Sliding of a 43S ribosomal complex from the recognized AUG codon triggered by a delay in eIF2-bound GTP hydrolysis

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SUPPLEMENTARY DATA

	10th	11th	12th
EMCV	<*IRES*>-CCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAAT	**ATGGCCACAACC**	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC
ΔE012	GGGAATTCGTGGTTTTCCTTTGAAAAACACGATGATAAT	**ATGGCCACAACC**	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE12	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGGCCACAACC**	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE1	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGGCCACAACC**	taGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC
ΔE2	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAATtaGGCCACAACC	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**	
ΔE	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAATtaGGCCACAACCtaGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC		
ΔE02	GGGAATTCGTGGTTTTCCTTTGAAAAACACGATGATAATtaGGCCACAACC	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**	
ΔE112	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGataat**atggccgATAAT**	**ATGGCCACAACC**	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE121	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGGCCACAACC**	**ATGGAAgataatatggccACAACCACGGAACGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE122	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGGCCACAACC**	**ATGGaaacaaccatggAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE1del2	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGG------CC**	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE1ins2	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGGCCACaaccgccacAACC**	**ATGGAACAAGAGACTTGCGCGCACTCTCTCACTTTTGAGGAATGCCCAAAATGCTCTGCTCTAC**
ΔE12UMBRA	GGGAATTCGTGGTTTTCCTTTGAAAAACACGtaGATAAT	**ATGGCCAtCAACC**	**ATGGAACAA**TCTTCGCAAGTGGCAAAAGCTGCAACTCAGGGGGAGCTCCTCGAAGCTCTGTAC

Figure S1. Nucleotide sequences of ΔE012 plasmid and its derivatives. Only the fragments are shown that correspond to RNA transcripts (from the 5' end to the toe-printing primer annealing site, which is double-underlined). The EMCV coding region (with all extensions or modifications) is in **bold**. ATG codons within the initiation region are underlined. Nucleotides absent from the original ΔE012 sequence are shown in lower case letters. In ΔE12UMBRA, the umbravirus ORF3/4 region is outlined. Note that in the latter case "AUG11" and "AUG12" are in different reading frames due to additional "T" insertion between them.

GL3-Fluc 64 nt *GGGAGCTTATCGATACCGTCGACCTCGAATCACTAGTCAGCTGGAATTCGTTGGTAAAGCCACC·····ATGGAAGACGCCAAAAACATAAAG...*

1uORF-Fluc1 73 nt *GGCTAGGCTTGGCATTCCGGTAGGCATGGCATTCCGGTACTGAATTGATCTGGAATTCGTTGGTAAAGCCACC·····ATGGAAGACGCCAAAAACATAAAG...*

1uAUG-Fluc1 81 nt *GGGAGCTTATCGATACCGTCGACCTCGAATCACTAGTCAGCTGGAATTCGTTGGTAAAGCCACCATGCCATCATCATCATCATGGAAGACGCCAAAAACATAAAG...*

CDK4 146 nt

*GG*GTGTGTCTATGGTCGGGCCCTCTGCGTCCAGCTGCTCCGGACCGAGCTCGGGTGTATGGGGCCGTAGGAACCGGCTCCGGGGCCCCGATAACGGGCCGCCCCCACAGCACCCCGGGCTGGCGTGAGGGTCTCCCTTGATCTGAGAATGGCT*AAGCTTGACGCCAAAAACATAAAG...*

CFTR 134 nt

*GG*AATTGGAAGCAAATGACATCACAGCAGGTCAGAGAAAAAGGGTTGAGCGGCAGGCACCCAGAGTAGTAGGTCTTTGGCATTAGGAGCTTGAGCCCAGACGGCCCTAGCAGGGACCCCAGCGCCCGAGAGACCATG*AAGCTTGACG CCAAAAACATAAAG...*

MDM2 307 nt

*GG*TAGGGGGCGCGCACCGAGGCACCGCGGCGAGCTTGGCTGCTTCTGGGGCCTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGAAGCCGAGCCCGAGGGGCGGCCGCGACCCCTCTGACCGAGATCCTGCTGCTTTCGCAGCCA GGAGCACCGTCCCTCCCCGGATTAGTGCGTACGAGCGCCCAGTGCCCTGGCCCGGAGAGTGGAATGATCCCCGAGGCCCAGGGCGTCGTGCTTCCGCGCGCCCCGTGAAGGAAACTGGGGAGTCTTGAGGGACCCCCGACTCCAAGC GCGAAAACCCCGGATGGTGAGGAGCAGGCAAATGTGCAATACCAACATGTCTAAGCTTGACGCCAAAAACATAAAG...

