Supporting Information

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SI Materials and Methods

Real-Time PCR. Real-time PCR was carried out by using the Stratagene MX3005P QPCR system. 2% of RNA from each gradient fraction was reverse transcribed by using SuperScript III (Invitrogen) and was analyzed by using the QuantiTect SYBR Green PCR kit following the manufacturer's instructions (Qiagen). *Renilla* luciferase cDNA was amplified by using the primers 5′-GGAATTATAATGCTTATCTACGTGC-3′ and 5′-CTTGCGAAAAATGAAGACCTTTTAC-3′. Firefly luciferase cDNA was amplified by using the primers 5′-CTCACTGAGACTACATCAGC-3′ and 5′-TCCAGATCCACAACCTTCGC-3′. Actin cDNA was amplified by using the primers 5′-GGCATGGGTCAGAAGGATT-3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′.

Sucrose Density Gradient Centrifugation and RNA Detection. Sucrose density gradient centrifugation was used to separate ribosomes into polysomal and subpolysomal fractions. Cells (6×10^6) were incubated with 0.1 mg/ml cycloheximide for 3 min at 37°C, washed in PBS containing 0.1 mg/ml cycloheximide, and lysed in lysis buffer [15 mM Tris·HCl (pH 7.4), 15 mM $MgCl_2$, 0.15M NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, and 1 mg/ml heparin]. The nuclei and debris were removed by centrifugation at 12,000 × g for 5 min, and the supernatants were loaded onto 10–50% sucrose gradients composed of lysis buffer lacking Triton X-100. The gradients were sedimented at 38,000 rpm for 120 min in a SW41 rotor at 4°C (221,777 × *g*). Fractions of equal volume were collected from the top of the gradient directly into guanidine·HCl, at a final concentration of 4M, using an ISCO fraction collector system. RNA was precipitated by addition of 2 volumes of ethanol and resuspended in 1 mM Tris·HCl (pH 8). All fractions were subjected to DNase I treatment to remove contaminating plasmid DNA. Quantification of northern analysis was preformed on "QuantityOne" HD analysis software from Bio-Rad after scanning on Bio-Rad molecular Imager FX.

Electroporation. Transfections were carried out by using the Nucleofector II from Amaxa Biosystems. HeLa cells (1×10^6) were resuspend in the Nucleofector Solution R and mixed with 1 μg of DNA. The DNA-cell mix was then transferred into an cuvette, and transfection was carried out by using Nucleofector program I13. The appropriate number of cells were then plated onto a 24 well plate and harvested 48 h after transfection.

Generation of the pRLSV40L78. Oligonucleotides containing the let-7A miRNA binding sites were cloned into the XbaI site of the plasmid pRL-SV40 from Promega (see below). This was repeated four times to generate pRLSV40L78.

Oligonucleotide: XhoI EcoRV XbaI. 5′-CTAGCTCGAG*AACTATACAACGTCTACCTCA*GATATC*AACTATACAACGTCTACCTCA*T-3′.

3′-GAGCTC*TTGATATGTTGCAGATGGAGT*CTATAG*TTGATATGTTGCAGATGGAGT*AGATC-5′.

agatctgcgcagcaccatggcctgaaataacctctgaaagaggaacttggttaggtaccttctgaggcggaaagaaccagctgtggaatgtgtgtcagttagggtgtggaaagtccccaggctccccagcaggcagaagtatgcaaagcatgcatctcaattagtcagcaaccatagtcccgcccctaactccgcccatcccgcccctaactccgcccagttccgcccattctccgccccatggctgactaatttttttttatttatgcagaggccgaggccgcctcggcctctgagctattccagaagtagtgaggaggctttttggaggcctaggcttttgcaaaaagcttgattcttctgacacaacagtctcgaacttaagctgcagaagttggtcgtgaggcactgggcaggtaagtatcaaggttacaagacaggtttaaggagaccaatagaaactgggcttgtcgagacagagaagactcttgcgtttctgataggcacctattggtcttactgacatccacctttgcctttctctccacaggtgtccactcccagttcaattacagctcttaaggctagagtacttaatacgactcactataggctagccaccatgacttcgaaagtttatgatccagaacaaaggaaacggatgataactggtccgcagtggtggggccagatgtaaacaaatgaatgttcttgattcatttattaattattatgattcagaaaaacatgcagaaaatgctgttatttttttacatggtaacgcggcctcttcttatttatggcgacatgttgtgccacatattgagccagtagcgcggtgtattataccagaccttattggtatgggcaaatcaggcaaatctggtaatggttcttataggttacttgatcattacaaatatcttactgcatggtttgaacttcttaatttaccaaagaagatcatttttgtcggccatgattggggtgcttgtttggcatttcattatagctatgagcatcaagatazhyagatcaaagcaatagttcacgctgaaagtgtagtagatgtgattgaatcatgggatgaatggcctgatattgaagaagatattgcgttgatcaaatctgaagaaggagaaaaaaatggttttggagaataacttcttcgtggaaaccatgttgccatcaaaaatcatgagaaagttagaaccagaagaatttgcagcatatcttgaaccattcaaagagaaaggtgaagttcgtcgtccaacattatcatggcctcgtgaaatcccgttagtaaaaggtggtaaacctgacgttgtacaaattgttaggaattataatgcttatctacgtgcaagtgatgatttaccaaaaatgtttattgaatcggacccaggattctttttccaatgctattgttgaaggtgccaagaagtttcctaatactgaatttgtcaaagtaaaaggtcttcatttttcgcaagaagatgcacctgatgaaatgggaaaatatatcaaaatcgttcgttgagcgagttctcaaaaatgaacaataattctagagcggccgcttcgagcagacatgataagatacattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttcaggggaggtgtgggaggttttttaaagcaagtaaaacctctacaaatgtggtaaaatcgataaggatccaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattccctttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatccccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctcacatggctcgac.

Generation of pRLTK and pRLTKL78. To create the constructs pRLTK and pRLTKL78, the TK promoter (sequence shown

below) was cut with BglII and HindIII from the plasmid pRL-TK (Promega) and cloned into the BglII and HindIII sites of pRLSV40 and pRLSV40L78, respectively.

