Supporting Information

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SI Materials and Methods

Plasmid Construction. A plasmid expressing MS2-β-gal was constructed by ligating FspI/HindIII-treated pCMV·SPORT β-gal (Invitrogen) with BspEI/mungbean nuclease/HindIII-treated pMS2-GFP. MS2-4GMC (an eIF4GI expression plasmid) was generated by amplifying a DNA fragment corresponding to amino acids 623–1600 of eIF4GI from pSK eIF4GI by PCR, treating with HindIII and SacII, and cloning the resulting fragment into HindIII/SacII-digested pEGFP-C1 (Clontech). The resulting plasmid (pEGFP-C1-4GMC) was treated with SacII, T4 DNA polymerase, and HindIII, and ligated to KpnI-, T4 DNA polymerase-, and HindIII-treated pMS2-GFP.

To generate plasmid pcDNA3.1 Fluc harboring MS2-binding sites and firefly luciferase, PCR-amplified firefly luciferase gene was digested with AflII and BamHI and then inserted into pcDNA3.1 vector treated with the same restriction enzymes. This plasmid (pcDNA3.1 Fluc) was used to generate pcDNA3.1 Fluc MS2 × 6, pcDNA3.1 MS2 × 12, and pcDNA3.1 MS2 × 24 harboring 6, 12 and 24 copies of MS2-binding sites, respectively. Synthetic DNA fragments with various copy number of MS2-binding sites were inserted between BamHI and EcoRV sites of pcDNA3.1 Fluc. Dual reporter construct pcDNA3.1 RF, which harbors *Renilla* luciferase and firefly luciferase genes consecutively, was constructed by inserting *Renilla* luciferase gene into the AflII site of pcDNA3.1 Fluc. pcDNA3.1 RF MS2 × 24 was generated similarly to pcDNA3.1 RF, but using pcDNA3.1 Fluc MS2 × 24 instead of pcDNA3.1 Fluc. A DNA fragment encoding the EMCV IRES was inserted between the NotI and XhoI sites of pcDNA3.1 Rluc vector to generate pcDNA3.1 Rluc EMCV harboring *Renilla* luciferase, followed by EMCV IRES.

Site-directed mutagenesis was performed to generate a modified EMCV IRES with a mutation at the eIF4G-binding region of the IRES element using a primer pair (forward: 5′-ACCCCATTGTATGGGATCTGTTCTGGGGCCTCGGTGCACAT-3′; reverse: 5′-ATGTGCACCGAGGCCCCAGAACAGATCCCATACAATGGGGT-3′). To block putative backward scanning of a 40S ribosomal subunit through the 3′ UTR of mRNA or prevent ribosome loading at the 5′ end of mRNA, a stable stem-loop (5′-GCCTAGGCCGGAGCGCCCAGATCTGGGCGCTCCGGCCTAGGC-3′, −46 kcal/mol) was inserted at the 3′ UTR or 5′ end, respectively.

Cell Culture. HEK293T cells were cultivated in DMEM (Gibco) supplemented with 10% (vol/vol) FBS (Clontech).

DNA and RNA Transfection. For translation analysis in cells, HEK293T cells were transfected with 2 μg of plasmid DNAs expressing MS2-GFP, MS2-β-gal, or MS2-4GMC, using Lipofectamine 2000 (Invitrogen). After culturing for 48 h, pMS2-GFP-, pMS2-β-gal-, and pMS2-4GMC-transfected cells were transfected with 200 ng of reporter RNAs (firefly luciferase) and 50 ng of *Renilla* luciferase RNAs, included as a control for transfection efficiency.

Reporter Assay. Luciferase activity in cells was measured after cultivating cells for an additional 4 h using the Promega Dual-Luciferase Reporter Assay System or Firefly Luciferase Assay System.

In Vitro Translation. In vitro-transcribed reporter RNAs were translated in vitro with RNase-untreated RRLs (Promega) as described previously (1).

RNA Analysis. Total RNAs were purified from transfected HEK293T cells using the TRIzol Reagent (Invitrogen). For real-time PCR, total RNAs (2 μg) were reverse-transcribed using the Improm-II Reverse-Transcription System (Promega), and the cDNAs were amplified by real-time PCR using SYBR premix Ex Taq (Takara).

Coimmunoprecipitation. To investigate whether MS2-GFP-4GMC protein expressed in 293T cell can make a complex with eIF3 and 40S ribosomal subunit as shown in Fig. S1*B*, pull-down experiments were performed with an anti-GFP antibody. MS2-GFP and MS2-GFP-4GMC were precipitated using anti-GFP antibody-conjugated beads. eIF3 and 40S ribosome were observed by Western blot analysis with an anti-eIF3b and anti-RpS6 antibody, respectively.

Comparison of the Empirically Measured Translation Efficiency with the Probability of Collision of Two Objects on an mRNA. Reporter DNAs, kindly provided by Dr. Vincent Mauro (2), were transfected into HEK293T cells, and luciferase activity was measured at 24 h after transfection. Luciferase activity was normalized to RNA levels measured by real-time PCR. Relative translation efficiencies of mRNAs containing 5′ UTRs of various lengths are depicted in Fig. 5*A*. The translation efficiency of mRNA containing 0.25 copy of the β-globin leader was set to 1 for comparison. The mRNA translation efficiency was the greatest at a 5′ UTR length of ∼70 nt. When the 5′ UTR length of mRNAs was calculated, 10 nt was added to the nucleotide number of the β-globin sequence, because an additional 10 nt are located upstream of the β-globin sequence.