PNRC2 414 nt

*GGG*CTGCCGACTCCATTTTGTCGGTAGAGGCAGAAGGAGAAGGTCGGGTTGTAGAAGCTGGGGTGGCCGGCAGCTCGCTCATCGGTGTTCGTGGGCTTTGTCGGTCCGTGCCTCGTCTCTCCCTGGAAAGGGAGGGAGGCTTCGACG TCGAGAGGGAGCCGCTGCCGCGTTAGTTCCGAGCTTGAAGTCACTAGGACTTCTCTCAAACTTGTGTGCTGAGGAGACTCAGATGTTGGCCTCAGCTCCTAGGCTGAACTCAGCAGATCGGCCCATGAAAACTTCTGTATTGAGACA AAGGAAGGGATCTGTCAGAAAGCAACACTTGTTATCTTGGGCTTGGCAGCAAGGAAGAGGACAGGTAGTGGAGATCCTGCAATCTGAAAAGCAGACTGAAAGGTGACAAAGAAGCTGACCATGGAAGACGCCAAAAACATAAAG...

eIF2D 123 nt

*GC*TTTTCGCGGCCGGGCCCCAGCATGGCTGCCCCCACGGCTGAGGGCCTGGCAGCTGCTGCGCCCTCGCTTTCTTGACATTCCCTGGCTTCTGTGCTCTCTTCCCCAGGCCACCCCAGCAGACATGTTTGCCAAGGCCTTTCGGGTC AAGTCCAACACGGCCATCAAG*CTGGCCACG*GAAGACGCCAAAAACATAAAG...

ATF4 285 nt

*GGG*TTTCTACTTTGCCCGCCCACAGATGTAGTTTTCTCTGCGCGTGTGCGTTTTCCCTCCTCCCCCGCCCTCAGGGTCCACGGCCACCATGGCGTATTAGGGGCAGCAGTGCCTGCGGCAGCATTGGCCTTTGCAGCGGCGGCAGCA GCACCAGGCTCTGCAGCGGCAACCCCCAGCGGCTTAAGCCATGGCGCTTCTCACGGCATTCAGCAGCAGCGTTGCTGTAACCGACAAAGACACCTTCGAATTAAGCACATTCCTCGATTCCAGCAAAGCACCGCAACC*ATGGAAGAC GCCAAAAAACATAAAG...*

UCP2 383 nt

*GGG*CACTGCGAAGCCCAGCTGCGCGCGCCTTGGGATTGACTGTCCACGCTCGCCCGGCTCGTCCGACGCGCCCTCCGCCAGCCGACAGACACAGCCGCACGCACTGCCGTGTTCTCCCTGCGGCTCGGACACATAGTATGACCATTA GGTGTTTCGTCTCCCACCCATTTTCTATGGAAAACCAAGGGGATCGGGCCATGATAGCCACTGGCAGCTTTGAAGAACGGGACACCTTTAGAGAAGCTTGATCTTGGAGGCCTCACCGTGAGACCTTACAAAGCCGGATTCCGGCAG AGTTCCTCTATCTCGTCTTGTTGCTGATTAAAGGTGCCCCTGTCTCCAGTTTTTCTCCATCTCCTGGGACGTAGCAGGAAATCAGCACCATGGAAGACGCCAAAAACATAAAG...

UCP2mut 383 nt

*GGG*CACTGCGAAGCCCAGCTGCGCGCGCCTTGGGATTGACTGTCCACGCTCGCCCGGCTCGTCCGACGCGCCCTCCGCCAGCCGACAGACACAGCCGCACGCACTGCCGTGTTCTCCCTGCGGCTCGGACACATAGTAgGACCATTA GGTGTTTCGTCTCCCACCCATTTTCTAgGGAAAACCAAGGGGATCGGGCCAgGATAGCCACTGGCAGCTTTGAAGAACGGGACACCTTTAGAGAAGCTTGATCTTGGAGGCCTCACCGTGAGACCTTACAAAGCCGGATTCCGGCAG AGTTCCTCTATCTCGTCTTGTTGCTGATTAAAGGTGCCCCTGTCTCCAGTTTTTCTCCATCTCCTGGGACGTAGCAGGAAATCAGCACCATGGAAGACGCCAAAAACATAAAG...