TK Promoter Sequence. gatctaaatgagtcttcggacctcgcgggggc-cgcttaagcggtggttagggtttgtctgacgcgggggagggggaaggaacgaaa-cactctcattcggaggcggctcggggtttggtcttggtggccacgggcacgcagaa-gagcgccgcgatcctcttaagcacccccccgccctccgtggaggcgggggtttg-gtcggcgggtggtaactggcgggccgctgactcgggcgggtcgcgcgccccagagt-gtgacctttcggtctgctcgcagacccccgggcggccgccgcggcggcgac-gggctcgctgggtcctaggctccatggggaccgtatacgtggacaggctctggag-catccgcacgactgcggtgatattaccggagaccttctgcgggacgagccgggt-cacgcggctgacgcggagcgtccgttgggcgacaaacaccaggacggggcacag-gtacactatcttgtcacccggaggcgcgagggactgcaggagcttcagggagtg-gcgcagctgcttcatccccgtggcccgttgctcgcgtttgctggcggtgtccccg-gaagaaatatatttgcatgtctttagttctatgatgacacaaacccgcccagcg-tcttgtcattggcgaattcgaacacgcagatgcagtcggggcggcgcggtcccag-gtccacttcgcatattaaggtgacgcgtgtggcctcgaacaccgagcgaccctg-cagcgacccgcttaaaagct.

Generation of pRLSV40-Enh and pRLSV40L78-Enh. To create the constructs pRLSV40-Enh and pRLSV40L78-Enh, the SV40 promoter without the enhancer region (sequence shown below) was cut with BglII and HindIII from the plasmid pGL3 (Promega) and cloned into the BglII and HindIII sites of pRLSV40 and pRLSV40L78, respectively.

gatctgcgatctgcatctcaattagtcagcaaccatagtcccgcccctaactc-cgcccatcccgcccctaactccgcccagttccgcccattctccgccccatcgct-gactaatttttttttatttatgcagaggccgaggccgcctcggcctctgagctattc-cagaagtagtgaggaggctttttggaggcctaggcttttgcaaaaagct.

Generation of pLSV. The construct pLSV, previously called pGL3′, was described by Stoneley *et al.* [Stoneley M, Paulin FE, Le Quesne JP, Chappell SA, Willis AE (1998) C-Myc 5′ untranslated region contains an internal ribosome entry segment. *Oncogene* 16:423–428]. Briefly, an EcoRI site was introduced between the HindIII site and the NcoI sites of pGL3 (Promega), using PCR. Subsequently, an oligonucleotide containing an EcoRV site, a PvuII site and an SpeI site was inserted at the EcoRI site, thereby creating a polylinker region between the SV40 promoter and the start codon of firefly luciferase [Stoneley *et al.* (1998)].

Generation of pLSVM3′ (myc 3′UTR). The sequence of the short human c-*myc* 3′UTR was amplified by using reverse transcriptase followed by PCR with the primers 5′-GATCACTAGT-GGAAAAGTAAGGAAAACGATT-3′ and 5′-GATCTCTA-GAAAAGTTATTTACATTTAATGG-3′. To create the construct pLM3′, the c-*myc* 3′UTR sequence was inserted into the XbaI site of pL, immediately downstream of the firefly luciferase ORF.

Human c-myc 3′UTR Sequence. ggaaaagtaaggaaaacgattccttct-aacagaaatgtcctgagcaatcacctatgaacttgtttcaaatgcatgatcaaat-gcaacctcacaaccttggctgagtcttgagactgaaagatttagccataatgta-aactgcctcaaattggactttgggcataaaagaacttttttatgcttaccatctttt-ttttttctttaacagatttgtatttaagaattgtttttaaaaaatttaagatttacac-aatgtttctctgtaaatattgccattaaatgtaaataacttt.

Generation of pLTK and pLTKM3. To create the constructs pLTK and pLTKM3, the TK promoter (sequence shown below) was cut with BglII and HindIII from the plasmid pRL-TK (Promega) and cloned into the BglII and HindIII sites of pL and pLM3, respectively.

TK Promoter Sequence. gatctaaatgagtcttcggacctcgcgggggccg-cttaagcggtggttagggtttgtctgacgcgggggagggggaaggaacgaaa-cactctcattcggaggcggctcggggtttggtcttggtggccacgggcacgcagaa-gagcgccgcgatcctcttaagcacccccccgccctccgtggaggcgggggtttg-gtcggcgggtggtaactggcgggccgctgactcgggcgggtcgcgcgccccagagt-gtgacctttcggtctgctcgcagacccccgggcggccgccgcggcggcgac-gggctcgctgggtcctaggctccatggggaccgtatacgtggacaggctctggag-catccgcacgactgcggtgatattaccggagaccttctgcgggacgagccgggt-cacgcggctgacgcggagcgtccgttgggcgacaaacaccaggacggggcacag-gtacactatcttgtcacccggaggcgcgagggactgcaggagcttcagggagtg-gcgcagctgcttcatccccgtggcccgttgctcgcgtttgctggcggtgtccccg-gaagaaatatatttgcatgtctttagttctatgatgacacaaacccgcccagcg-tcttgtcattggcgaattcgaacacgcagatgcagtcggggcggcgcggtcccag-gtccacttcgcatattaaggtgacgcgtgtggcctcgaacaccgagcgaccctgcag-cgacccgcttaaaagct.

Generation of pLP2 and pLP2M3. To create the constructs pLP2 and pLP2L78, the myc P2 promoter was amplified by using primers 5′ TTTATAATGCGAAGATCTGGACGGCTGAGG 3′ and 5′ ATTAAAAGGCAAGCTTACTTCGGTGCTTAC 3′. The PCR product was cloned into the BglII and HindIII sites in plasmids pL and pLM3 thus creating pLP2 and pLP2M3, respectively.

