SUPPLEMENTAL MATERIAL

The mechanism of translation initiation on Type 1 picornavirus IRESs

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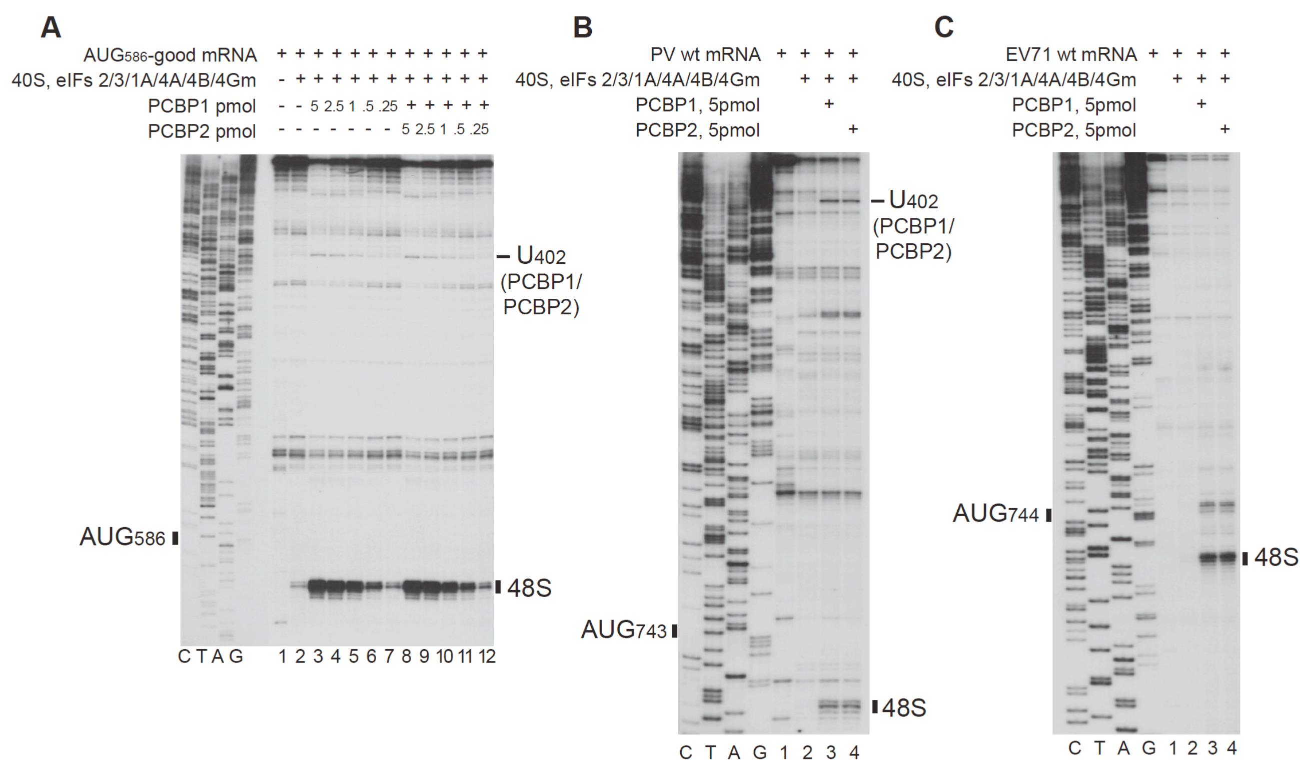

Figure S1. Equivalent activity of PCBP1 and PCBP2 in promoting initiation on Type 1 IRESs. Toeprinting analysis of 48S complex formation (A) at AUG_{586} on PV AUG_{586}-Good mRNA, (B) at AUG_{743} on PV *wt* mRNA and (C) at AUG_{744} on EV71 *wt* mRNA. Reaction mixtures contained 40S subunits, Met-$tRNA^{Met}_{i}$, initiation factors and amounts of PCBP1 or PCBP2 as indicated. The positions of initiation codons are indicated on the left and toe-prints caused by binding of PCBP1 or PCBP2 and by 48S complexes are shown on the right.