(CA)uORF-Fluc 37 nt *GGAACAACAACAACAACAAAATGACACAAACACACTGATGGAAGACGCCAAAAACATAAAG...*

(CA)-Fluc 37 nt *GGAACAACAACAACAACAAAAgtACACAAACACACTGATGGAAGACGCCAAAAACATAAAG...*

Figure S2. cDNAs of reporter mRNAs used for the *in vitro* translation assay. Artificial and reporter sequences are *italized*, CDSs are shown in **bold**, uORFs are shadowed, Firefly luciferase sequence is in yellow, start sites are shown in **green**, the corresponding stop codons are in **red**. The 5' UTR lengths are indicated.

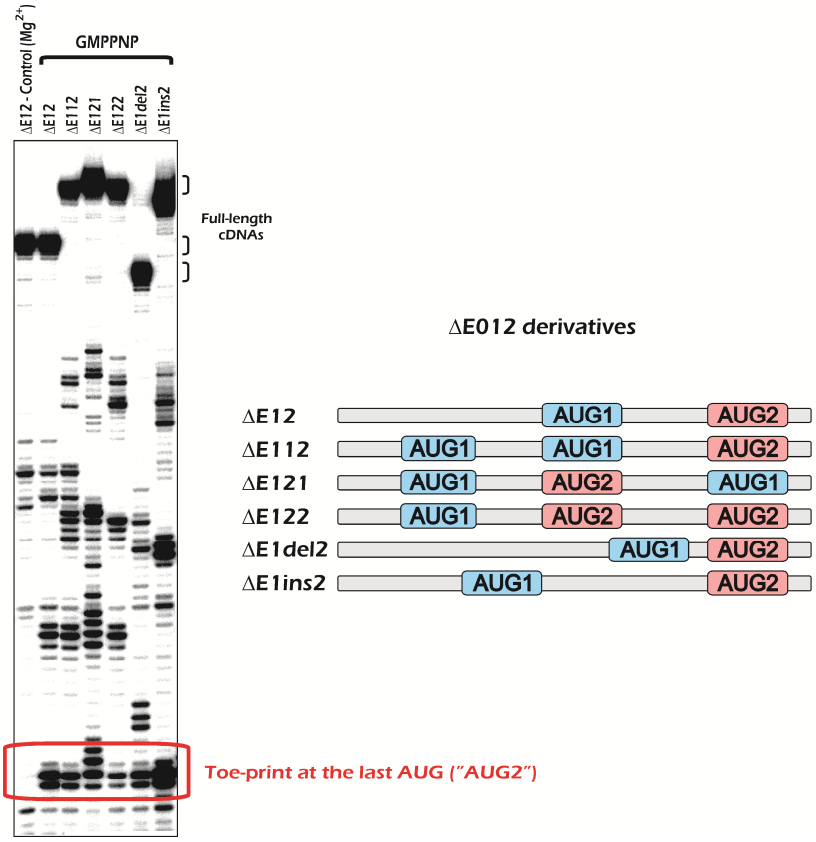

Figure S3. Toe-printing analysis of 48S complexes assembled in RRL on mRNAs with different combinations of ΔE012-derived initiating regions. m^7G-capped mRNAs were incubated in RRL with 2 mM GMPPNP·Mg for 10 min. The 48S complexes are detected at the last AUG codons of the ΔE012 mRNA derivatives irrespective of a number of preceding AUG codons. Schematic representation of the initiation regions is shown on the right.