Generation of pRFL78 and pRHCVFL78. To create the constructs pRFL78 and pRHCVFL78, eight copies of let-7a targets were cloned into the 3′UTR of the dicistronic construct pRF and pRHCVF [Stoneley *et al.* (1998)] on the FseI site.

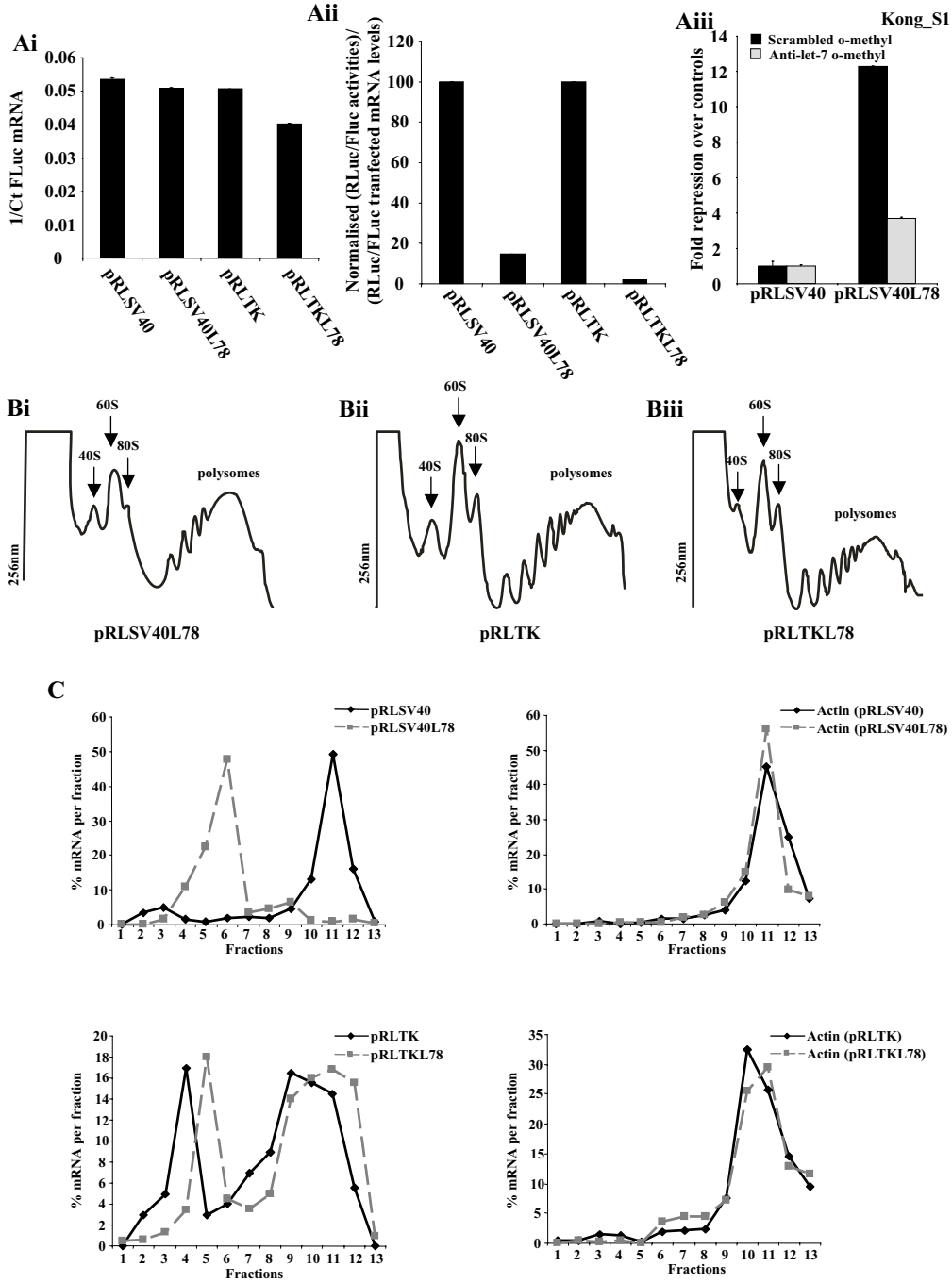

Fig. S1. (*A*) Normalization of luciferase expression to transfection efficiency shows a similar degree of translation repression to the data when normalized to mRNA levels, as in Fig. 1*C*. (*Ai*) Quantification by *qRT*-PCR of the firefly luciferase mRNA cotransfected into HeLa cells with *Renilla* luciferase (Fig. 1*B*), as a measure of transfection efficiency. (*Aii*) Normalization of *Renilla* luciferase activity in HeLa cells to transfection efficiency (*Ai*) for data from Fig. 1*B*. [*Renilla* luciferase activity/firefly luciferase activity)/(*Renilla* luciferase mRNA level (Fig. 1*C*))/(Firefly luciferase mRNA level (*Ai*)]. (*Aiii*) 2′-*O*-methyl oligonucleotides directed against let-7 restore translation efficiency. HeLa cells were transfected with either pRLSV40 or pRLSV40L78 along with either control scrambled 2′-*O*-methyl oligonucleotides or 2′-O-methyl oligonucleotides directed against let-7 at final concentration of 200 nM, as indicated. β-Galactosidase activity acted as a transfection control and values calculated as fold repression against control. (*B*) Accompanying polysome profiles from Fig. 1*D*. (*Bi*) Sucrose density profile of HeLa cells transfected with pRLSV40L78, ribosomal subunits and polysomes locations are marked above the A256-nm trace (Fig. 1*Dii Lower*). (*Bii*) Sucrose density profile of HeLa cells transfected with pRLTK, ribosomal subunits and polysome locations are marked above the A256-nm trace (Fig. 1*Diii Upper*). (*Biii*) Sucrose density profile of HeLa cells transfected with pRLTKL78, ribosomal subunits and polysome locations are marked above the A256-nm trace (Fig. 1*Diii Lower*). Note: 20% less material was loaded onto this gradient. (*C*) Quantification of the northern analysis from Fig. 1*D* for both *Renilla* luciferase and actin mRNAs across the polysome gradient, expressed as a percentage per fraction of total signal. (*D*) Removal of the enhancer element reduces the transcript levels to below that of the TK promoter without affecting the mode of miRNA repression. (*Di*) qRT-PCR of total *Renilla* luciferase and actin mRNA levels in HeLa cells transfected with pRLSV40, pRLTK or pRLSV40-enh. Total RNA was prepared and subjected to qRT-PCR with primers specific to both *Renilla* luciferase and actin. *Renilla* luciferase mRNA levels were normalized to actin. (*Dii*) Sucrose density profile of HeLa cells transfected with pRLSV40-enh (*Left*) or pRLSV40L78-enh (*Right*) (Fig. 1*E*). Ribosomal subunits and polysome locations are marked above the A256-nm trace. (*Diii*) qRT-PCR of polysome association of actin mRNA levels in HeLa cells from Fig. 1*E* and *Dii*. (*Div*) qRT-PCR of total *Renilla* mRNA levels in HeLa cells transfected with pRLSV40-enh or pRLSV40L78-enh normalized to actin total mRNA levels. (*Dv*) Percentage luciferase activity of HeLa cells transfected as in *Div* expressed as *Renilla* luciferase activity/Firefly luciferase activity. (*E*) Data from Fig. 1*E* expressed as 1/Ct.

