percentage of each lysate present in the assay.

replaced by MDL. This level of enhancement remained relatively constant even when MDL was the only lysate present.

When HSW ribosomes of WG were gradually replaced by MDL HSW ribosomes in a WG S-100 background, the overall efficiency of translation dropped accordingly, as expected. The fold-enhancement dropped from 5.2 when only WG HSW Rib were present to 1.5 when only MDL HSW Rib were present (Fig. 9A). In a MDL S-100 background, the predicted drop in translational efficiency was observed when HSW MDL ribosomes were gradually replaced by WG HSW ribosomes (Fig. 9B); but again the fold-enhancement followed the ribosome type. The 1.5 fold-enhancement that was observed when only MDL HSW Rib were present gradually increased to a 4.7 fold-enhancement when only WG HSW Rib were present (Fig. 9).

In the next two experiments, the ribosome type was constant, but one S-100 type was gradually replaced by the other. In the experiment in which HSW Rib of WG were used, the initial fold-enhancement in WG S-100 was 5.6 (Fig. 10A). As the WG S-100 was gradually replaced with MDL S-100, there was the usual drop in translational efficiency but the fold-enhancement did not alter

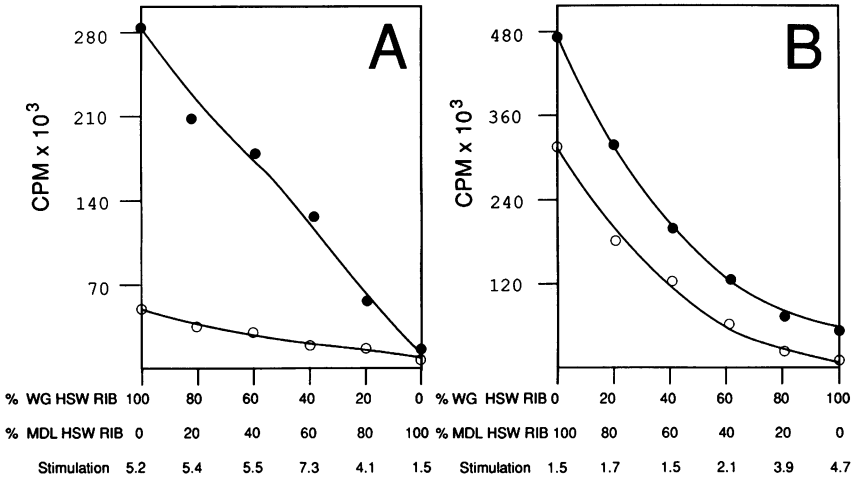

Fig. 9. The effect of gradually exchanging WG high salt-washed ribosomes with high salt-washed rabbit reticulocyte ribosomes in a WG S-100 background (A) and the effect of exchanging high salt-washed rabbit reticulocyte ribosomes with WG ribosomes in a MDL S-100 background (B) on Ω-associated enhancement. O , CAT mRNA; ● , Ω-CAT mRNA. X-axis scale represents the percentage of each ribosome type present in the assay.

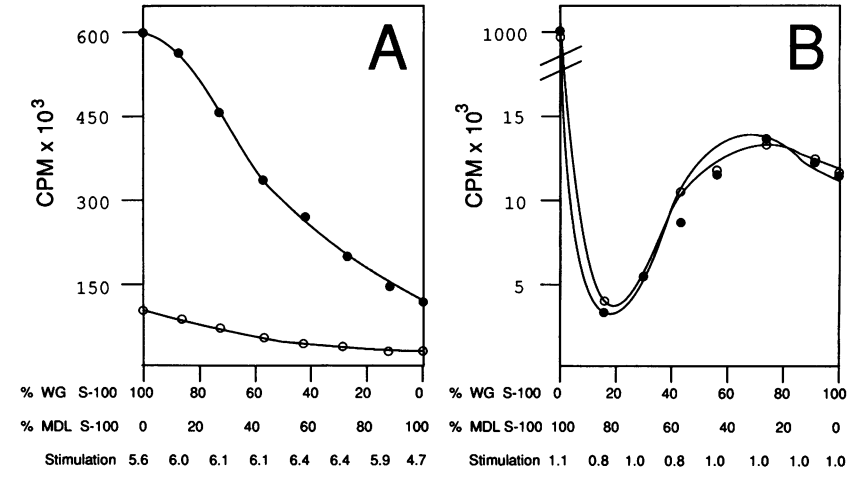

Fig. 10. The effect of gradually exchanging WG S-100 with MDL S-100 on WG high salt-washed ribosomes' ability to respond to Ω (A) and the effect of exchanging MDL S-100 with WG S-100 on MDL high salt-washed ribosomes' ability to respond to Ω (B). O , CAT mRNA; ● , Ω-CAT mRNA. X-axis scale represents the percentage of each S-100 type present in the assay.

significantly, varying from 4.7 fold to 6.4 fold. When MDL HSW ribosomes were used, the translational efficiency dropped dramatically as soon as the MDL S-100 began to be replaced by small amounts of WG S-100 (Fig. 10B), yet the translational ratio remained within the range of 0.8 to 1.1 fold, typical for that observed with MDL translations.

