N1-methyl-pseudouridine in mRNA enhances translation through eIF2α-dependent and independent mechanisms by increasing ribosome density

SUPPLEMENTARY DATA

Contents:

Figure S1

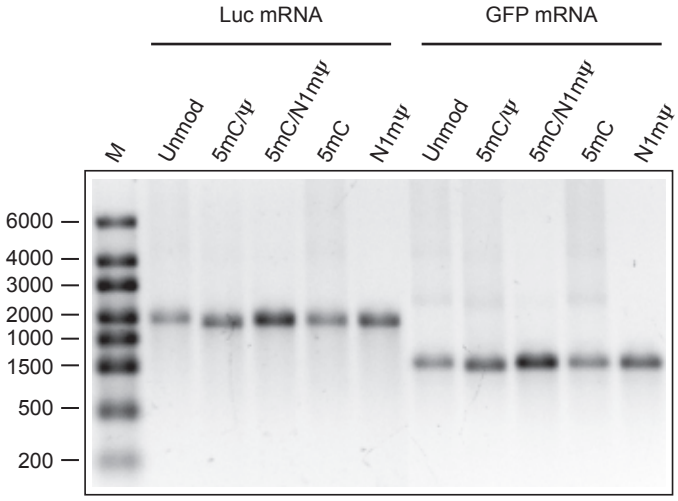
Luc mRNA
GFP mRNA
M
Unmod
5mC/ψ
5mC/N1mψ
5mC
N1mψ
Unmod
5mC/ψ
5mC/N1mψ
5mC
N1mψ
6000
4000
3000
2000
1000
1500
500
200

Figure S1. Analysis of mRNA integrity. The indicated unmodified and modified Luc or GFP mRNAs were analysed by denaturing 1% agarose gel electrophoresis. RNA bands were visualised by ethidium bromide