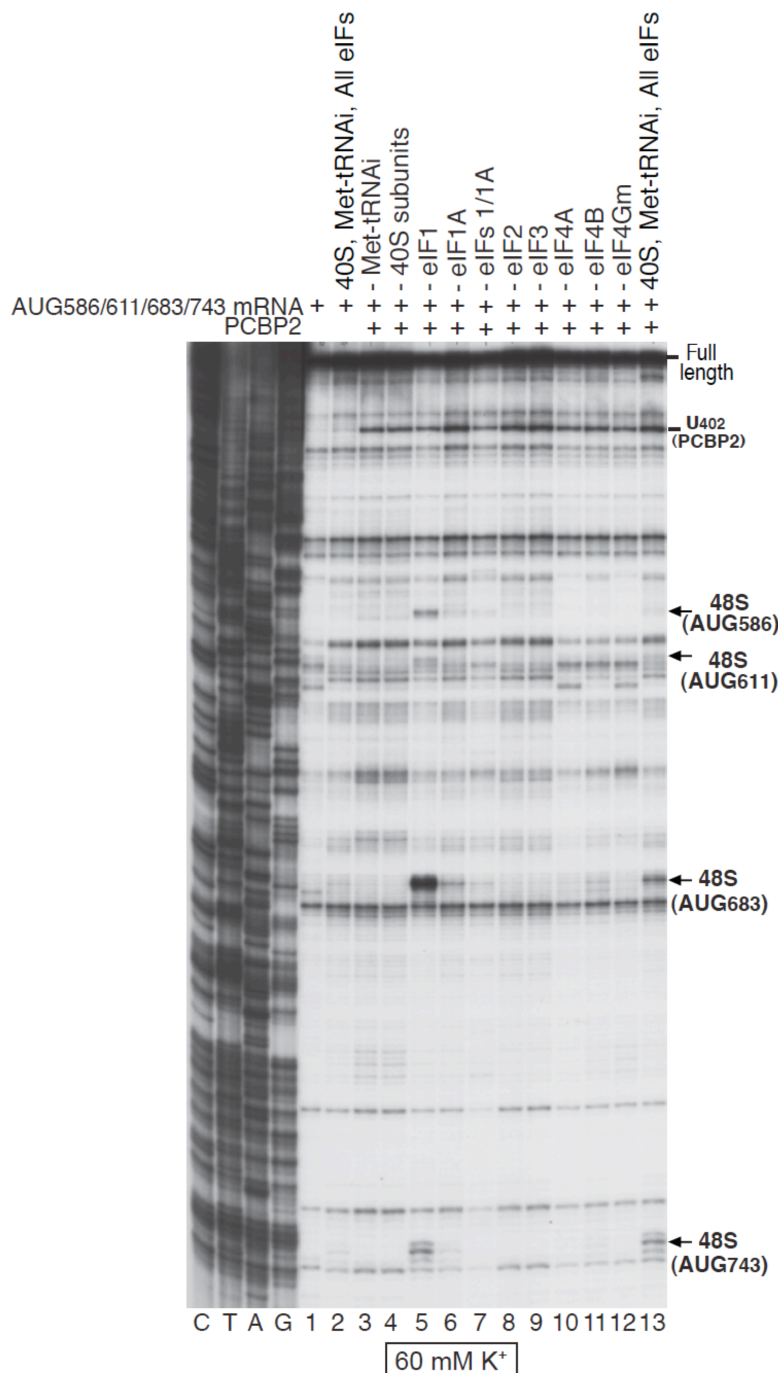

Figure S2. Factor requirements for 48S complex formation at AUG triplets at positions 586, 611, 683 and 743 of the PV IRES.

Toeprinting analysis of 48S complex formation on $AUG_{586/611/683/743}$ mRNA (upper panel in Fig. 2F of the main text). Reaction mixtures contained 40S subunits, Met-tRNA$_i^{Met}$ and indicated sets of eIFs and PCBP2. Toeprints caused by 48S complexes assembled at AUG_{586}, AUG_{611}, AUG_{683} and AUG_{743} are shown on the right.