To correlate experimental data and mathematically predicted values of translation efficiency, we need to know the Kuhn length of the 5′ UTR (parameter "l" in the equation shown in Fig. S7*B*). The Kuhn length of the 5′ UTR, which is difficult to estimate by sequence analyses alone (3), was estimated from the appropriate experimental data (Fig. 5*A*). According to the formula given in Fig. S7*B*, the maximum interaction probability occurs at the point where site separation is 1.62 n (Fig. 5*B*), and ∼70 nt was estimated to be the most efficient 5′ UTR length by nonlinear regression analysis of the data (Fig. 5*A*); thus, the Kuhn length of the β-globin 5′ UTR is 43.8 nt (1.62 n = 70 nt). The length per base of RNA is 0.65 nm (3); thus, the predicted Kuhn length of the β-globin 5′ UTR is 28.47 nm. The calculated probability of interaction based on this assumption (x-axis) and the relative translation efficiency determined experimentally (y-axis) are plotted in Fig. S7*C*.

1. Soto Rifo R, Ricci EP, Décimo D, Moncorgé O, Ohlmann T (2007) Back to basics: The untreated rabbit reticulocyte lysate as a competitive system to recapitulate cap/poly(A) synergy and the selective advantage of IRES-driven translation. *Nucleic Acids Res* 35(18):e121.
2. Chappell SA, Edelman GM, Mauro VP (2006) Ribosomal tethering and clustering as mechanisms for translation initiation. *Proc Natl Acad Sci USA* 103(48):18077–18082.
3. Rippe K (2001) Making contacts on a nucleic acid polymer. *Trends Biochem Sci* 26(12):733–740.