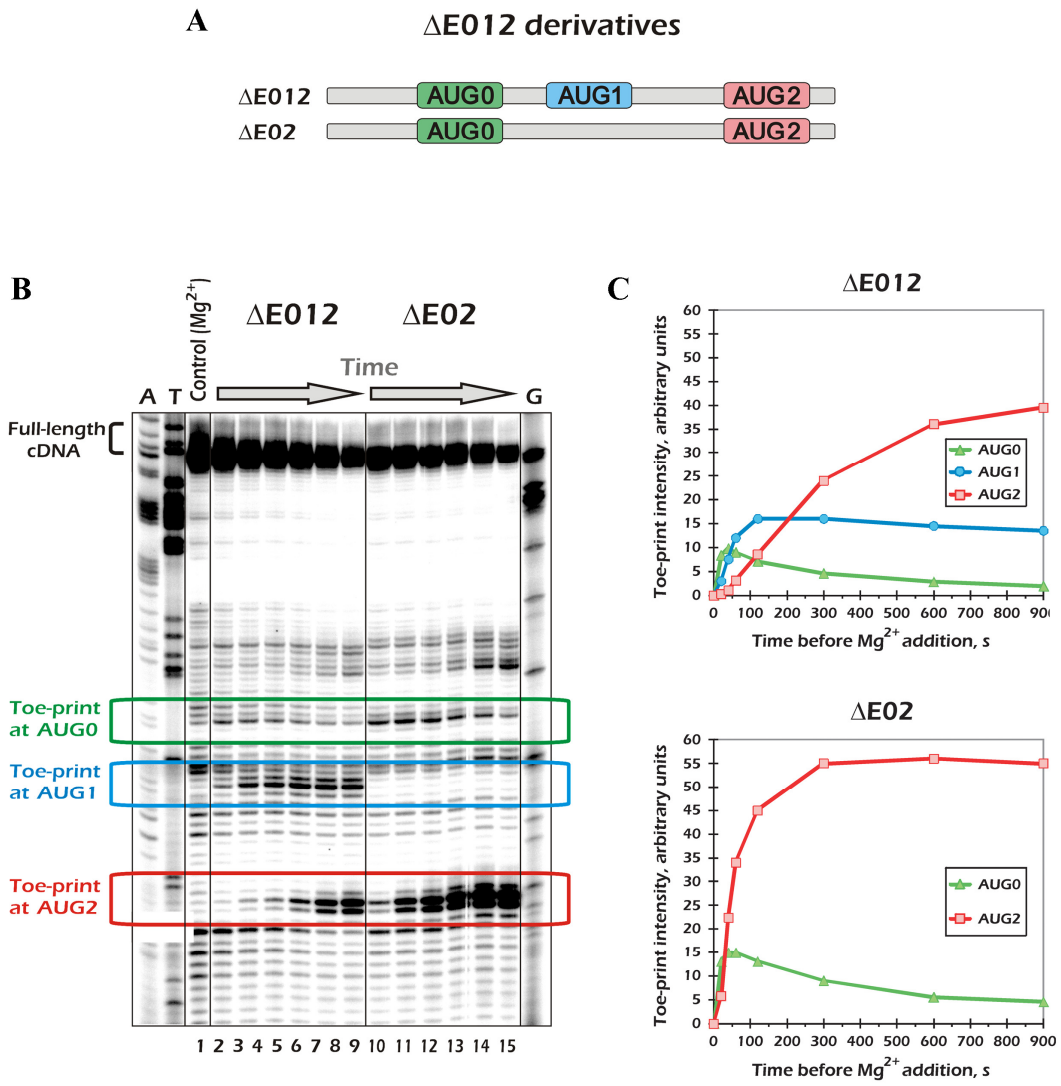

Figure S4. Time course of 48S formation at AUG codons of the ΔE012 and ΔE02 mRNAs assayed by kinetic toe-printing technique. (**A**) Schematic representation of the mRNA's initiation regions. (**B**) Sequencing gel with results of the kinetic toe-printing assay. (**C**) Quantification of toe-print signals corresponding to the 48S complexes at the three AUG codons. The values were normalized to the overall signal densities in the corresponding lanes.