staining. A negative image is shown. The positions of high range RNA markers (200 to 6000 bases; MBI) are indicated on the left.

Figure S2

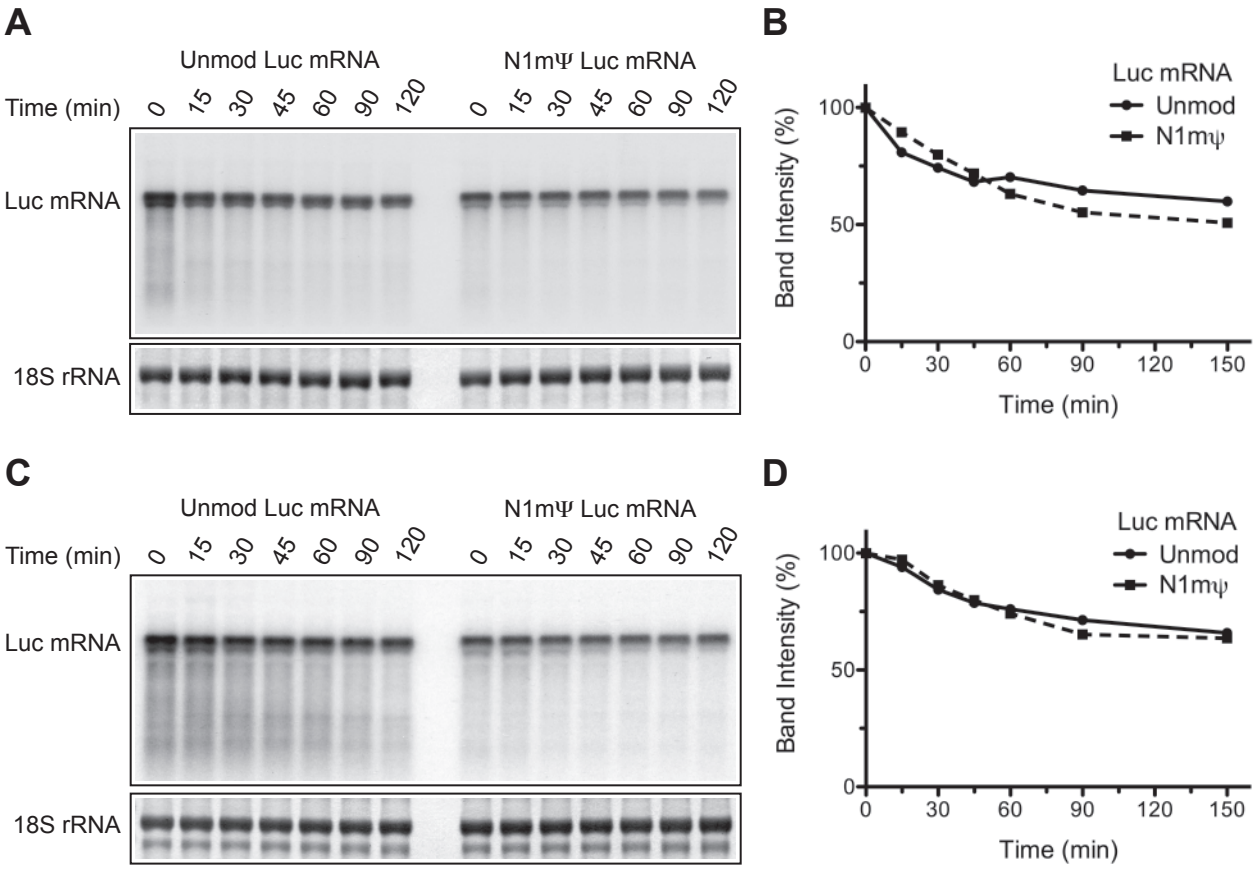
A
Unmod Luc mRNA
N1mΨ Luc mRNA
Time (min)
0 15 30 45 60 90 120
0 15 30 45 60 90 120
Luc mRNA
18S rRNA
B
Luc mRNA
Unmod
N1mψ
Band Intensity (%)
100
50
0
0 30 60 90 120 150
Time (min)
C
Unmod Luc mRNA
N1mΨ Luc mRNA
Time (min)
0 15 30 45 60 90 120
0 15 30 45 60 90 120
Luc mRNA
18S rRNA
D
Luc mRNA
Unmod
N1mψ
Band Intensity (%)
100
50
0
0 30 60 90 120 150
Time (min)