DISCUSSION

Ω, when present at the 5'-end of a mRNA enhances its translational efficiency *in vitro* and *in vivo*. It should be noted that this enhancement is markedly less in an *in vitro* assay system such as WG lysate (4-8 fold) than seen in an *in vivo* system such as electroporated tobacco protoplasts (60-320 fold (9)). This difference between *in vitro* and *in vivo* systems has also been observed for the stimulatory effect of a cap (m^7GpppG) on the translation of a mRNA; the effect can be at least an order of magnitude greater *in vivo* than *in vitro* (22). However, the *in vitro* systems are more amenable to manipulation and control of assay conditions than whole cells.

The mechanism by which Ω stimulates translation is unknown although several possibilities are unlikely based on present evidence. First, Ω's ability to bind two ribosomes simultaneously in the presence of sparsomycin is not the reason for its ability to enhance translation in plants, although this mechanism can not be ruled out for *Xenopus* oocytes (13). Second, the enhancement is specific to Ω as unrelated sequences of equal length do not necessarily result in enhancement (11). Third, Ω does not seem to increase mRNA stability. Indeed, the time course experiment between CAT and Ω-CAT mRNA (Fig. 3) demonstrated that translation of Ω-CAT ceased well before that of CAT mRNA. Moreover, the Ω-containing form of the CAT mRNA exhibited greater sensitivity to micrococcal nuclease than CAT mRNA without Ω. Fourth, Ω does not function to promote internal initiation, as Ω loses the ability to enhance translation if it is not positioned at the 5' terminus (23). Moreover, when the sequence Ω itself is circularized, it can no longer bind 80S ribosomes (24).

EDTA had an equal effect on either the CAT or Ω-CAT mRNAs,

suggesting that the mechanism by which Ω brings about enhancement cannot overcome the need for Mg^{++}. In contrast, there was a significant difference between CAT and Ω-CAT mRNA in their responses to EGTA. Low concentrations of EGTA (0.1 mM) actually stimulated overall translation but higher concentrations (>1 mM) were detrimental to CAT mRNA translation. The presence of Ω improved markedly the ability of mRNA to continue to be translated under high EGTA levels. It is doubtful that the high levels of EGTA are only required to chelate Ca^{++}, as the Ca^{++} levels in WG are normally low. It is possible that the high EGTA concentration is chelating Mg^{++}, but as EDTA equally suppressed CAT and Ω-CAT mRNA translation, this would not account for the significant differential seen between the two forms of the mRNA in the presence of EGTA. In contrast to these findings in the WG system, EGTA had little effect on translation in rabbit reticulocyte lysate.

If the greater translational efficiency associated with Ω was the result of a more efficient interaction with eIFs, supplementation of lysates with eIF-containing fractions might reduce the observed translational efficiency differential. No such reduction was observed in either WG or rabbit reticulocyte lysate. Supplementation of rabbit reticulocyte lysate with rabbit reticulocyte eIFs did produce a modest increase in the enhancement associated with Ω, although the eIF concentration was present up to nine times the physiological level.

Interestingly, as a group, cannot functionally replace rabbit reticulocyte eIFs in a MDL lysate. Nor can the rabbit reticulocyte eIFs replace the WG eIFs in a WG lysate. In fact, the B fraction from WG lysate, which is known to contain eIF-2, eIF-4A, eIF-5, along with EF-1α , EF-2, and EF-1βγ (21), dramatically inhibited translation in unfractionated MDL. The eIF-containing fraction from MDL was also moderately inhibitory to unfractionated WG lysate. In addition, Abramson et al. (23) have observed that certain wheat germ eIFs (e.g. 4A and 4F) are functionally equivalent in either WG lysate or MDL, whereas other WG eIFs are less able to replace their MDL eIF counterparts.

The ability of Ω to enhance translation was greatly affected by the ribosome concentration. In either lysate system,

enhancement was not observed until the ribosomal concentration reached 0.05X to 0.1X its physiological level. Enhancement continued to increase along with the ribosome concentration. When the ribosomal fraction of each lysate was substituted for the other, the enhancement followed the ribosomal fraction. Further such substitutions between the two translation systems delineated the high salt-washed ribosomal fraction as the Ω-responsive element of a lysate. As a high salt-wash removes the Ω eIFs which are associated with the ribosomes, it is very likely that it is the ribosome itself which is responding to the sequence, although it can not be ruled out that there is some ribosomal associated factor, previously unknown, which is resistant to removal by the high salt wash procedure. What is clear is that the Ω-responsive component of a lysate is not found in the eIF-fraction or the S-100 fraction. If it is the ribosome type which is responding differently to Ω, then this would demonstrate that the WG and rabbit reticulocyte ribosomes are not functionally equivalent in their interaction with mRNAs.

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*To whom correspondence should be addressed

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