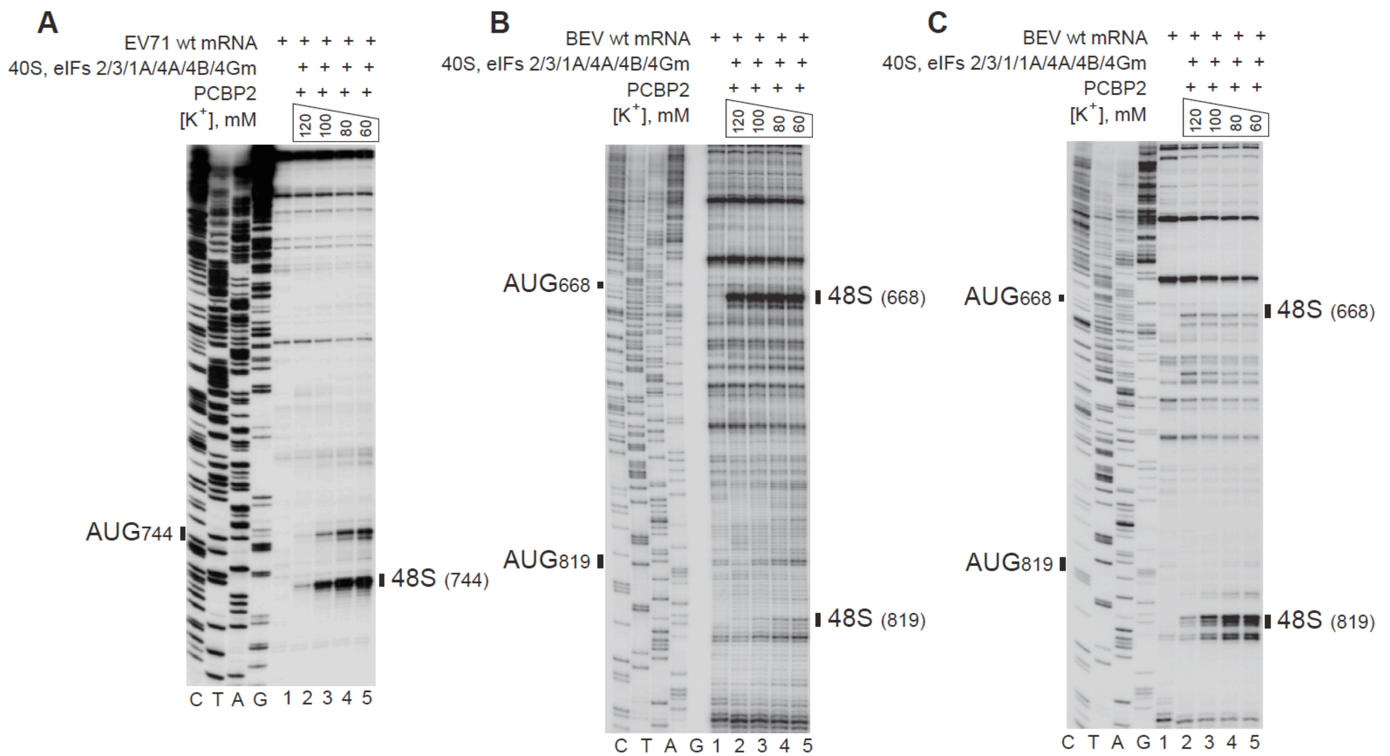

Figure S3. [K^+] dependence of 48S complex formation on EV71 and BEV Type 1 IRESs. Toeprinting analysis of 48S complex formation on (A) EV71 *wt* mRNA, and (B, C) BEV *wt* mRNA. 48S complexes were assembled at the indicated K^+ concentrations. Reaction mixtures contained 40S subunits, Met-$tRNA^{Met}_i$, initiation factors and PCBP2 as indicated. Toe-prints caused by 48S complexes assembled on (A) EV71 AUG_{744}, and (B, C) BEV AUG_{668} and AUG_{819} are shown on the right.

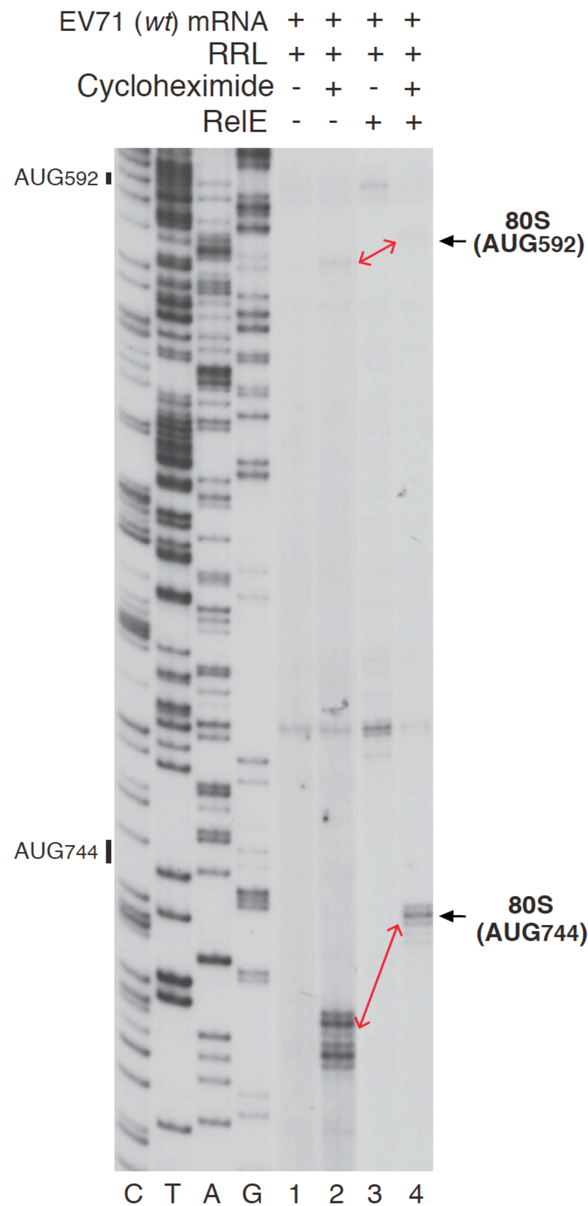

Figure S4. Utilization of AUG_{592} and AUG_{744} of the EV71 IRES in a cell-free translation extract. Analysis of 80S ribosomal complexes assembled on *wt* EV71 mRNA in RRL in the presence of cycloheximide. After incubation, ribosomal complexes were separated by sucrose density gradient centrifugation, and fractions corresponding to 80S ribsosomes were assayed by toe-printing (lanes 1-2) or RelE cleavage (lanes 3-4). Positions of ribosomal complexes formed at AUG_{592} and AUG_{744} are shown on the right.