Figure S2. Incorporation of N1mΨ into Luc mRNA does not alter its stability in Krebs extract. Unmodified or N1mΨ-incorporated Luc mRNAs (4 μg/ml) were used to program untreated (**A** and **B**) or RNase-treated (**C** and **D**) Krebs extracts. Total RNA was isolated at the indicated times from the aliquots of the reaction mixtures. (**A** and **C**, top panels) Northern blot analysis of Luc mRNA integrity. (**A** and **C**, bottom panels) 18S ribosomal RNA (rRNA) visualised by staining. Note that RNase treatment results in partial cleavage of 18S rRNA, as previously reported (1). Quantification of Luc mRNA signals from panels **A** and **C** are shown on panels **B** and **D**, respectively (data are given after correction for the levels of intact 18S rRNA). Band intensities of Luc mRNAs recovered at time 0 were set at 100%.

REFERENCE

1. Holmberg,L. and Nygard,O. (1997). Mapping of nuclease-sensitive sites in native reticulocyte ribosomes - an analysis of the accessibility of ribosomal RNA to enzymatic cleavage. *Eur. J. Biochem.*, **247**, 160-168.

Figure S3

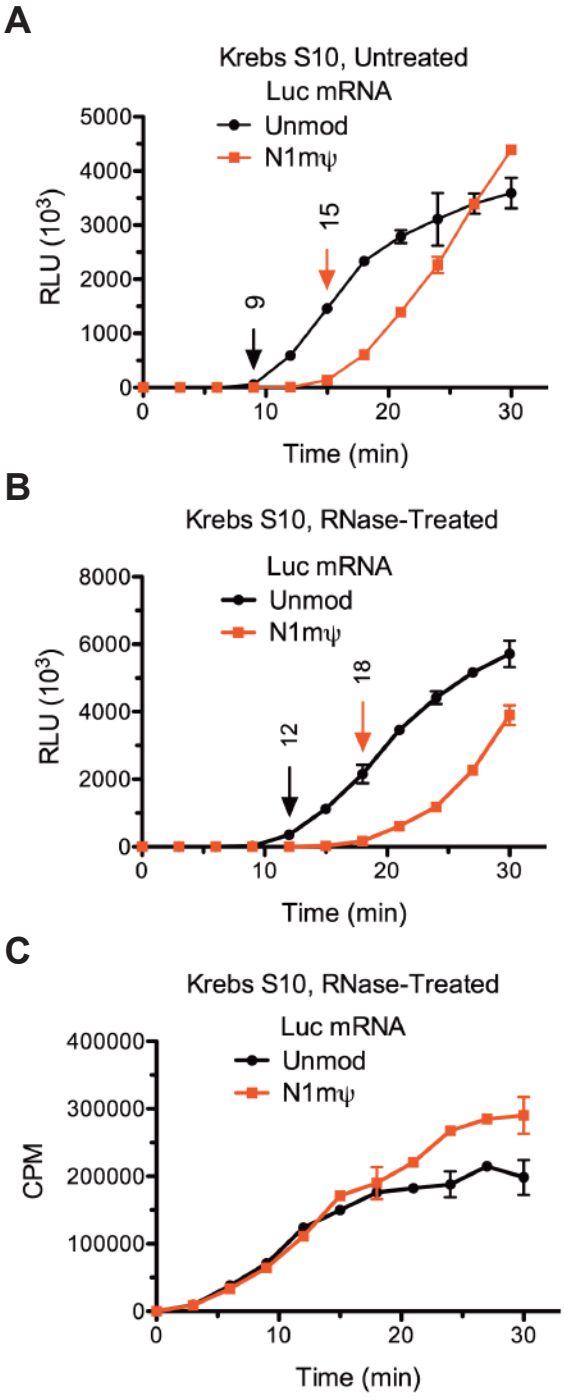
A
Krebs S10, Untreated
Luc mRNA
Unmod
N1mψ
RLU (10^3)
5000
4000
3000
2000
1000
0
9
15
0
10
20
30
Time (min)
B
Krebs S10, RNase-Treated
Luc mRNA
Unmod
N1mψ
RLU (10^3)
8000
6000
4000
2000
0
12
18
0
10
20
30
Time (min)
C
Krebs S10, RNase-Treated
Luc mRNA
Unmod
N1mψ
CPM
400000
300000
200000
100000
0
0
10
20
30
Time (min)

Figure S3. High-resolution kinetics of translation of unmodified and N1mΨ-modified Luc mRNA in Krebs extracts. (**A** and **B**) Kinetics of synthesis of active luciferase in untreated (**A**) or RNase-treated (**B**) Krebs extracts supplemented with Luc and N1mΨ-Luc mRNAs. Extracts receiving the mRNAs (4 µg/ml) were incubated at 30°C. Aliquots (1 µl) of the reaction mixtures were withdrawn in 3-min intervals and assayed for luciferase activity. Arrows indicate the time points of first appearance of luciferase activity. (**C**) Kinetics of ^{35}S-methionine incorporation in RNase-treated Krebs extracts (12.5 µl) supplemented with Luc or N1mΨ-Luc mRNAs (4 µg/ml). Incorporation in samples incubated without mRNA is subtracted. Data are means of two replicates ± SD. Incorporation of N1mΨ in Luc mRNA, while delaying active luciferase production, does not decrease the initial rate of ^{35}S-methionine incorporation (compare panels **B** and **C**).