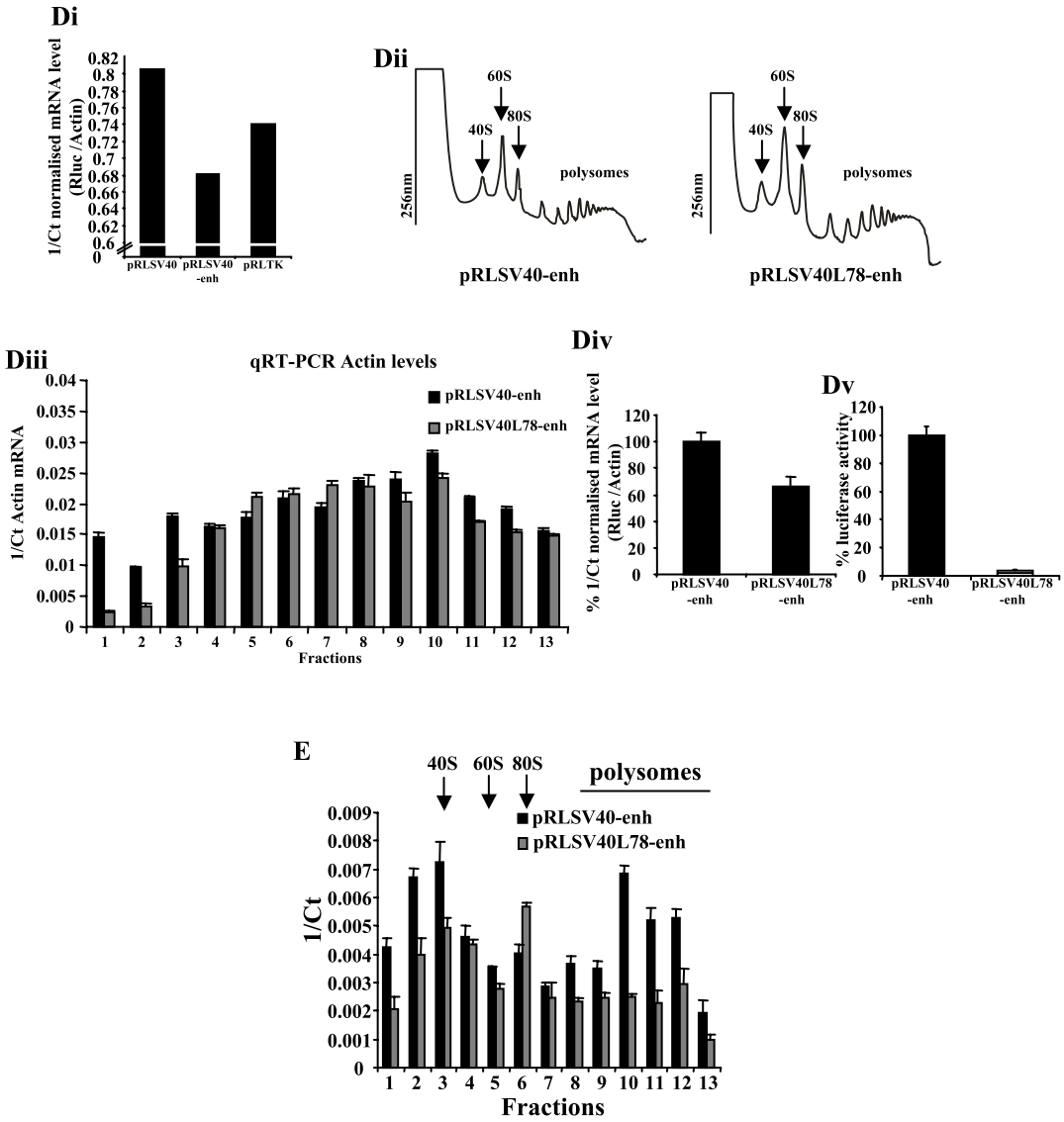

Fig. S1. (*Continued*)