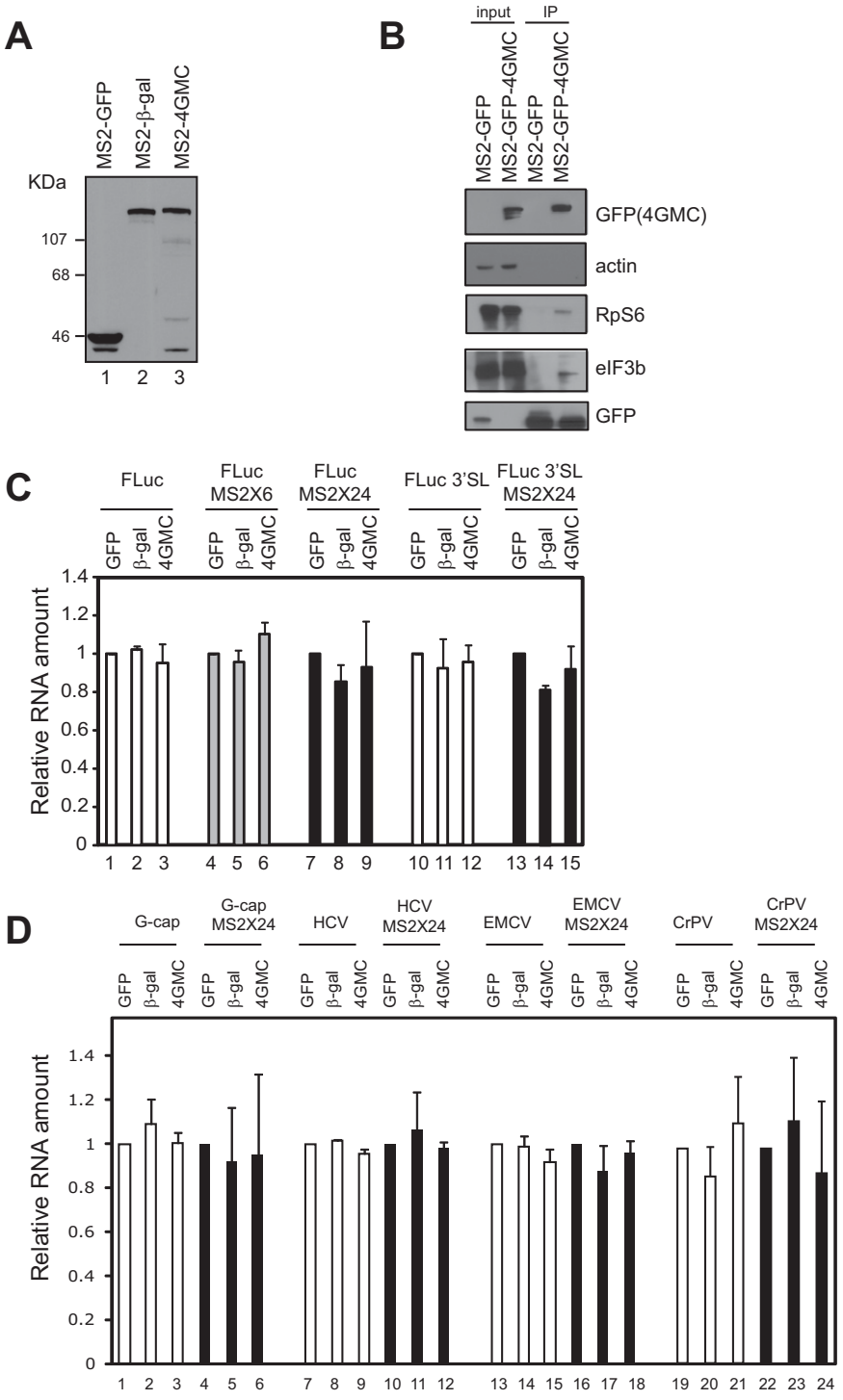

Fig. S1. Activation of translation by eIF4G tethered to the 3′ UTR of reporters harboring various 5′ UTRs. (*A*) Expression of fusion proteins in mammalian cells was monitored by Western blot analysis with an antibody against GFP. (*B*) MS2-GFP-4GMC protein associates with eIF3 and 40S ribosome. The GFP-sized protein shown on panel GFP in lane MS2-GFP-4GMC is likely a proteolytic cleavage product of MS2-GFP-4GMC fusion protein. (*C*) The levels of reporter RNAs in the cells transfected with plasmids and RNAs used in Fig. 1*C* were quantified by real-time PCR. (*D*) Total RNA from cells transfected with RNA and plasmids used in Fig. 1*E* was extracted, and reporter RNA concentrations were measured by real-time PCR.

