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Supplemental Data

Translation Initiation on Mammalian mRNAs with Structured 5′UTRs Requires DExH-Box Protein DHX29

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

Vectors for expression of recombinant eIF4A, eIF4B, eIF1, eIF1A, eIF5, eIF5B, PTB and *E. coli* methionyl tRNA synthetase, and for transcription of $tRNA^{Met}_i$, CAA-GUS mRNA, mRNA containing two AUG triplets flanked by CAA repeats, neutrophil cytosolic factor 2 (NCF2) mRNA and mRNAs containing CrPV IRES, *wt* and Δ Domain II CSFV IRESs, SPV9 IRES and EMCV IRES have been described (Evstafieva et al., 1991; Wilson et al., 1990; Pestova et al., 1996, 1998a, b, 2000; Kolupaeva et al., 2000; Wilson et al., 2000; Pestova and Hellen, 2001; Lomakin et al., 2006; Pisarev et al., 2006; Gauss et al., 2006; de Breyne et al., 2008). The vector for transcription of Ser/Thr protein phosphatase CDC25 mRNA (Sadhu et al., 1990) was obtained from Origene (Rockville, MD) (Genbank Acc. No. NM_001790). Vectors for transcription of mRNAs containing GC-rich stems inserted into the 5'-UTR were derived from the CAA-GUS plasmid by PCR (the inserted sequences are shown in Fig. 1C**)**. Vectors for transcription of CAA-Stem3,4-MVHC-STOP mRNAs were made by inserting appropriate DNA sequences flanked by an upstream T7 promoter between EcoRI and HindIII sites of pUC57 (CelTek Genes). Vectors for transcription of CAA-GUS mRNA and its derivatives, of NCF2 mRNA, of CDC25 mRNA and of CAA-Stem3,4-MHVC-STOP mRNAs were linearized by restriction with SalI, EcoN1, BspHI and HindIII, respectively. IRES-containing mRNAs were linearized and transcribed as described (Pestova et al., 1996, 1998b, Kolupaeva et al., 2000; Wilson et al., 2000; de Breyne et al., 2008). All mRNAs except those containing IRESs were transcribed using T7 polymerase in the presence of 5.6 mM anti-reverse cap analog m7(3'-O-methyl)-G(5')ppp(5')G (Ambion). A vector containing *Xenopus laevis* 18S rDNA (Borovjagin and Gerbi, 2004) was used to generate sequence ladders for identification of sites of DHX29-mediated protection from chemical/enzymatic modification in rabbit 18S rRNA.

Purification of initiation factors, ribosomal subunits and aminoacylation of initiator tRNA

40S and 60S ribosomal subunits, eIF2, eIF4F, eEF1H and eEF2 were purified from rabbit reticulocyte lysate (Pestova et al., 1996; Pestova and Hellen, 2003; Pisarev et al., 2007). eIF3 was purified from rabbit reticulocyte lysate or from HeLa cells (Pestova et al., 1996; Unbehaun et al., 2004). Rabbit aminoacyl-tRNA synthetases were purified, and native total rabbit tRNA (Novagen) was aminoacylated with Met, Val, His and [^{35}S[Cys as described (Pestova and Hellen, 2003). Yeast 40S subunits were purified as described (Galkin et al., 2007). Recombinant eIF1, eIF1A, eIF4A, eIF4B, eIF5, eIF5B, PTB and *E. coli* methionyl tRNA synthetase were expressed in *E. coli* BL21 (DE3) and purified as described (Pestova et al., 1996, 1998a, 2000; Lomakin et al., 2006). *In vitro* transcribed $tRNA^{Met}_i$ was aminoacylated using recombinant *E. coli* methionyl tRNA synthetase as described (Lomakin et al., 2006).