Figure S4

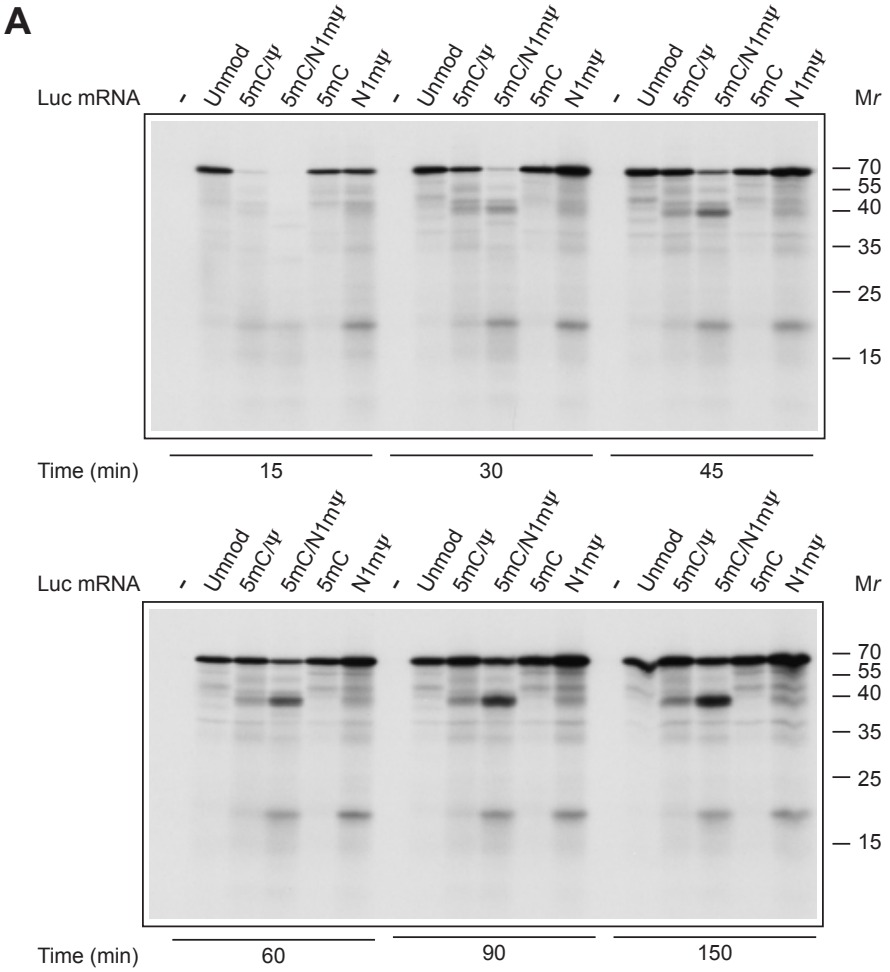
A
Luc mRNA
-
Unmod
5mC/ψ
5mC/N1mψ
5mC
N1mψ
Mr
70
55
40
35
25
15
Time (min)
15
30
45
60
90
150
B
Input
IP: α-Luc N-t
IP: α-Luc C-t
C
Luc mRNA
Unmod
5mC/ψ
5mC/N1mψ
5mC
N1mψ
CPM
150000
100000
50000
0
0
30
60
90
120
150
Time (min)

Figure S4. Time course of synthesis of translation products in RRL programmed with unmodified or modified Luc mRNAs. (**A**) Unmodified or 5mC/Ψ, 5mC/N1mΨ, 5mC and N1mΨ-incorporated Luc mRNAs (4 µg/ml) were programmed in RNase-treated RRL in the presence of ^{35}S-methionine. At the indicated time points, aliquots of the reaction mixtures were withdrawn, and translation was stopped by the addition of SDS-sample buffer. SDS-PAGE analysis of the ^{35}S-methionine labelled translation products is presented. (**B**) 5mC/N1mΨ Luc mRNA was translated in RRL in the presence of ^{35}S-methionine. The products of translation were immunoprecipitated with the antibodies directed against the N-terminal portion (N-t) or C-terminal portion (C-t) of luciferase and resolved by SDS-PAGE as described in Materials and methods. (**C**) ^{35}S-methionine incorporation in 1-µl aliquots of samples from panel **A.** Data are given after subtraction of the values of endogenous incorporation and are means of two measurements ± SD. The presence of 5mC/Ψ and 5mC/N1mΨ in Luc mRNA, while delaying the appearance of the full-size luciferase protein (**A**), does not decrease the initial rate of ^{35}S-methionine incorporation (**C**).