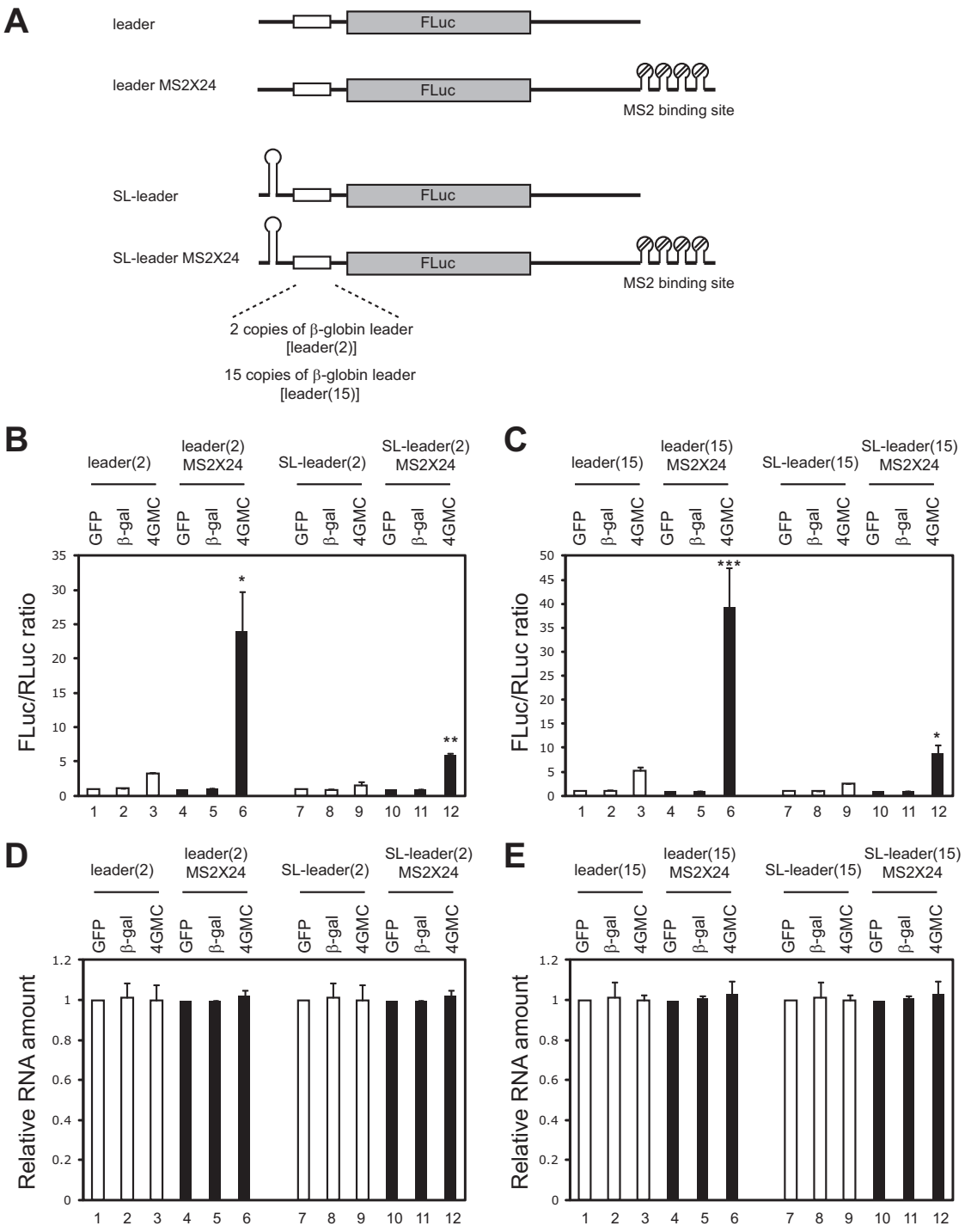

Fig. S2. Translation activation by eIF4G tethered to the 3′ UTR of reporter mRNAs harboring a stable stem-loop at the 5′ end. (*A*) Schematic diagrams of reporter mRNAs used in this study. (*B* and *C*) Luciferase activities of reporters with two copies of the β-globin leader sequence (~100 nt; *B*) or with 15 copies of the β-globin leader sequence (~780 nt; *C*) with (lanes 7–12) or without (lanes 1–6) a stable stem-loop at the 5′ UTRs of mRNAs. Firefly luciferase activity was normalized to *Renilla* luciferase activity expressed from a capped mRNA cotransfected as a transfection efficiency control. Error bars reflect SD in three independent experiments. (*D* and *E*) Levels of reporter RNAs in the cells transfected with plasmids and RNAs were measured by real-time PCR. $*P < 0.0005$; $**P < 1.5 \times 10^{-5}$; $***P < 0.0002$ compared with reporter RNA without an MS2 sequence in a 4GMC-transfected cell.

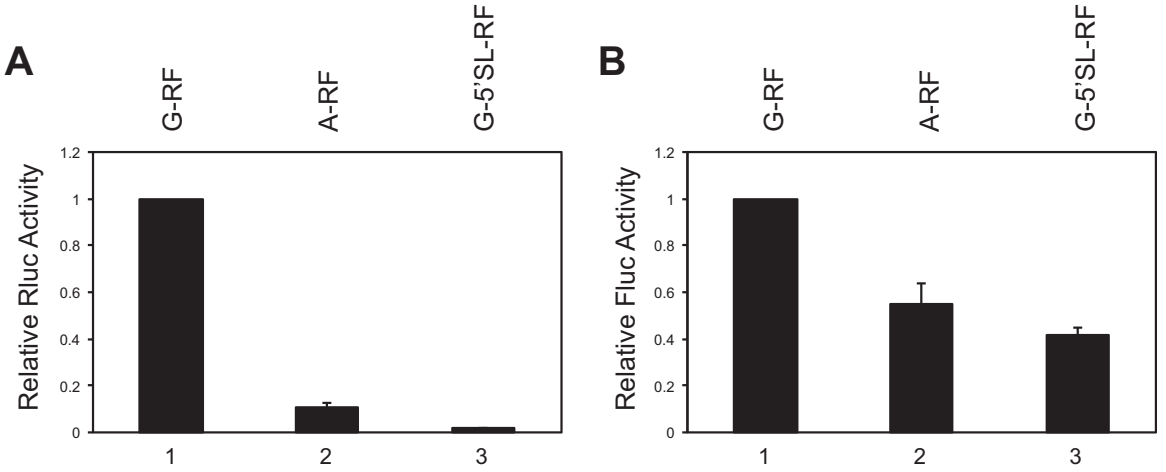

Fig. S3. Comparison of translation efficiencies of the reporter RNAs used in Fig. 2. *Renilla* (*A*) and Firefly luciferase (*B*) activities from G-RF, A-RF, and G-5′SL-RF in cells expressing GFP protein were normalized to β-galactosidase activity from a plasmid cotransfected as a transfection efficiency control. The normalized luciferase activity from G-RF was set to 1 for comparison (lane 1 for lanes 2 and 3). *Renilla* luciferase activity, which is produced from the first cistron, was decreased by 10-fold when the G cap is replaced by the A cap (compare lanes 1 and 2 in *A*) and by 50-fold when a stable stem-loop is inserted at the 5′ end of the reporter (compare lanes 1 and 3 in *A*). In contrast, firefly luciferase activity, which is produced from the second cistron, was reduced only by approximately twofold by the A-capping (compare lanes 1 and 2 in *B*) or by the insertion of a stable stem loop (compare lanes 1 and 3 in *B*). These data indicate that translation of second cistron is largely independent of translation of first cistron.