Purification of native DHX29

DHX29 was purified from the 0-40% ammonium sulphate precipitation fraction of the 0.5 M KCl ribosomal salt wash from 2 liters of rabbit reticulocyte lysate (RRL) (Green Hectares, Oregon, WI) prepared as described (Pisarev et al., 2007). The pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.1 mM EDTA) containing 100 mM KCl and applied to a DEAE (D52) column equilibrated with buffer A + 100 mM KCl. The fraction containing DHX29 was eluted in the flow-through fraction with buffer A + 100 mM KCl. This fraction was applied to a phosphocellulose (P11) column equilibrated with buffer A + 100 mM KCl. Step elution was done with buffer A containing 100, 200, 300, 400 and 500 mM KCl. DHX29 eluted at 300-400 mM KCl. This fraction was dialyzed overnight against buffer B (20 mM HEPES, pH 7.5, 5% glycerol, 2 mM DTT, 0.1 mM EDTA) containing 100 mM KCl and then applied to a FPLC MonoS HR 5/5 column. Fractions were collected across a 100-500 mM KCl gradient. DHX29 eluted at ~300 mM KCl. DHX29-containing fractions were dialyzed overnight against buffer C (20 mM Tris-HCl, pH 7.5, 5% glycerol, 2 mM DTT, 0.1 mM EDTA) containing 100 mM KCl and then applied to a FPLC MonoQ HR 5/5 column. Fractions were collected across a 100-500 mM KCl gradient. DHX29 eluted at ~250 mM KCl. DHX29-containing fractions were dialyzed overnight against buffer containing 20 mM Tris-HCl, pH 7.5, 5% glycerol and 100 mM KCl, then diluted 5-fold with 20 mM phosphate buffer, pH 7.5 with 5% glycerol and applied to a hydroxyapatite column pre-equilibrated in the same phosphate buffer. Fractions were collected across a 20-500 mM phosphate buffer gradient. Apparently homogenous DHX29 eluted at ~300 mM phosphate buffer. The identity of DHX29 was confirmed by LC-nanospray tandem mass spectrometry of peptides derived by in-gel tryptic digestion at the Rockefeller University Proteomics Resource Center.

Attempts to express recombinant full-length DHX29, its central helicase domain and/or its C-terminal domain in *E. coli* did not yield soluble proteins.

Toe-printing analysis

48S complexes were assembled on capped *in vitro* transcribed (CAA)-GUS mRNA, its derivatives containing GC-rich stems or two AUG triplets, (CAA)-Stem-MVHC-STOP mRNAs, NCF2 mRNA, CDC25 mRNA and native β-globin mRNA (Invitrogen) (Figures 1, 2). Reaction mixtures (40 μl) containing 1 pmol mRNA, 3.5 pmol 40S subunits, 10 pmol eIF2, 10 pmol eIF3, 10 pmol eIF4A, 10 pmol eIF4B, 2.5 pmol eIF4F, 20 pmol eIF1, 20 pmol eIF1A, 0.2 pmol DHX29 and 5 pmol Met-tRNA$^{Met}_i$ (as indicated in Figures) were incubated for 10 minutes at 37^{o}C in buffer D (20 mM Tris pH 7.5, 100 mM KAc, 1 mM DTT, 2.5 mM $MgCl_2$, 0.25mM spermidine) supplemented with 1 mM ATP and 0.2 mM GTP. Assembled initiation complexes were analyzed by primer extension using AMV reverse transcriptase (AMV-RT) and appropriate [^{32}P]-labeled primers complementary to coding regions of mRNAs (Pisarev et al., 2007). cDNA products were resolved in 6% polyacrylamide sequencing gels. To assay elongation on (CAA)-Stem-MVHC-STOP mRNAs, assembled 48S initiation complexes were supplemented with 8 pmol eIF5, 8 pmol eIF5B, 5 pmol 60S subunit, 15 μg total native tRNA aminoacylated with Met, Val, His and [^{35}S]Cys, 5 pmol eEF1H and 5 pmol eEF2 and incubated at 37^{o}C for an additional 20 min. Elongation complexes were assayed either by toe-printing as described above, or by centrifugation through 10-30% sucrose density gradients (SDGs) in a Beckman SW55 rotor at 53,000 rpm for 1h 15min with subsequent monitoring of [^{35}S]MVHC tetrapeptide formation.