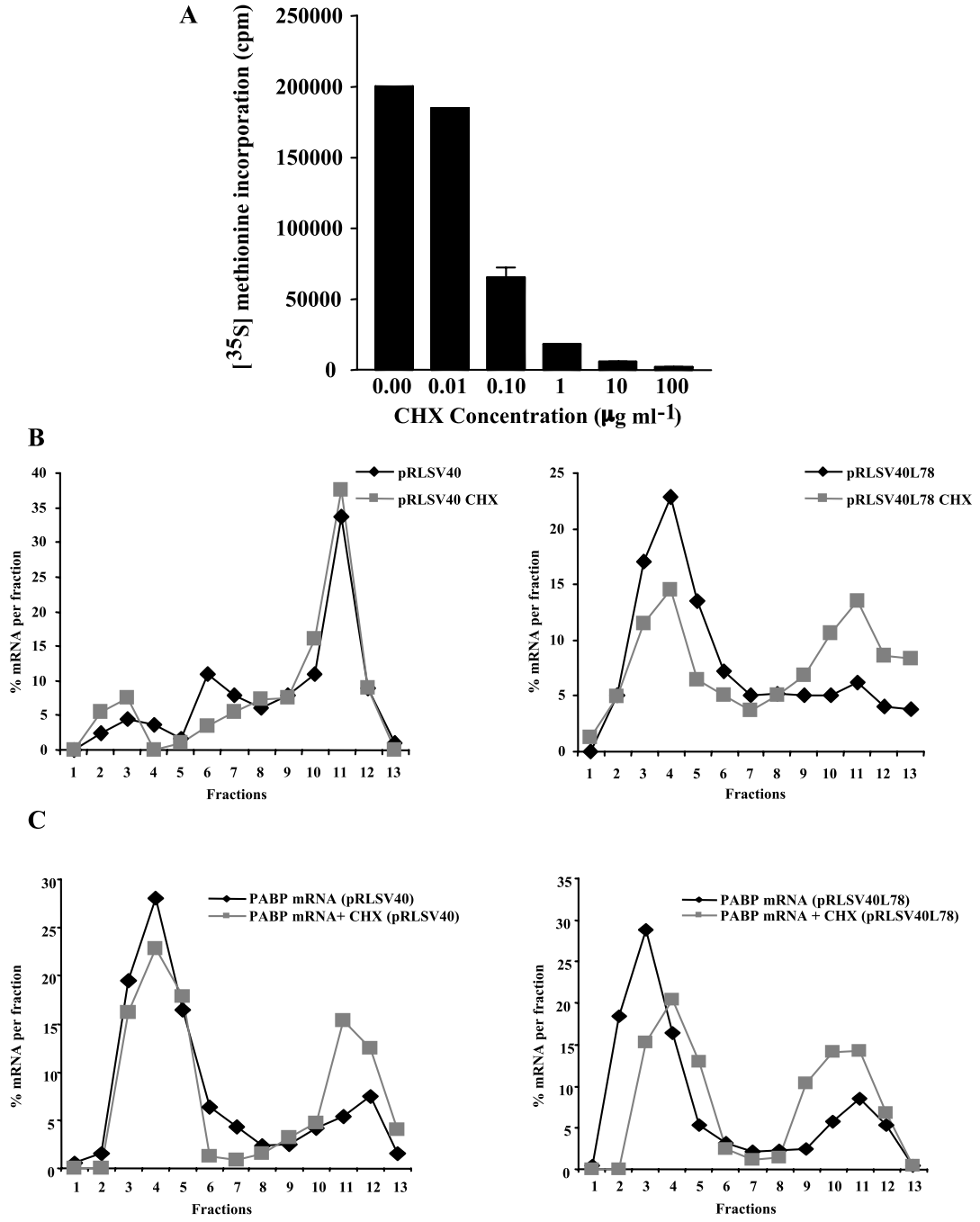

Fig. S2. (*A*) Concentration-response course to determine the inhibitory effect of cycloheximide on translation. HeLa cells were treated with cycloheximide at the concentration indicated for 30 min. Five minutes after cycloheximide treatment, [^{35}S]methionine was added to the cells. Cells were harvested and the amount of protein synthesis determined by TCA precipitation followed by scintillation counting. (*B*) Quantification of northern analysis of *Renilla* luciferase mRNA in each fraction shown in Fig. 2*A*, expressed as a percentage of total *Renilla* luciferase mRNA detected. (*C*) Quantification of northern analysis of PABP mRNA in each fraction shown in Fig. 2*A* expressed as a percentage of total PABP mRNA detected. Transfected constructs are indicated in brackets. (*Di*) Quantification of northern analysis of *Renilla* luciferase mRNA in each fraction shown in Fig. 2*B* expressed as a percentage of total of *Renilla* luciferase mRNA detected. (*Dii*) Quantification of northern analysis of actin mRNA in each fraction shown in Fig. 2*B* expressed as a percentage of total actin mRNA detected. Transfected constructs are indicated in brackets.