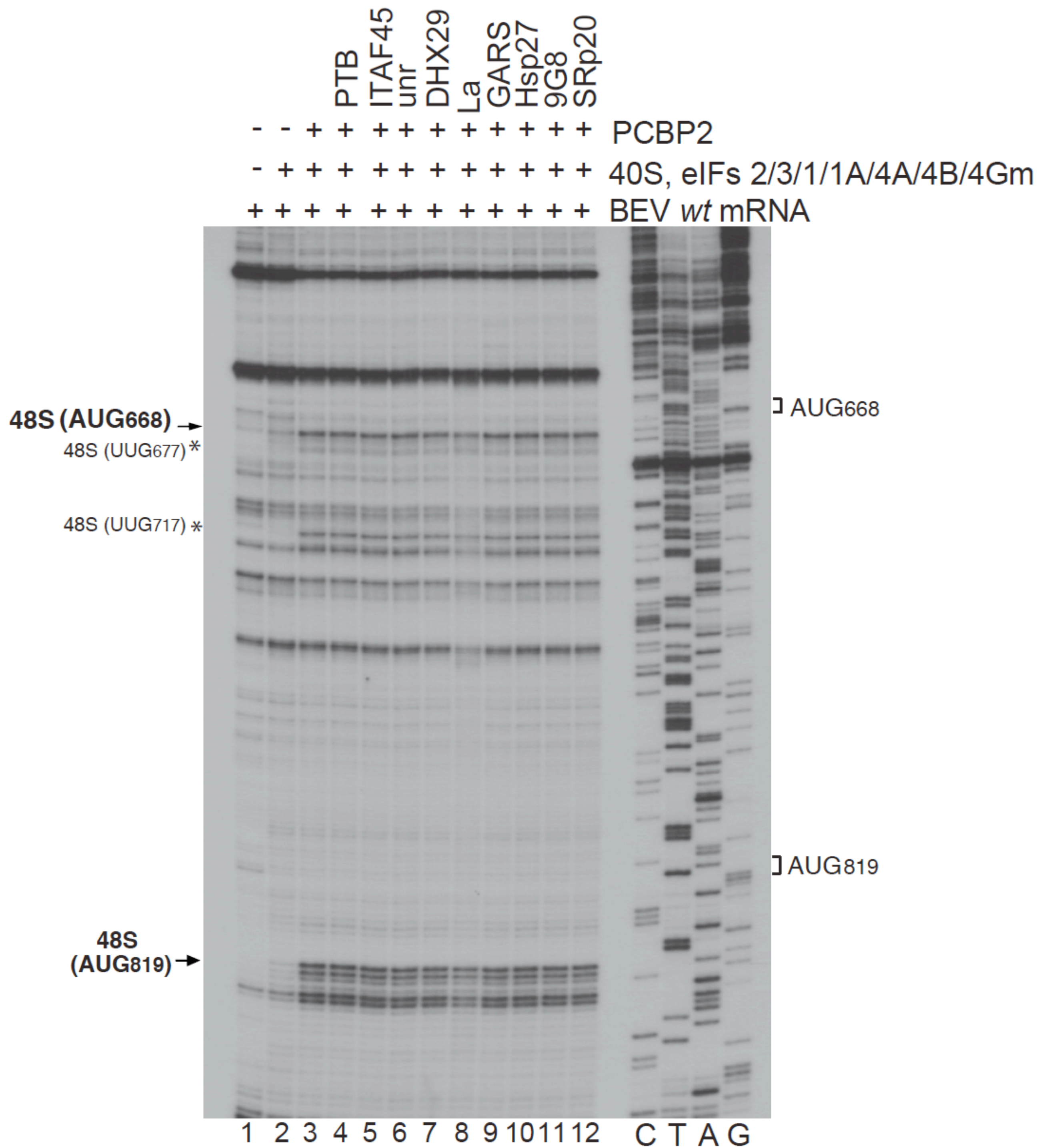

Figure S5. 48S complex formation on the *wt* BEV IRES in the presence of different ITAFs.
48S complex formation was assayed by toe-printing. Reaction mixtures contained 40S subunits, Met-tRNA$_i^{Met}$ and indicated sets of eIFs and ITAFs. Asterisks show toe-prints caused by 48S complexes assembled on near-cognate initiation codons. Toe-prints caused by 48S complexes assembled on AUG triplets are indicated by arrows.