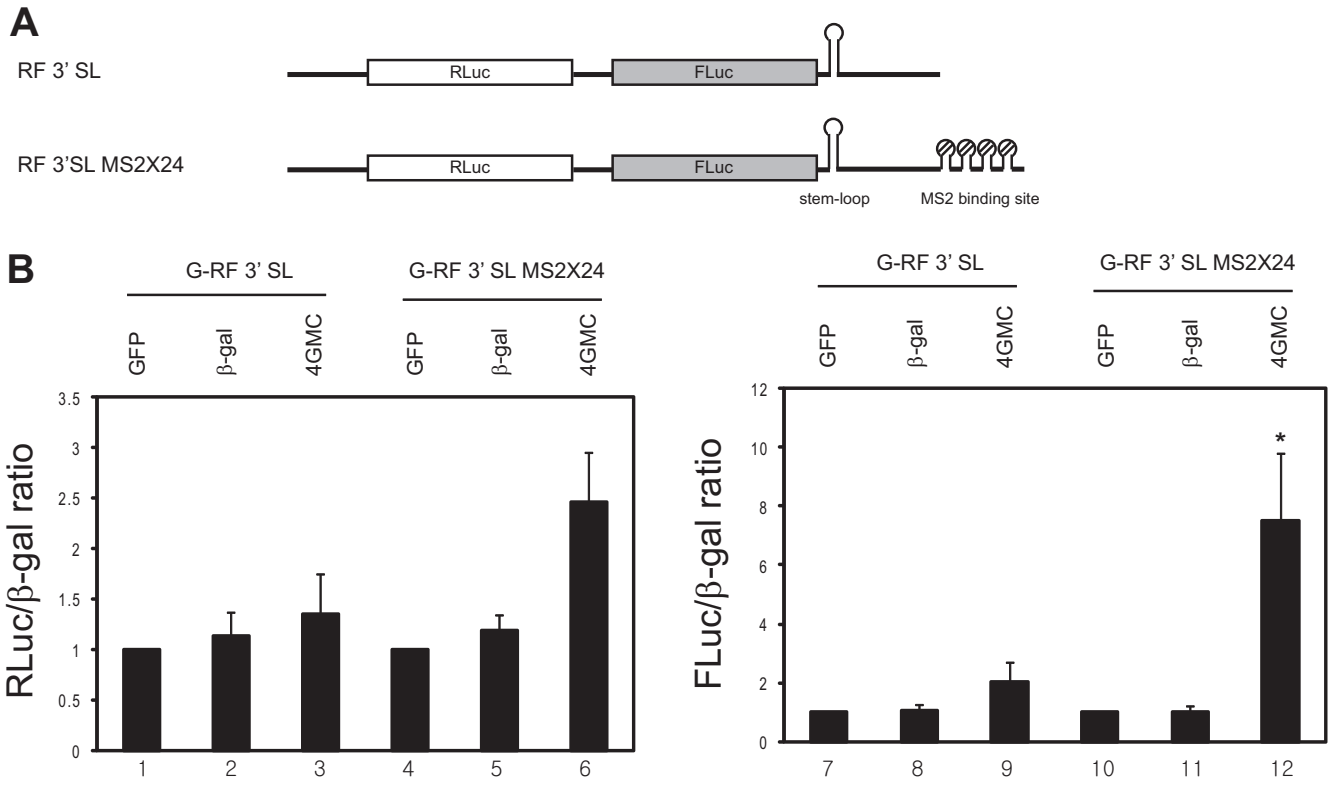

Fig. S4. 5′ end-independent translational activation by eIF4G tethered downstream of a reporter gene is not mediated by 3′ to 5′ backward scanning of ribosome. (*A*) Schematic representations of dicistronic reporters harboring a stable stem-loop between the second cistron and MS2 binding sequences to block putative ribosomal backward scanning. (*B*) m^7G-capped dicistronic reporters harboring a 3′ stem loop were transfected into cells producing different kinds of MS2-fused proteins, and then luciferase activities were measured. Firefly and *Renilla* luciferase activities were normalized by the activity of β-galactosidase from a plasmid cotransfected as a transfection efficiency control. The normalized firefly luciferase activity (lanes 1–6) and *Renilla* luciferase activity (lanes 7–12) of reporter without an MS2-binding site in cells overproducing MS2-GFP proteins were set to 1 for comparison (lane 1 for lanes 1–6 and lane 7 for lanes 7–12). *$P < 0.02$ compared with lane 3. Translational enhancement by the 3′-tethered eIF4G was observed from both reporters residing at the first and second cistrons even in the presence of a stable stem-loop at the 3′ UTR that would block putative ribosomal backward scanning. The results indicate that translational enhancement by the 3′-tethered eIF4G does not occur by ribosomal backward scanning.