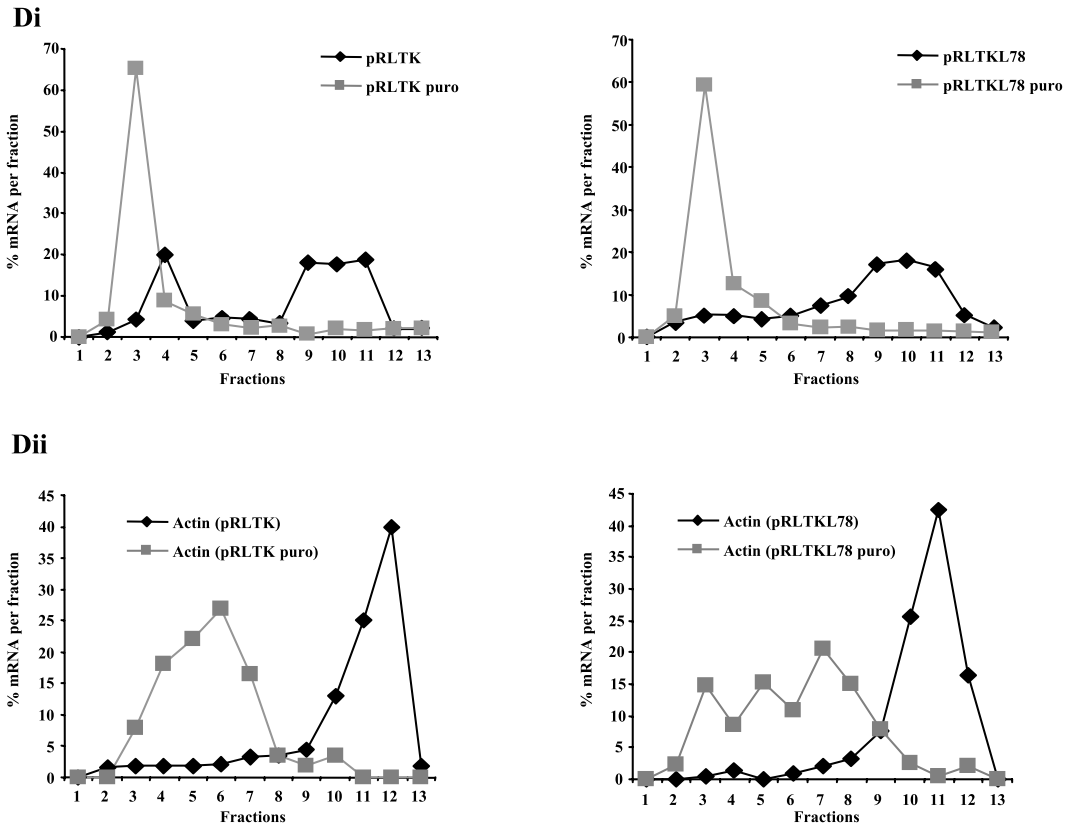

Fig. S2. (*Continued*)