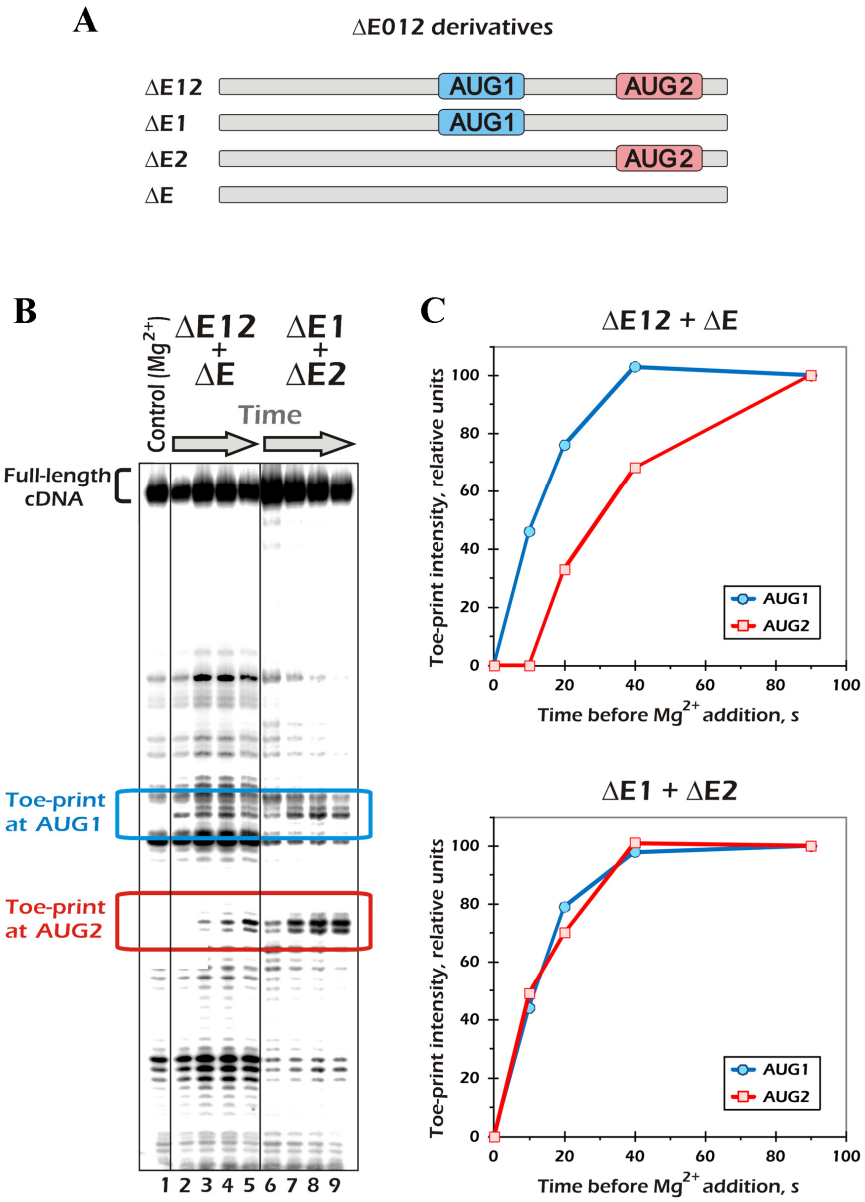

Figure S5. Time course of 48S formation at two AUG codons of different ΔE012 mRNA derivatives assayed by kinetic toe-printing technique. (**A**) Schematic representation of initiation regions of the four ΔE012 mRNA derivatives used in this experiment. Note that ΔE mRNA does not possess any AUG codon in this region. (**B**) Sequencing gel with results of the kinetic toe-printing assay. The indicated equimolar mRNA mixtures were incubated in RRL in the presence of GMPPNP for different times (lanes 2 and 6 – 10 s, 3 and 7 – 20 s, 4 and 8 – 40 s, 5 and 9 – 90 s), then the reaction was stopped by elevating the Mg^{2+} concentration. The ΔE mRNA was added as a ballast to ensure the equal molar "concentration" of AUG1 and AUG2 in all cases. Positions of the toe-prints are indicated. (**C**) Quantification of toe-print signals corresponding to the 48S complexes at the AUG codons. The values were normalized to the overall signal densities in the corresponding lanes and then expressed in percents of the maximum values for each complex.