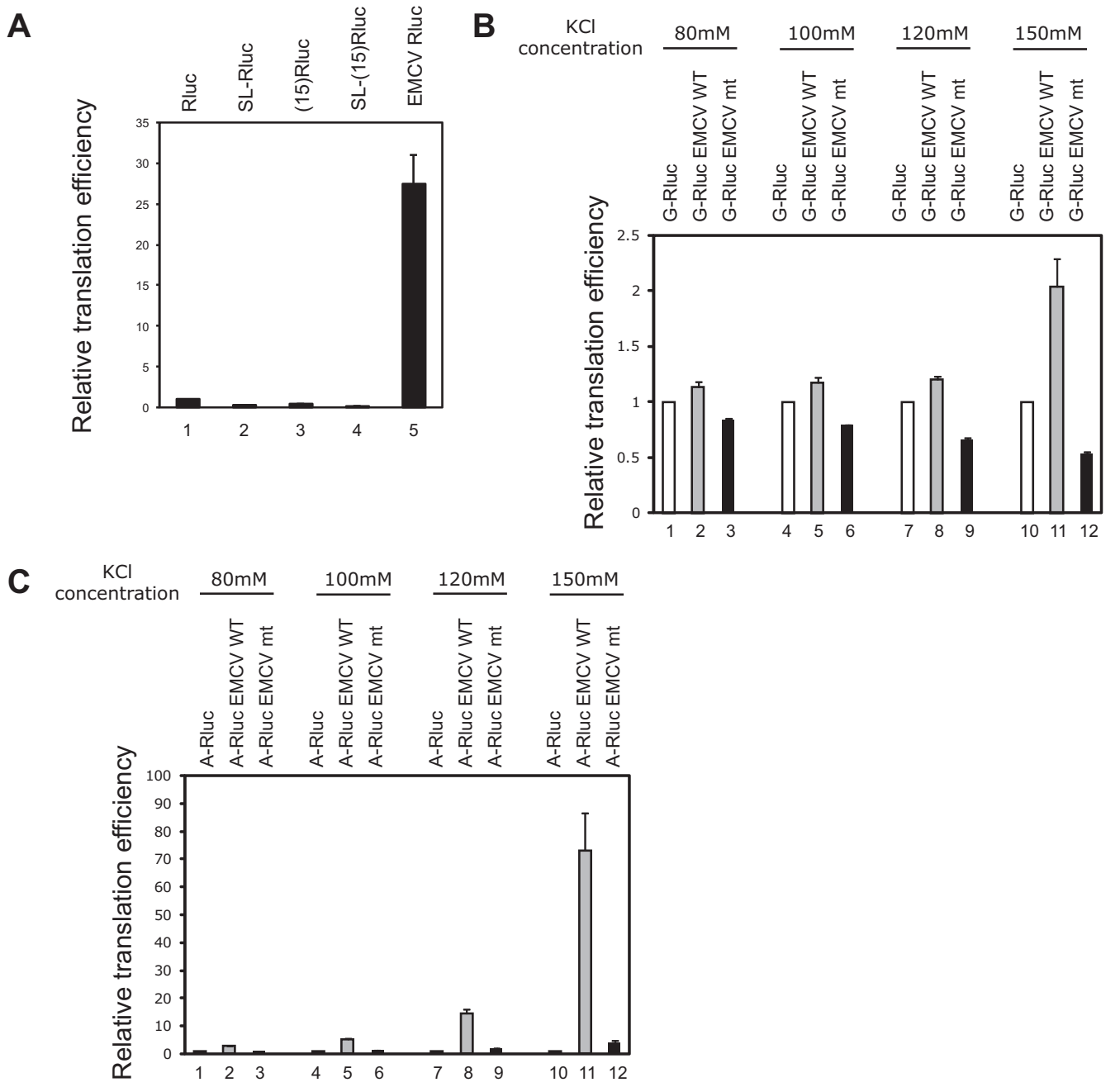

Fig. S5. Effects of salt concentration and various sequences at the 5′ UTR on translation. (*A*) Translation efficiencies of various reporters used in Fig. 3 (lanes 1–4) were investigated using nuclease-untreated RRLs in the presence of 150 mM KCl. The effect of the EMCV IRES at the 5′ UTR on translation was monitored as well (lane 5). (*B* and *C*) Reporters were subjected to in vitro translation with nuclease-untreated RRLs at various KCl concentrations. Relative translation efficiencies of m^7G-capped (*B*) or A-capped (*C*) mRNAs were monitored at different salt concentrations. Relative translation efficiencies of the reporter without EMCV IRES at each salt concentration was set to 1 for comparison (lanes 1, 4, 7, and 10 for lanes 1–3, 4–6, 7–9, and 10–12, respectively).