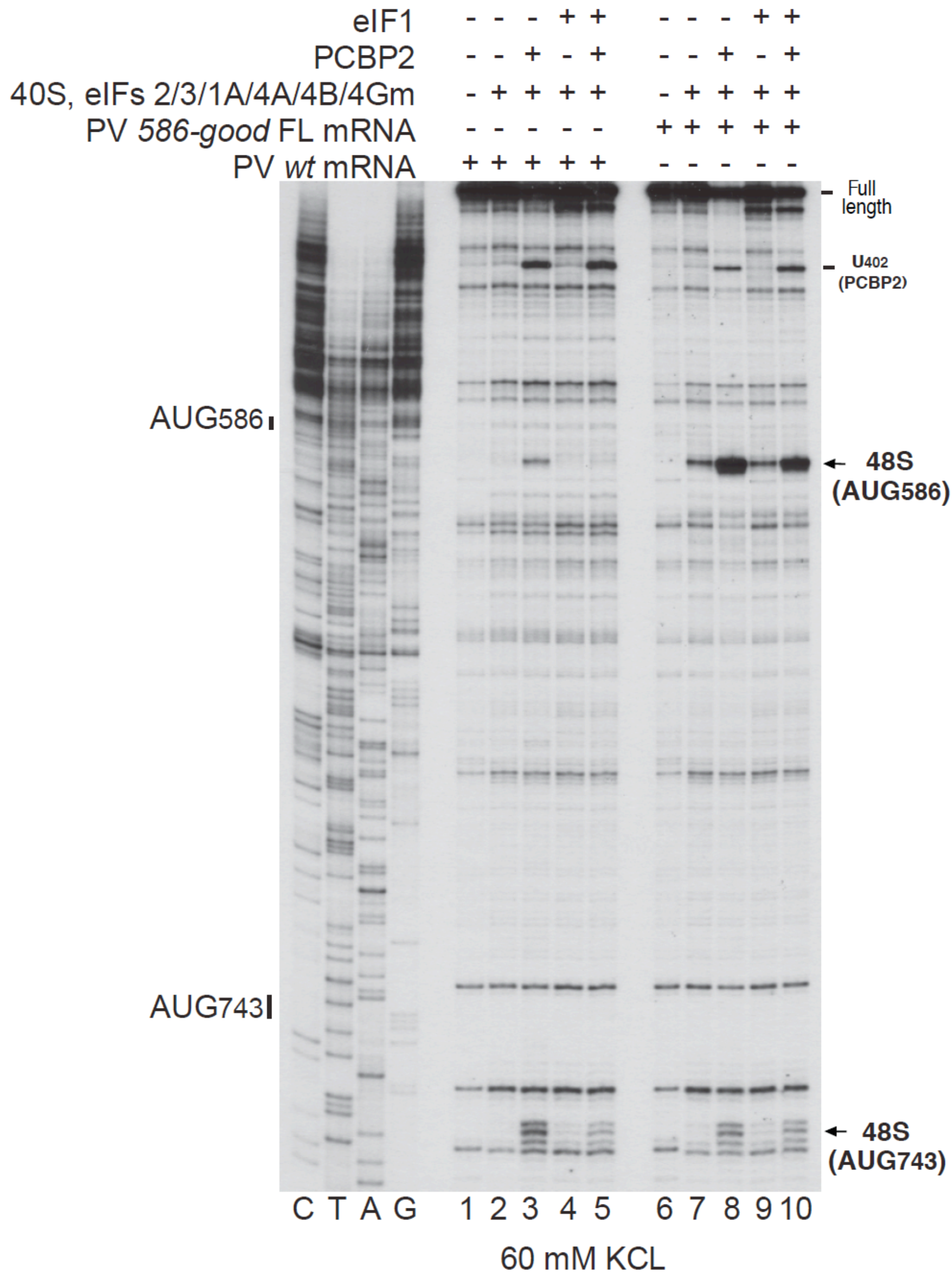

Figure S6. 48S complex formation on the *wt* and full-length AUG$_{586\text{-}good}$ PV IRESs.
48S complex formation was assayed by toe-printing. Reaction mixtures contained 40S subunits, Met-tRNA$_i^{Met}$ and indicated sets of eIFs and ITAFs. Toe-prints caused by 48S complexes assembled on AUG$_{586}$ and AUG$_{743}$ are indicated by arrows.

Figure S7. Activity of mutant forms of PCBP2 in promoting initiation on the poliovirus IRES. Toeprinting analysis of 48S complex formation at AUG_{586} on PV AUG_{586}-good mRNA. 48S complexes were assembled at 100 mM K^+. Reaction mixtures contained 40S subunits, Met-$tRNA^{Met}_i$, initiation factors and mutant forms of PCBP2 as indicated. The position of AUG_{586} is indicated on the left and toeprints caused by 48S complexes are shown on the right.

Enterovirus A

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EV71          MVTIKELLPYSYWIGHPVSNRAIVYLFVGFTPLTLETLHTLNYIILLNTKRW-APRSPHSDPARMRIPTQPRKAPL
CVA6          MVTIERLLPYSYWIGHPVNNRALIYLFVGFFPLTLRVVETLKYILILNTRKW-VHKFQHKSPGHTRQEI-------
CVA8          MVTIEELLPYSYWIGHPVCNRAIIYLFVGFVPLTYRSLNTLQHILILNTRKW-GHKFQHRNPAVMRLV--------
CVA12         MVTIEKLLPYSYWIGHPVTNRAIVYLFVGYVPLNYKVVSTLKYILTLNCRKW-EHKSRHKGQGHTRLATSPQKVP-
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Enterovirus B

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CVB3          MVTIERLLPYSYWIGHPVTNRAIIYLFVGFIPLSLKEVKTLQFIVKLNTAKWELKYQRKRLGHMRPG--------
EV2           MVTITELLPYSYWIGHPVTNRAIIFLFVGFVPLTVTSFTTLNYILSLNTARWEPKFPHRKSALMKLNSILATTRQ
EV11          MVTIERLLPYSYWIGHPVTNRAIVFLFVGFIPLNYKVLNTLNLIIAFNTIKWELKCQHRRLGPTKLG--------
HEV107        MVTIDKLLPYSYWIGHPVTNRAIVYLFVGFIPLNYKVLETLNFILTLNTAKWELKYQRRRLGHMRLA--------
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Enterovirus C

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PV1S          MVTITDCYHKANWIGHPVKVRFIIYLFAGFAPLSVFTLSTISTVISIRQLYHNGCSGFITESGRT----------
CAV17         MVTIIDCYHKANWIGHPVRIRSIIYLFIGFTPLTTYTYDLIRVNLLLRHLHHNGSSSFLTKSRCP----------
CAV20         MVTIIDCYHKANWIGHPVKARLTIYLLVGLTTLNTFILGVTCIAIRIKQLHHNGCASFITESGCTREH-------
HEVC96        MVTIIDCYHNANWIGHPVIYKHIVYLFIGFTPLSSFTLHLTRVVLLTRQWCQHGSSGIITESWSS----------
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Enterovirus E

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BEV2[3A]      MVTIVEICDLATLPLNIALYYLVAFHKTSDIPSSYIDLLVYLNLKHKLEGLRWEHN-------------------
BEV2[BHM26]   MVTIAEICDLATLPLNIALYYLIAFHKTSDIPSSYIDLLVYLNLKHKLGRLKWVHK-------------------
BEV[RM2]      MVTIVEICPLATLPLNIGLYYLVAFYKTSDIPSSYIDLLVFLNLKYRLQIAKWERK-------------------
BEV[Jena38]   MVTIAEICELATLPLNIALYYLVAFHKTSDIPSTYITLLVYLNLKHKLDNTKWEHK-------------------
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Enterovirus F

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BEV-56        MVTLTDICEFARLPLNIALYYFVAFHKTTQLTVELVDFAVFLNLTYKKLNNGSPNEQEHRRKSHHWYLRHRRLHH
BEV-SL305     MVTLTDIREFARLPLNIALYYLVSFHKTTELSVELINFAVFLNQIHKRTCNGSTNEQKHSRKPHHWYIRHWRIN-
BEV-D3        MVTLTDICEFARLPLNIALYYLIAFHKTTQFTVELIDFAVYLNIL-HKRNNGSPNEQKHSRQPYHWHVRHQRVNH
BEV-VD        MVTLTDICEFARLPLNIALYYLIAFHKTTQLSLELIDFAVYLNRI-HKLNNGSPIEQKYRRQPHYRDLRYRRINH
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Enterovirus G