Figure S5

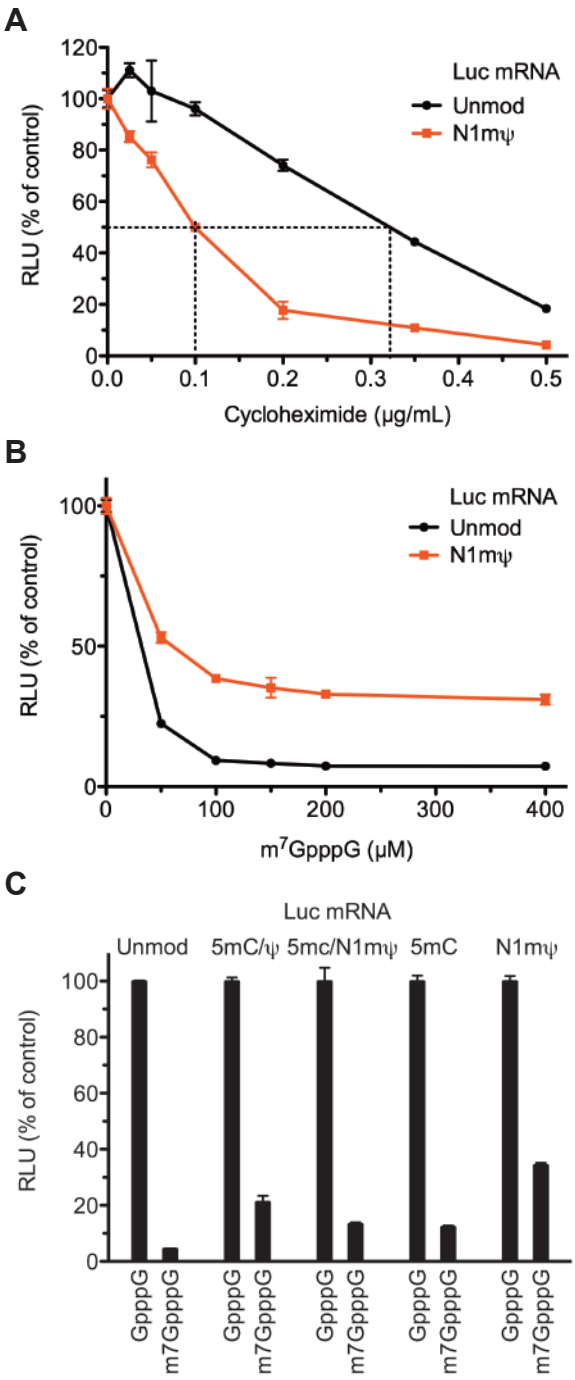
A
RLU (% of control)
Cycloheximide (µg/mL)
Luc mRNA
Unmod
N1mψ
B
RLU (% of control)
m7GpppG (µM)
Luc mRNA
Unmod
N1mψ
C
Luc mRNA
Unmod
5mC/ψ
5mc/N1mψ
5mC
N1mψ
RLU (% of control)
GpppG
m7GpppG

Figure S5. Unmodified and N1mΨ-modified Luc mRNAs exhibit different sensitivity to translation inhibitors in Krebs extract. (**A**) Incorporation of N1mΨ in Luc mRNA accentuates the sensitivity of translation to the inhibitor of elongation. Luc and N1mΨ-Luc mRNAs (0.8 µg/ml) were translated in an RNase-treated Krebs extract at 30°C for 150 min. Cycloheximide was present in the translation samples at the indicated concentrations. The values for luciferase synthesis were normalised to the translation levels of Luc and N1mΨ-Luc mRNAs in the minus cycloheximide control samples (set as 100%). The IC_{50} cycloheximide concentrations determined were 0.1 µg/ml and 0.32 µg/ml for N1mΨ-Luc and Luc mRNAs, respectively. (**B** and **C**) Nucleoside modifications in Luc mRNA increase the resistance of translation to inhibition by a cap analogue. (**B**) Luc and N1mΨ-Luc mRNAs were translated as described for panel (**A**). Prior to mRNA addition, the extracts were pre-incubated at 30°C for 2 min with the indicated concentrations of the cap analogue m^7GpppG. The values were normalized to the translation levels of Luc and N1mΨ-Luc RNAs in the water control samples (set as 100%). In the absence of inhibitors, the template activity of N1mΨ-Luc mRNA scored ~3.2-fold (**A**) and ~3.5-fold (**B**) higher than Luc mRNA. (**C**) Unmodified or 5mC/Ψ, 5mC/N1mΨ, 5mC and N1mΨ-incorporated Luc mRNAs were translated as in **A**. Prior to mRNA addition, the extracts were pre-incubated at 30°C for 2 min with either active (m^7GpppG) or inactive (GpppG) cap analogue (0.2 mM). Luciferase synthesis from each mRNA was normalized in reference to a value of 100% for the GpppG-containing control samples. Data are means from three samples ± SD.