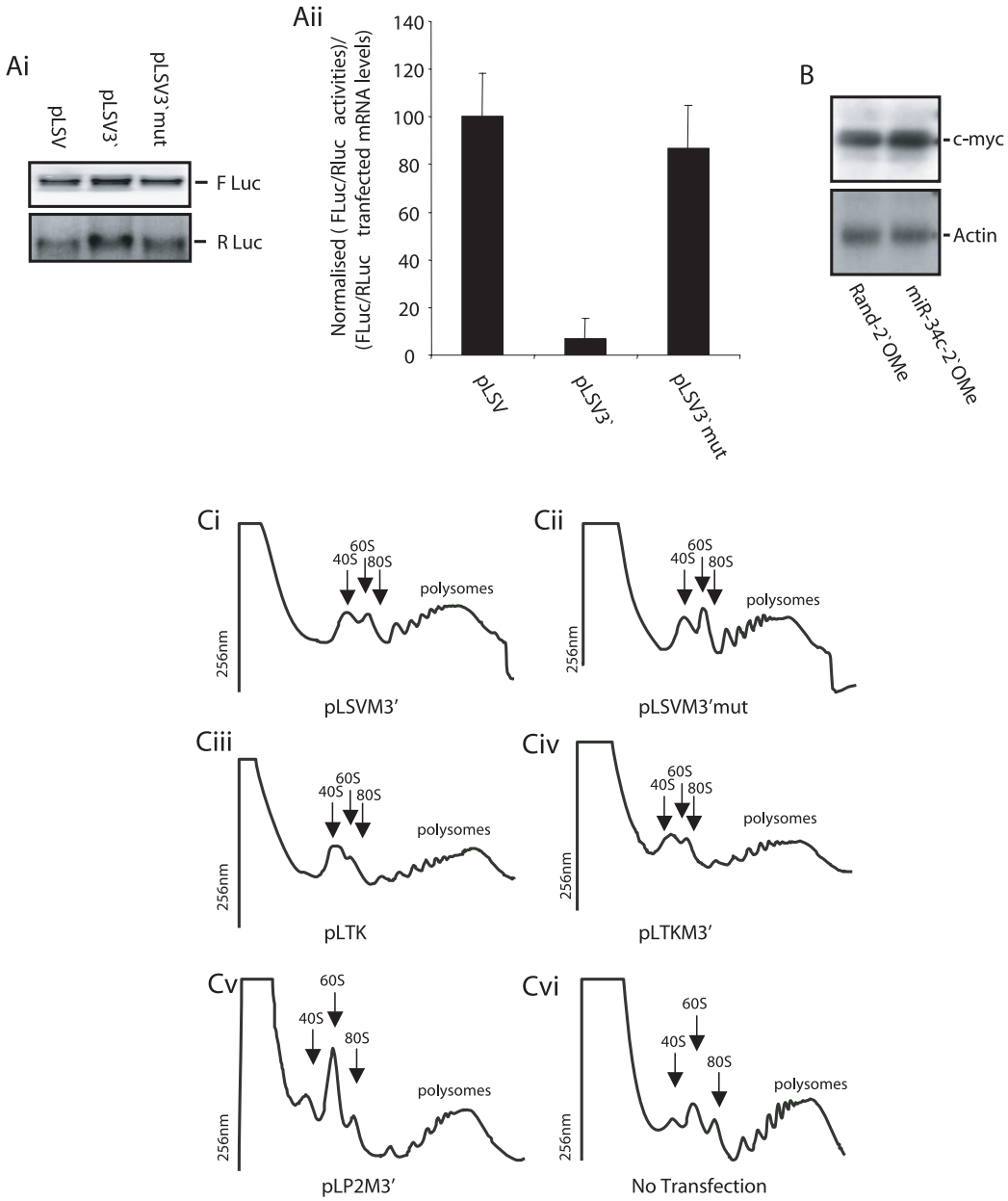

Fig. S3. (*A*) Normalization of *Renilla* luciferase activity to transfection efficiency shows a similar degree of translation repression to that seen in Fig. 3*Bii*, when data were normalized to *Renilla* mRNA. (*Ai*) Northern analysis of total Firefly and *Renilla* luciferase mRNA levels from HeLa cells transfected as in Fig. 3*Bi*. (*Aii*) Normalized values for luciferase activity in HeLa cells from Fig. 3*Bi*. [Firefly luciferase activity/*Renilla* luciferase activity)/(Firefly luciferase mRNA level (*Ai*))/(*Renilla* luciferase mRNA level (*Ai*)]. (*B*) *c-myc* mRNA level do not change after treatment of cells with 2′-*O*-methyl oligonucleotides directed against miR-34c. Northern analysis was performed on cells transfected with 2′-*O*-methyl oligonucleotides directed against miR-34c or a control oligonucleotide. Membranes were subsequently probed with radiolabeled DNA derived from c-myc and actin as indicated. (*C*) Accompanying polysome profiles from Fig. 3*F*. (*Ci*) Sucrose density profile of HeLa cells transfected with pLSVM3′. Ribosomal subunits and polysome locations are marked above the A256 trace. (*Cii*) Sucrose density profile of HeLa cells transfected with pLSVM3′mut. Ribosomal subunits and polysome locations are marked above the A256 trace. (*Ciii*) Sucrose density profile of HeLa cells transfected with pLTK. Ribosomal subunits and polysome locations are marked above the A256 trace. (*Civ*) Sucrose density profile of HeLa cells transfected with pLTKM3′. Ribosomal subunits and polysome locations are marked above the A256 trace. (*Cv*) Sucrose density profile of HeLa cells transfected with pLP2M3′. Ribosomal subunits and polysome locations are marked above the A256 trace. (*Cvi*) Sucrose density profile of control HeLa cells, no transfection. ribosomal subunits and polysome locations are marked above the A256 trace. (*D*) Actin mRNA distribution across polysome gradients accompanying Fig. 3*F*. Membranes from Fig. 3*F* were hybridized for actin mRNA. Transfected constructs are indicated. (*Ei*) Quantification of northern analysis of firefly luciferase mRNA in each fraction shown in the Fig. 3*F Upper*, expressed as a percentage of total firefly luciferase mRNA detected. (*Eii*) Quantification of northern analysis of actin mRNA in each fraction shown in *C*, expressed as a percentage of total actin mRNA detected. These profiles correspond to gradients in the Fig. 3*F Upper*, as indicated. (*Eiii*) Quantification of northern analysis of firefly luciferase or *c-myc* mRNA shown in the Fig. 3*F Lower* expressed as a percentage of the total of that mRNA detected. (*F*) qRT-PCR of total firefly luciferase mRNA levels normalized to actin mRNA levels from HeLa cells transfected with either pLTK, pLTKM3′, pLP2, or pLP2M3′.

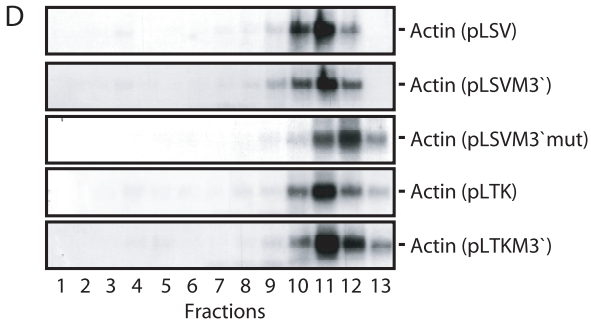

Fig. S3. (*Continued*)

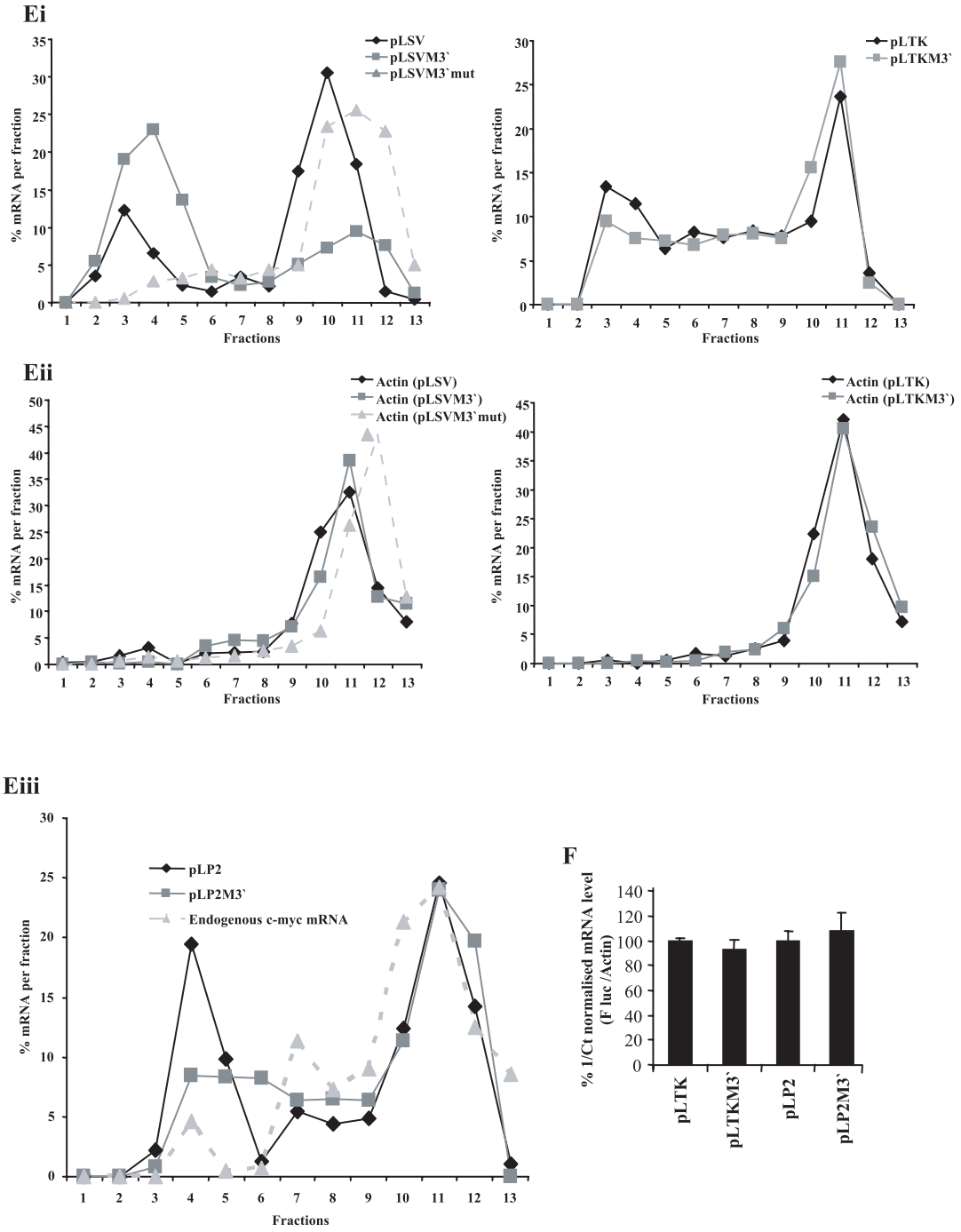

Fig. S3. (*Continued*)