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PEV9[CH]      MVTTISCYHKAFGLATWKRLSVFNIVRLSRLPIKQALYYLVSFYPKEEITEFHLQLLLSLSTRWECK
PEVB[KOR]     MVTTISCYHKAFGLATWKKLSVFDIVRLSRLPIKQSLYYLISFYPQEEISEFHLQLLLTLSARWVCK
PEV10         MVTTISCYHKAFGLATWKKLSVFDIVRLSRLPTKQALLLLIAFYPSEVITDFHLKLLLTVAVKWEHK
PEV14         MVTTYSCYHKAFGLAIRKELTVCSLIALSKFPLRKAIYLLISFYRLDKISSAHIDLLIQLHQTWVCK
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Figure S8.

Sequences of polypeptides encoded by the cryptic ORF initiating at the 3'-border of Type 1 IRESs of members of the species Enterovirus A, B, C, E, F and G. Identical residues within these polypeptides are in bold; sequences highlighted in blue overlap the initiation codon for the viral polyprotein. For each species, alignment was done using Clustal-W and sequences that are representative of the diversity within the species.

Enterovirus A: EV71 = Human enterovirus 71 sub_strain:BrCr-TR (AB204852); CVA6 = Human coxsackievirus A6 strain Gdula (AY421764); CVA8 = Human coxsackievirus A8 strain Donovan (AY421766); CVA12 = Human coxsackievirus A12 strain Texas-12 (AY421768).

Enterovirus B: CVB3 = Human coxsackievirus B3 strain Nancy (JN048468); EV2 = Human echovirus 2 strain Cornelis (AY302545); EV11 = Human echovirus 11 strain Kust/86 (AY167105); HEV107 = Human enterovirus 107 strain TN94-0349 (AB426609).

Enterovirus C: PV1S = Human poliovirus Type 1 Sabin (AY184219); CAV17 = Human coxsackievirus A17 strain G12 (AF499639); CAV20 - Human coxsackievirus A20 strain IH- 35 (AF465514); HEVC96 = Human enterovirus 96 isolate FIN05-2 (FJ751915).

Enterovirus E: (BEV2[3A] = Bovine enterovirus type 2 strain 3A (AY508697); BEV[BHM26] = Bovine enterovirus type 2 strain BHM26 (HQ917060); BEV[RM2] = Bovine Enterovirus strain RM-2 (X79369); BEV[Jena38] = Bovine enterovirus isolate Jena 38/02 (DQ092788)).

Enterovirus F: BEV-56 = Bovine enterovirus isolate 56/59/1 (DQ092778); BEV-SL305 = Bovine enterovirus isolate SL305 (AF123433); BEV-D3 = Bovine enterovirus isolate D 3/98 (DQ092790); BEV-VD = Bovine enterovirus isolate VD 2860/1-99 (DQ092774).

Enterovirus G: PEV9[CH] = Porcine enterovirus 9 isolate Ch-ah-f1 (HM131607); PEVB[KOR] = Porcine enterovirus B isolate PEV-B-KOR (JQ818253); PEV10 = Porcine enterovirus 10 strain LP 54 (AF363455); PEV14 = Porcine enterovirus 3H strain swine/K23/2008/HUN (HQ702854).

MATERIALS AND METHODS

Plasmids

Expression vectors used were for His_6-tagged eIF1 and eIF1A (Pestova et al. 1998a), *wt* eIF4A and eIF4B (Pestova et al. 1996), eIF4A-S42C single cysteine mutant (de Breyne et al. 2009), *wt*, cysteine-less and a T829C variant of $eIF4GI_{736–1115}$ (‘eIF4Gm’) (Kolupaeva et al. 2003), $eIF4GI_{653-1599}$, $eIF4GI_{736–1008}$ and $eIF4GI_{736–988}$ (Lomakin et al. 2000), eIF4H (Doepker et al. 2004), DHX29 (Skabkin et al. 2010), *Escherichia coli* methionyl tRNA synthetase (Lomakin et al. 2006), human glycyl-tRNA synthetase (GARS) (Seburn et al. 2006), Hsp27 (Lelj-Garolla and Mauk, 2005), $ITAF_{45}$ (Pilipenko et al. 2000), PTB1 (Hellen et al. 1993), La (Horke et al. 2002), unr-5 and unr+5 isoforms (Anderson et al. 2007).

Human PCBP1 and PCBP2 coding sequences were amplified by PCR from pQE30-PCBP1 and pQE30-PCBP2, respectively (Blyn et al. 1996) and inserted between Nde1 and HindIII sites of pET28b (Novagen) to yield pET28b-PCBP1 and pET28b-PCBP2. Cysteine residues in PCBP2 were removed or added by mutagenesis of pET28b-PCBP2 (NorClone Biotech, London, Ontario).

The vector pET16b-9G8 for expression of N-terminally His_6-tagged 9G8 was made by inserting DNA corresponding to the 9G8 coding sequence into Nco1 and BamH1 sites of pET16b. The DNA was synthesized by GeneArt (Regensburg, Germany) using a gene sequence that had been optimized by DAPCEL, Inc. (Cleveland, OH) by selection of synonymous codons to ensure optimal co-translational folding in *E. coli*.