Figure S6

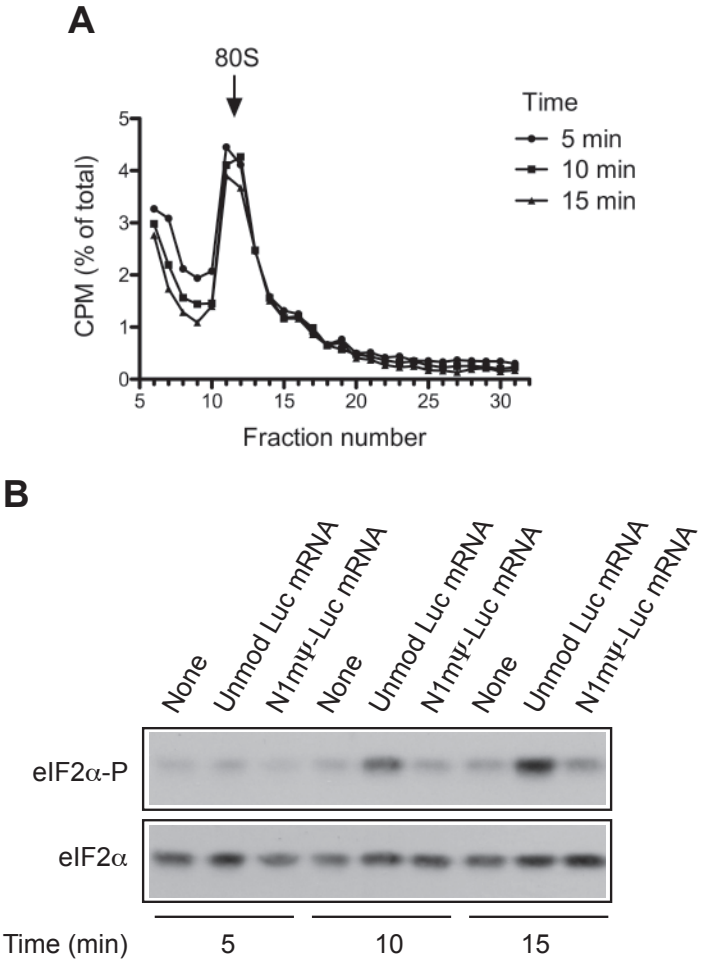
A
80S
Time
5 min
10 min
15 min
CPM (% of total)
Fraction number
B
None
Unmod Luc mRNA
N1mψ-Luc mRNA
eIF2α-P
eIF2α
Time (min)
5
10
15

Figure S6. Kinetic analysis of 80S initiation complex formation and eIF2α phosphorylation in Krebs extract. (**A**) Untreated Krebs extract supplemented with 3'-end labelled unmodified Luc mRNA was incubated at 30°C for the indicated times. 80S initiation complex formation was analysed as described for Figure 7C. Five top fractions of the gradients are omitted for greater clarity. (**B**) Untreated Krebs extract supplemented with unmodified or N1mΨ-modified Luc mRNA was incubated at 30°C for the indicated times. Phosphorylation of eIF2α in the absence (None) or presence of the indicated mRNAs was estimated as described for Figure 5B.

Figure S7

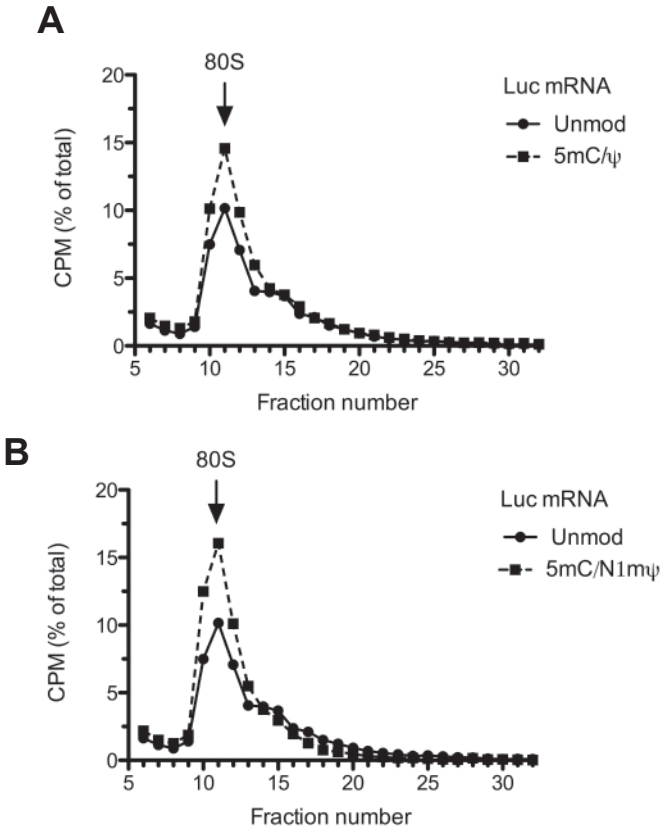
A
80S
Luc mRNA
Unmod
5mC/ψ
CPM (% of total)
Fraction number
B
80S
Luc mRNA
Unmod
5mC/N1mψ
CPM (% of total)
Fraction number