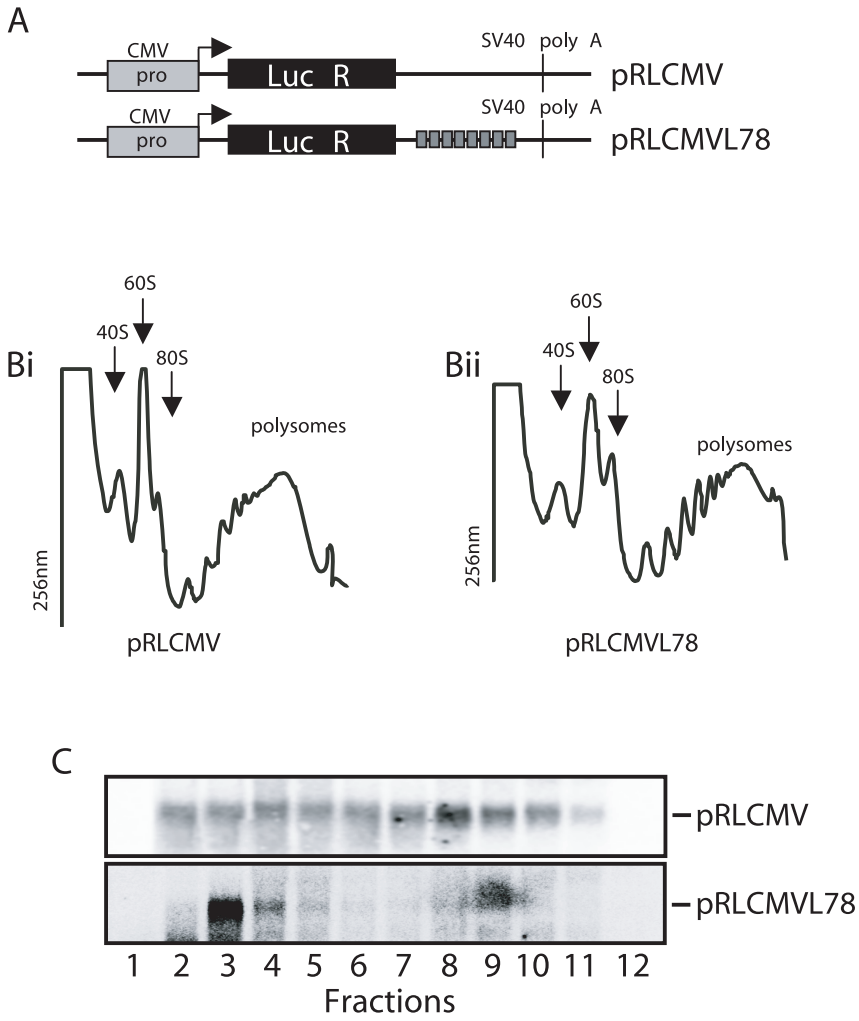

Fig. S4. CMV promoter leads to the production of mRNAs that undergo type I repression when targeted by let-7. (*A*) Diagrammatic representation of the pRLCMV and pRLCMVL78 reporter constructs. The SV40 promoter/enhancer elements from pRLSV40 and pRLSV40L78 were replaced with the CMV promoter element from pCDNA3.1 (Invitrogen), generating the constructs pRLCMV and pRLCMVL78. (*B*) HeLa cells (6×10^6) were transfected with constructs as indicated. Postnuclear lysates were prepared and subjected to sucrose density gradient centrifugation analysis i) Polysome trace from gradient analysis of cells transfected with the pRLCMV construct. ii) Polysome trace from gradient analysis of cells transfected with the pRLCMVL78 construct. (*C*) Northern blot analysis was then performed on equal volumes of RNA and membranes were probed with radiolabeled DNA derived from *Renilla* luciferase.

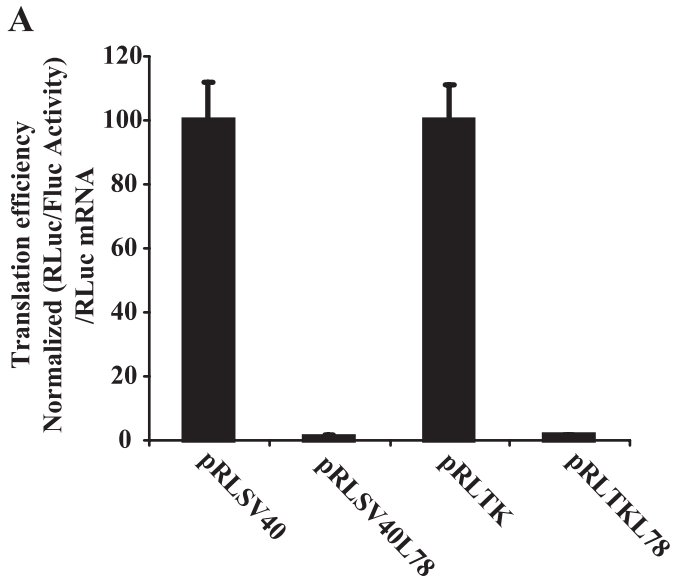

Fig. S5. The method of transfection does not significantly affect the degree of microRNA repression. (*A*) HeLa cells were transfected by using the constructs shown in Fig. 1*A* by electroporation, using Amaxa Nucleofector II and Kit R per the manufacturer's instructions (Amaxa Biosystems) (Fig. 1*A*). Lysates from transfected cells were assayed for luciferase activity, and *Renilla* luciferase levels were normalized to the transfection control, firefly luciferase. In parallel, total mRNA was prepared, quantified by real-time PCR and values were normalized to *Renilla* luciferase mRNA levels as a measure of translational efficiency. Experiments were performed in triplicate on three independent occasions.