A PV mRNA transcription vector containing the full-length 5’-UTR was derived from pMN25 (Nicklin et al. 1997) by deleting an AvrII-AvrII fragment from the P1 coding region, yielding a 180 amino acid long PV1M VP0 coding sequence (20.2 kDa).

An EV71 mRNA transcription vector was made (GenScript) by inserting DNA corresponding to a 5’-terminal T7 promoter, two G residues and a variant of EV71strain BrCr-TR nt.1–1661 (GenBank AB204852) followed by two UGA stop codons and 61 heterologous nucleotides (containing unique Xho1 and HindIII restriction sites) into pUC57. The EV71 sequence contained substitutions that introduced AUG triplets at positions corresponding to codons 296, 297, 299, 307, 308 and 309 of the 309 amino acid long EV71 VP0-ΔVP3 coding sequence (34 kDa).

A BEV mRNA transcription vector was made (GenScript) by inserting DNA corresponding to a T7 promoter, two G residues and BEV nt.1-1580 (GenBank: D00214.1) between Sal1 and BamH1 sites in pUC57. The BEV sequence contained a U85C substitution to stabilize the first cloverleaf and substitutions to introduce stop codons in place of codons 224 and 225 and AUG triplets in place of codons 150, 160, 164, 170, 177, 182, 190, 201 and 205 of the 223 amino acid long (24.4 kDa) BEV coding sequence.

Mutations in domains V and VI, and downstream regions of PV, BEV and EV71 transcription vectors were introduced by NorClone Biotech.

The poliovirus type 1 Mahoney (PV1M) transcription vectors used to generate “AUG_{586}-good” and

'$AUG_{586/611/683/743}$' mRNAs correspond to the previously described constructs pIRES-(AUG586)Δ3D (Pestova et al. 1994) and pIRESΔP1(611) (Hellen et al. 1994), respectively.

Transcription vectors for monocistronic CSFV and EMCV IRES-containing mRNAs have been described (Pestova et al., 1996, 1998b).

BEV, CSFV, EMCV, EV71 and PV mRNAs were transcribed *in vitro* using T7 or T3 polymerase, as appropriate.

Purification of initiation factors, ribosomal subunits and aminoacylation of tRNA

40S ribosomal subunits, eIF2, eIF3 and eIF4F were purified from RRL (Pisarev et al. 2007). Recombinant eIF1, eIF1A, eIF4A, eIF4B, $eIF4GI_{653-1599}$, $eIF4G_{736-1015}$ ('eIF4Gm'), $eIF4G_{736-1008}$, $eIF4G_{736-988}$, eIF4Gm-T829C, eIF4Gm-Cys-less, *E. coli* methionyl tRNA synthetase and DHX29 were expressed and purified as described (Pisarev et al. 2007; Skabkin et al. 2010). Native total tRNA (Promega) was aminoacylated with *E. coli* methionyl tRNA synthetase as described (Pisarev et al. 2007).

Expression and Purification of recombinant ITAFs

Recombinant His_6-tagged unr(-5) and unr(+5) isoforms with or without exon 5 sequences were expressed in 1 liter of *E. coli* BL21 (DE3) after induction by 1 mM IPTG for 4 hours at 37°C. They were purified by affinity chromatography on Ni-NTA agarose, followed by FPLC on a MonoQ HR5/5 column. Fractions were collected across a 50 to 500 mM KCl gradient: His_6-Unr(-5) and His_6-Unr(+5) eluted at ~215 mM KCl.

Recombinant His_6-tagged PCBP2 was expressed in 1 liter of *E. coli* BL21 (DE3) after induction by 0.2 mM IPTG for 3 hours at 30°C. PCBP2 was purified by affinity chromatography on Ni-NTA agarose, followed by FPLC on a MonoQ HR5/5 column. Fractions were collected across a 30 to 500 mM KCl gradient: His_6-PCBP2 eluted at ~90 mM KCl. His_6-tagged PCBP1 was expressed and purified in an analogous manner.

Recombinant His_6-tagged GARS was expressed in 1 liter of *E. coli* BL21 (DE3) after induction by 0.2 mM IPTG for 5 hours at 30°C. His_6-GARS was purified by affinity chromatography on Ni-NTA agarose, followed by FPLC on a MonoQ HR5/5 column. Fractions were collected across a 50 to 500 mM KCl gradient: His_6-GARS eluted at ~ 200 mM KCl.

Recombinant Hsp27 was expressed and purified by precipitation with 35% ammonium sulfate and ion-exchange chromatography, essentially as described (Behlke et al. 1991), followed by FPLC on a MonoQ HR5/5 column and gel filtration using a G-200 Sephadex column.

Recombinant La was expressed in 1 liter of *E. coli* BL21 (DE3) after induction by 1 mM IPTG for 4 hours at 37°C, and was purified by affinity chromatography on Ni-NTA agarose, followed by FPLC on a MonoS HR5/5 column, using a 100 to 500 mM KCl gradient. Fractions containing La eluted between 130-180 mM KCl. They were dialyzed and then applied to a FPLC MonoQ HR5/5 column. Fractions were collected

across a 50 to 500 mM KCl gradient: La eluted at ~240 mM KCl.

Recombinant His-tagged 9G8 was expressed in 1 liter of *E. coli* BL21 (DE3) after induction by 0.2 mM IPTG for 4 hours at 37°C, and was purified by affinity chromatography on Ni-NTA agarose. 50 mM L-glutamate and 25 mM L-arginine were maintained in all 9G8 purification buffers (Golovanov et al. 2004). Eluted protein was further purified by FPLC on a Mono Q HR5/5 column. Fractions were collected across a 50 to 500 mM KCl gradient: 9G8 eluted at ~250 mM KCl.