Figure S7. Incorporation of 5mC/Ψ or 5mC/N1mΨ nucleoside modifications in Luc mRNA stimulates the assembly of 80S ribosome initiation complexes in RRL. Untreated and cycloheximide (0.6 mM)-supplemented RRLs were incubated with 3'-end labelled 5mC/Ψ-Luc (**A**), 5mC/N1mΨ-Luc (**B**) and unmodified Luc mRNAs at 30°C for 15 min. 80S initiation complexes were resolved by centrifugation of the reaction mixtures through sucrose gradients as described for Figure 7F. The proportions of 5mC/Ψ-Luc (**A**) and 5mC/N1mΨ-Luc (**B**) mRNAs engaged in 80S initiation complexes (fractions 10-12) were 1.6-fold and 1.9-fold higher, respectively, than the proportion of Luc mRNA.

Figure S8

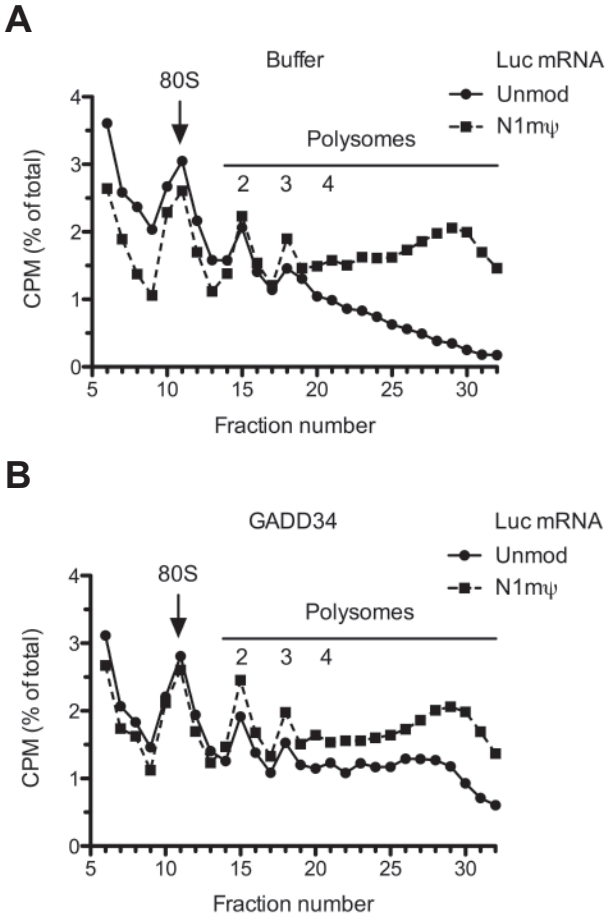
A
Buffer
Luc mRNA
Unmod
N1mψ
80S
Polysomes
2 3 4
CPM (% of total)
Fraction number
B
GADD34
Luc mRNA
Unmod
N1mψ
80S
Polysomes
2 3 4
CPM (% of total)
Fraction number

Figure S8. Inhibition of eIF2α phosphorylation selectively increases polysome formation on unmodified Luc mRNA as compared to N1mΨ-Luc mRNA in RRL. Untreated RRL supplemented with 3'-end labelled unmodified or N1mΨ-incorporated Luc mRNA were incubated at 30°C for 15 min in the presence of control buffer (**A**) or GADD34 (7 μg/ml; **B**). Polysome analysis was performed as described for Figure 7D. Addition of GADD34 preferentially increases the recruitment of ribosomes by Luc mRNA as compared to N1mΨ-Luc mRNA. This lessens the difference between Luc and N1mΨ-Luc mRNA with regard to their polysome distribution. Five top fractions of the gradients are omitted for greater clarity.