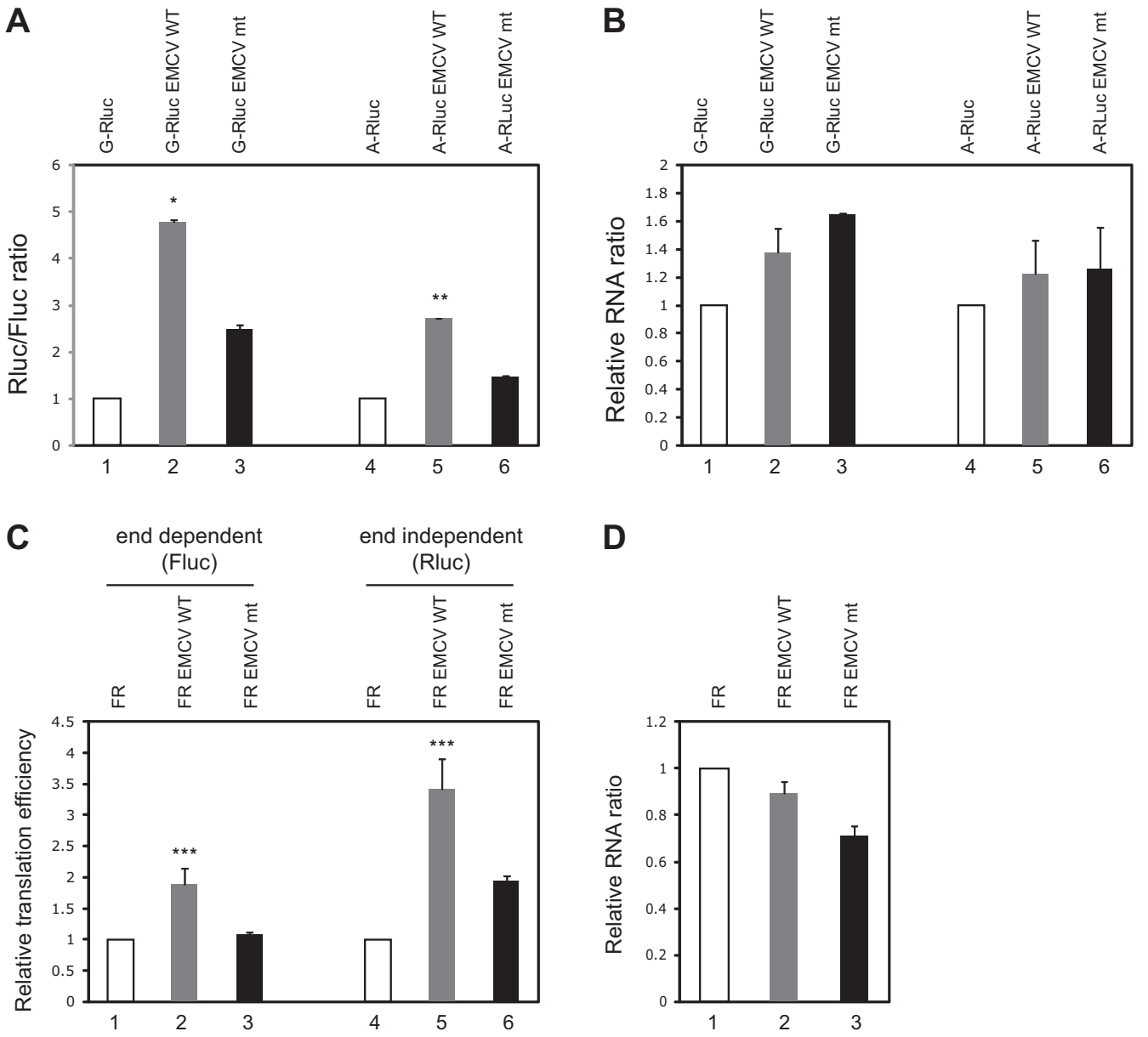

Fig. S6. EMCV IRES stimulates translation of upstream genes in vivo. (*A*) m^7G-capped or A-capped RNA reporters described in Fig. 3*A* were transfected into HEK293T cells together with m^7G-capped Fluc reporters as a transfection control. At 4 h after transfection, cells were harvested and luciferase activity was measured. Relative translation efficiencies of the reporters without EMCV IRES were set to 1 (lane 1 for lanes 1–3 and lane 4 for lanes 4–6). (*B*) The levels of reporter RNAs in the cells transfected with reporter RNAs were quantified by real-time PCR. (*C*) Dual reporters harboring WT or mt EMCV IRES described in Fig. 4*A* were transfected into HEK293T cells. At 4 h after transfection, cells were harvested and firefly and *Renilla* luciferase activities were measured to monitor 5′ end-dependent translation (Fluc) and 5′ end-independent translation (Rluc). Translation efficiencies of the reporters without EMCV IRES were set to 1 for comparison (lane 1 for lanes 1–3 and lane 4 for lanes 4–6). (*D*) The levels of reporter RNAs in the transfected cells were quantified by real-time RT-PCR. $*P < 3.25 \times 10^{-8}$; $**P < 7.2 \times 10^{-10}$; $***P < 0.005$ compared with reporters without EMCV IRES.