Recombinant PTB1 and $ITAF_{45}$ were expressed and purified as described (Kolupaeva et al., 2000). Recombinant human GST-Srp20 was from Abnova (Taipei City, Taiwan).

Assembly and analysis of ribosomal complexes

To assemble 48S complexes, 1 pmol *wt* or mutant picornaviral IRES-containing mRNAs was incubated with 2 pmol 40S subunits, 4 pmol Met-$tRNA^{Met}_{i}$, and indicated combinations of 4 pmol eIF2, 3 pmol eIF3, 10 pmol eIF4A, 5 pmol eIF4B, 5 pmol eIF4H, 5 pmol $eIF4G_{653-1599}$, $eIF4G_{736-1115}$, $eIF4G_{736-1008}$, $eIF4G_{736-988}$, 2.5 pmol eIF4F, 10 pmol eIF1, 10 pmol eIF1A and 5 pmol of PCBP2 for 10 min at 37°C in 20 μl buffer A (20mM Tris pH 7.5, 60-120mM KCl, 1mM DTT, 2.5mM $MgCl_2$) with 0.25mM spermidine and supplemented with 1mM ATP and 0.4 mM GTP. PCBP1 (5 pmol), PTB (5 pmol), $ITAF_{45}$ (5 pmol), 9G8 (2 pmol), Hsp27 (2 pmol), GARS (2 pmol), unr+5 (2 pmol), unr-5 (2 pmol), DHX29 (0.4 pmol), La (5 pmol) or GST-SRp20 (3 pmol) were included in reactions as indicated in the text. Assembled 48S complexes were analyzed by toeprinting using avian myeloblastosis virus reverse transcriptase (AMV RT) and ^{32}P-labelled primers as described (Pisarev et al. 2007). cDNA products were resolved in 6% polyacrylamide sequencing gels.

Directed hydroxyl radical cleavage

eIF4Gm-T829C, eIF4A-S42C, PCBP2 mutants containing varying numbers of cysteine residues (3000 pmol) and native eIF3 (500 pmol) were derivatized with Fe(II)-BABE by incubation with 1mM Fe(II)-BABE in 100 μl buffer containing 80mM HEPES (pH 7.5), 300mM KCl and 10% glycerol for 30 min at 37°C (Kolupaeva et al. 2003). Derivatized proteins were separated from unincorporated reagent by buffer exchange on Microcon YM-30 filter units. To investigate hydroxyl radical cleavage, 5 pmol of *wt* or mutant PV, EV71 or CSFV IRES-containing mRNAs were incubated at 37°C for 10 min in 50 μl buffer B (20mM HEPES pH 7.6, 100mM KCl, 2.5mM $MgCl_2$ and 5% glycerol) with 10 pmol [Fe(II)-BABE]-eIF4Gm-T829C (in the presence/absence of 20 pmol unmodified eIF4A, eIF3, GARS or unr-5), 2 pmol [Fe(II)-BABE]-eIF3 (in the presence/absence of 20 pmol unmodified eIF4Gm and/or eIF4A), 10 pmol [Fe(II)-BABE]-eIF4A-S42C (in the presence/absence of 20 pmol unmodified $eIF4G_{736-1115}$, $eIF4G_{736-1008}$ or $eIF4G_{736-988}$), or 10 pmol [Fe(II)-BABE]-PCBP2. To generate hydroxyl radicals, reaction mixtures were supplemented with 0.05% H_2O_2 and 5mM ascorbic acid and incubated on ice for 10 min. Reactions were quenched by adding 20mM thiourea. Sites of hydroxyl radical cleavage were determined by primer extension using AMV RT and appropriate [^{32}P]-labeled

primers. cDNA products were resolved in a 6% sequencing gel.

In vitro translation

PV, EV71 and BEV mRNAs were translated using either the Flexi RRL System (Promega) or the Cell-free Protein Expression System (Takara Bio). In mixed lysate experiments, RRL (Promega) was supplemented with 30% (v/v) Cell-free Protein Expression System (Takara Bio). Reaction mixtures (20 µl) containing 0.3 µg RNA and 0.5 mCi/ml [^{35}S]methionine (43.5 TBq/mmol) were incubated for 60 min at 32°C. Translation products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography and quantification on a phosphoimager.

Analysis of ribosomal complex formation on PV and EV71 mRNAs in RRL.

EV71 *wt* or PV 586/611/683/743 mRNA was 5'-end labeled with ^{32}P using T4 Polynucleotide Kinase. [^{32}P]mRNA (5pmol) was incubated in nuclease-treated RRL (100 µl) (Promega) in the presence or absence of cycloheximide (100 µM), as indicated, at 37°C for 15 min. The reaction mixture was then loaded on to a 10-30% sucrose density gradient prepared in buffer A containing 60 mM KCl, and subjected to centrifugation in a Beckman SW55 rotor at 53,000 rpm for 75 minutes. 80S ribosomal fractions containing [^{32}P]mRNA were either analyzed directly by toe-printing using AMV RT and ^{32}P-labelled primers as described (Pisarev et al. 2007), or incubated with RelE (0.5 pmol) at 37°C for 30 minutes, phenol-extracted and then analyzed by primer extension using AMV RT. cDNA products were resolved in 6% polyacrylamide sequencing gels.

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