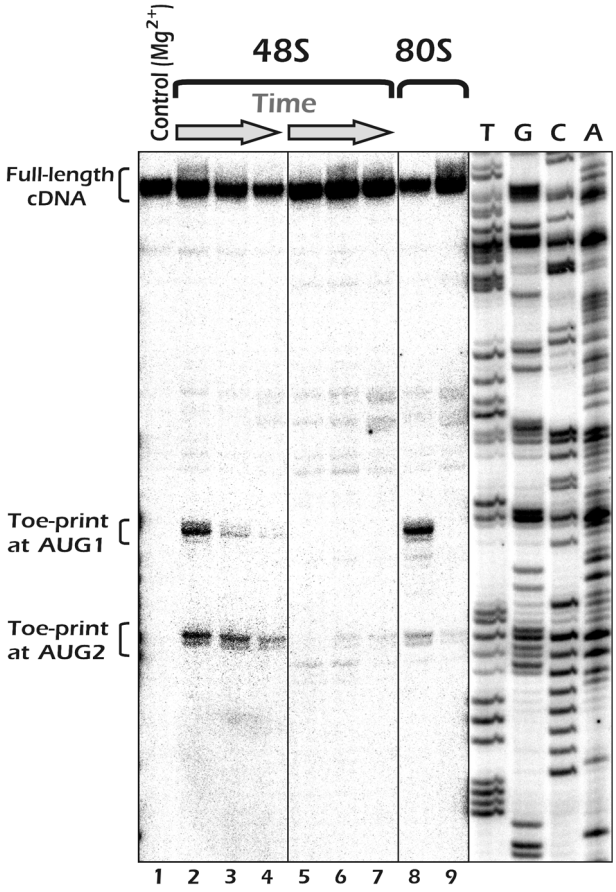

Figure S6. Uncapped transcript ΔE12 is unable to form initiation complexes efficiently. m^7G-capped (lanes 1-4 and 8) or uncapped (lanes 5-7 and 9) mRNAs were incubated in RRL in the presence of GMPPNP (lanes 1-7) or cycloheximide (lanes 8-9) for different times (lanes 2 and 5 – 20 s, 3 and 6 – 60 s, 4 and 7-9 – 600 s). The reaction was stopped by elevating the Mg^{2+} concentration. Sequencing lanes obtained with the same primer and the corresponding cDNA are shown on the right.

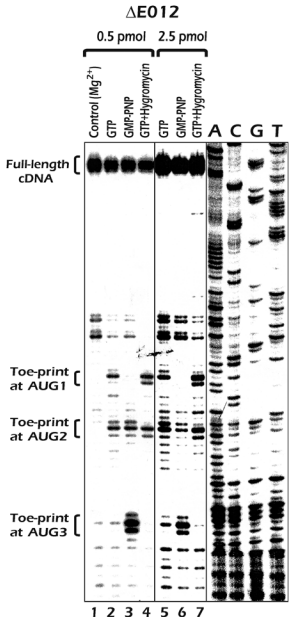

Figure S7. Toe-printing analysis of ribosomal complexes assembled in RRL on the ΔE012 mRNA at different RRL to mRNA ratio. The ΔE012 mRNA was incubated in RRL with 15 mM $Mg(OAc)_2$ (lane 1), 2 mM GTP·Mg/GMPPNP·Mg (lanes 2/3 and 5/6), or 2 mM GTP·Mg and 1 mg/ml hygromycin B (lanes 4 and 7). The latter antibiotic arrested translation at the stage of 80S similarly to cycloheximide {Dmitriev, 2003 #96}, and GTP·Mg was added in this case just to be sure that the concentrations of all components except the hygromycin B were the same in the reaction mixtures.

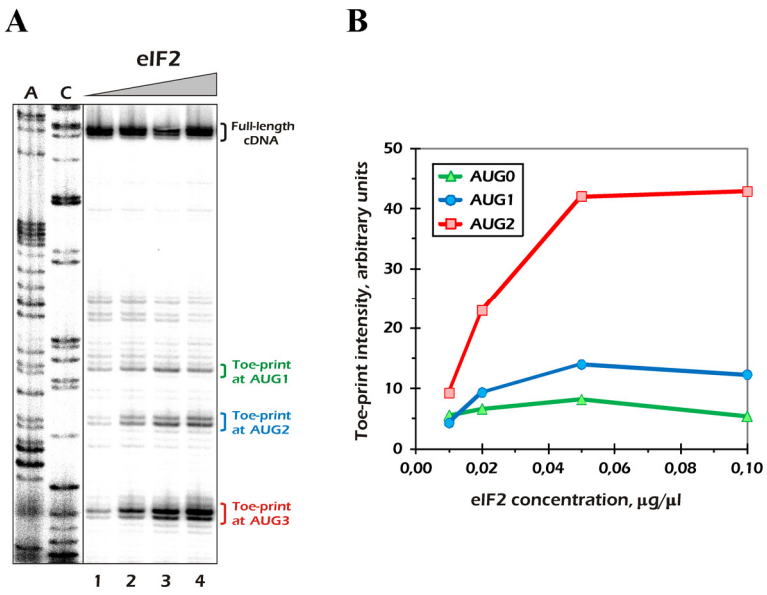

Figure S8. Effect of the increasing concentration of eIF2 on ribosomal complexes assembled from purified components on the ΔE012 mRNA. (**A**) Sequencing gel with results of the toe-printing assay. The reaction mixtures contained all individual components necessary for the 48S complex reconstitution (40S, Met-tRNA$_i$, eIF1, eIF1A, eIF3, eIF4A, eIF4B, eIF4F) and the increasing amounts of eIF2 (lane 1 – 0,01 µg/µl; 2 – 0,02 µg/µl; 3 – 0,05 µg/µl; 4 – 0,1µg/µl). The reaction was stopped after 10 min incubation by addition of a high concentration of Mg(OAc)$_2$. (**B**) Quantification of toe-print signals corresponding to the 48S complexes at the three AUG codons. The values were normalized to the overall signal densities in the corresponding lanes.