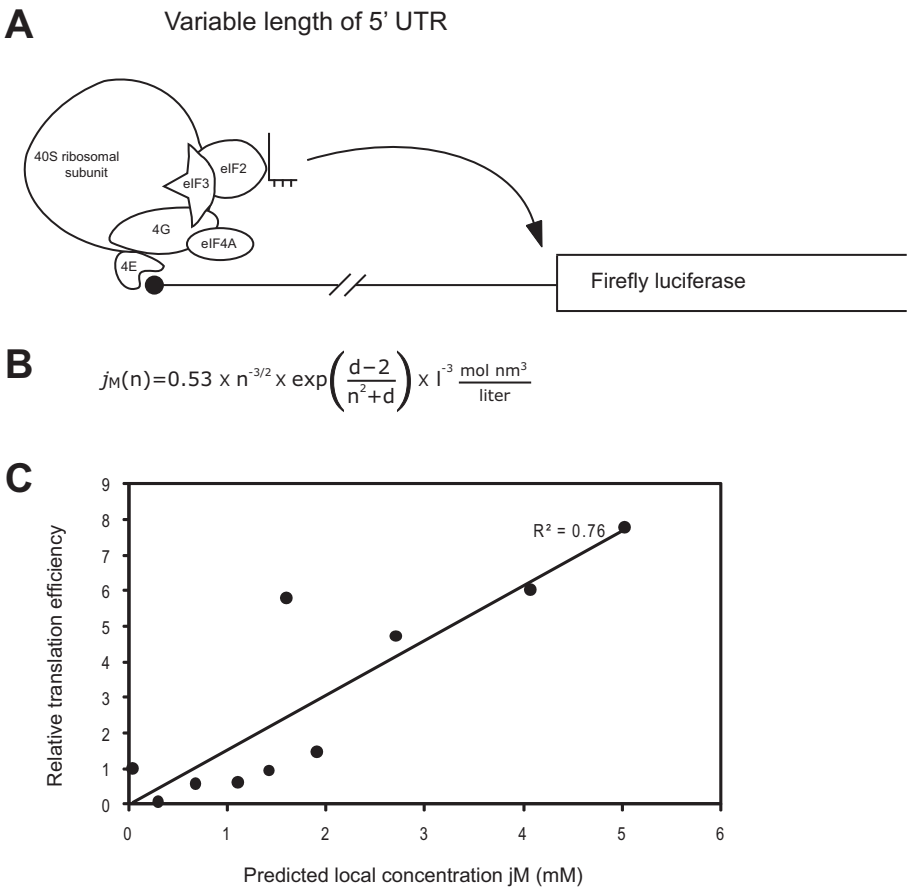

Fig. S7. Theoretical analysis of the effect of 5′ UTR length on translation efficiency. (*A*) In cap-dependent translation, eIF4E (a cap-binding protein) and eIF4G (a scaffold protein that links the eIF4F complex and eIF3) are recruited to the cap structure of mRNA, followed by recruitment of the eIF3-associated 40S ribosomal subunit, which results in 43S ribosome complex formation (1). The 43S ribosome complex "searches for" the initiation codon before commencement of translation. We theoretically analyzed the effect of the distance between the cap structure and the initiation codon from the perspective of RNA looping using capped mRNAs with various 5′ UTR lengths. 4E and 4G denote eIF4E and eIF4G, respectively. (*B*) The equation predicting the probable density, *j*M (local concentration), of two sites separated by a distance, *n* (number of Kuhn segments) in a linear polymer, where *l* is the statistical segment length and *d* is a parameter describing two effects that facilitate contacts at small values of *n*. The first is the effect of the binding site of a protein or protein complex, and the second is the effect of an intrinsically curved region within the nucleic acid polymer. (*C*) The correlation between empirically measured translation efficiency (Fig. 5*A*) and theoretically estimated translation efficiency (Fig. 5*B*) of mRNAs with various 5′ UTR lengths are plotted in the graph. A strong linear correlation was observed between the two parameters ($R^2 = 0.76$), indicating that RNA looping governs translation initiation of the mRNAs tested in vivo.

1. Jackson RJ (2005) Alternative mechanisms of initiating translation of mammalian mRNAs. *Biochem Soc Trans* 33(Pt 6):1231–1241.

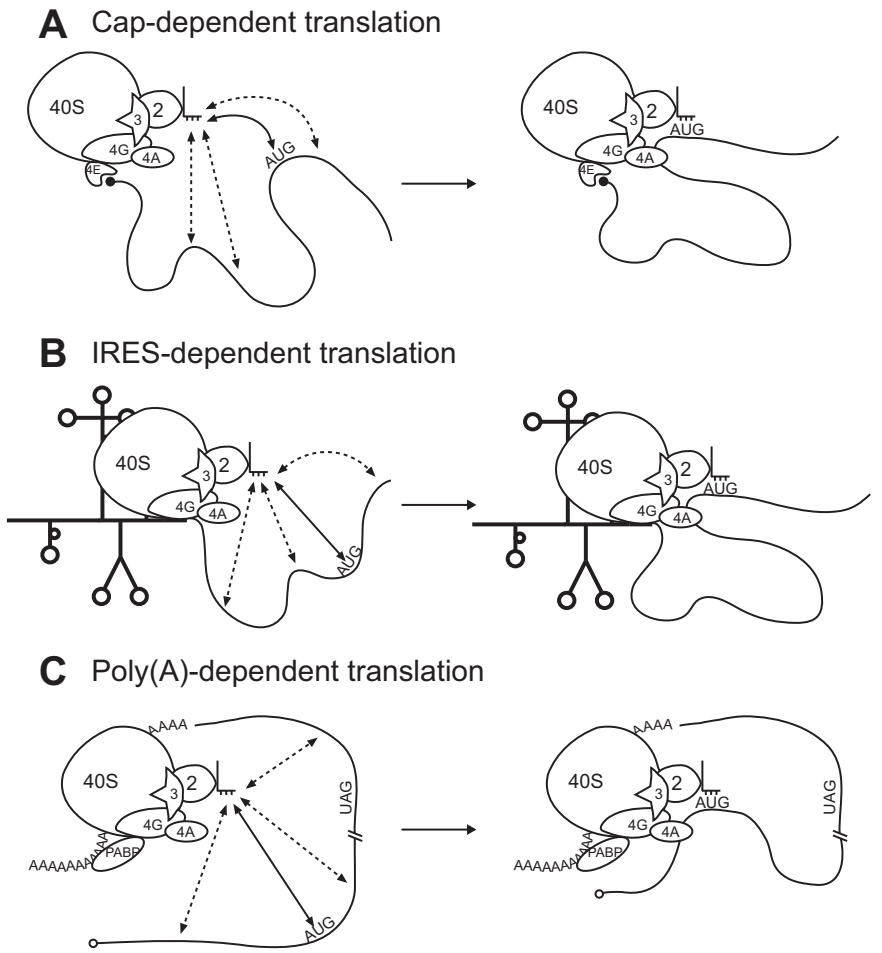

Fig. S8. Hypothetical models of translation initiation by RNA looping. (*A*) The cap structure at the 5′ end of an mRNA may facilitate translation through RNA looping. A similar model was proposed by Chappell et al. (2) to explain the result shown in Fig. 5*A*, given that it could not be explained by the scanning hypothesis. (*B*) IRES elements in the 5′ UTR of an mRNA may facilitate translation through RNA looping. (*C*) The poly(A) tail at the 3′ end of an mRNA may facilitate translation through RNA looping. The black and white circles in *A* and *C* represent G-cap and no cap, respectively.