To assemble initiation complexes on CrPV, SPV9, EMCV, *wt* and Δ domain II CSFV IRESs (Figure 7), 1 pmol mRNA was incubated with 2.6 pmol 40S subunits, 10 pmol eIF2, 10 pmol eIF3, 10 pmol eIF4A, 10 pmol eIF4B, 2.5 pmol eIF4F, 20 pmol eIF1, 20 pmol eIF1A, 0.3 or 3 pmol DHX29, 4 pmol PTB and 5 pmol Met-tRNA$^{Met}_i$ (as indicated in Figures) for 10 minutes at 37^{o}C in 40 μl reaction mixtures containing buffer D supplemented with 1 mM ATP and 0.2 mM GTP. Initiation complexes were assayed by toe-printing as described above.

To investigate the requirement for NTP hydrolysis by DHX29 for its activity in stimulating 48S complex formation (Figure 5), 48S complexes were assembled on CAA-GUS Stem-1 mRNA. Reaction mixtures (40 μl) containing 0.5 pmol CAA-GUS Stem-1 mRNA, 0.5 pmol SDG-purified 43S complexes

(assembled with eIFs 2, 3, 1 and 1A in the presence of GMPPNP), 20 pmol eIF1 and 20 pmol eIF1A (to compensate for the loss of these factors from 43S complexes during SDG centrifugation), and 0.1 pmol DHX29 were incubated for 10 minutes at 37^{o}C in buffer D in the presence/absence of 0.5 mM ATP, GTP, CTP, UTP, AMPPNP or GMPPNP (as indicated in the Figure). After incubation, the concentration of Mg^{2+} was elevated to 20 mM to prevent further initiation. Primer extension was also done at the elevated 20 mM Mg^{2+} concentration to avoid any possibility of initiation that might occur during primer extension when DHX29 would have a chance to hydrolyze dNTPs.

To investigate the ability of DHX29 to participate in multiple rounds of initiation (Figure 6), 48S complexes were assembled on CAA-GUS Stem-1 mRNA using SDG-purified 43S complexes containing different amounts of DHX29. Reaction mixtures (40 μl) containing 0.5 pmol CAA-GUS Stem-1 mRNA, 0.6 pmol SDG-purified 43S complexes (assembled with eIFs 2, 3, 1, 1A and with/without different amounts of DHX29), 0.6 pmol SDG-purified 40S/eIF3/$(CUUU)_9$ complexes, 20 pmol eIF1 and 20 pmol eIF1A (to compensate for the loss of these factors from 43S complexes during SDG centrifugation) were incubated for 10 minutes at 37^{o}C in buffer D with 1 mM ATP and 0.2 mM GTP. Assembled initiation complexes were analyzed by primer extension, as described above.

For toe-printing analysis of 48S complexes assembled on β-globin mRNA in RRL (Figure 2A), 0.2 μg globin mRNA (Invitrogen) was incubated in 15 μl RRL (Promega) in the presence of 2 mM GMPPNP for 10 min at 30^{o}C. The reaction mixture was diluted with buffer D to 40 μl final volume prior to primer extension, which was done essentially as described above.

Analysis of ribosomal binding of DHX29

To investigate ribosomal association of DHX29 (Figures 3A-D), 20 pmol DHX29 was incubated with 30 pmol 40S subunits, 30 pmol 60S subunits, 30 pmol 80S ribosomes, 20 pmol yeast 40S subunits, a combination of 25 pmol 40S subunits, 50 pmol eIF2, 30 pmol eIF3, 100 pmol eIF1, 100 pmol eIF1A and 50 pmol Met-tRNA$^{Met}_{i}$, or a combination of 25 pmol 40S subunits, 25 pmol eIF3 and 50 pmol $(CUUU)_9$ RNA in 200 μl reaction mixtures containing buffer E (20 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$) supplemented with 1 mM ATP and 0.2 mM GTP at 37^{o}C for 10 minutes, and subjected to centrifugation through 10-30% SDGs prepared in buffer E in a Beckman SW55 rotor at 53,000 rpm for 1h 15min. To investigate the nucleotide-dependence of ribosomal binding of DHX29 (Figure 3C), 20 pmol DHX29 was incubated with a combination of 35 pmol 40S subunits, 50 pmol eIF3 and 100 pmol $(CUUU)_9$ RNA in the presence/absence of 0.5 mM ATP, ADP or AMPPNP in 400 μl reaction mixtures containing buffer E at 37^{o}C for 10 minutes, and subjected to centrifugation through 10-30% SDGs prepared in buffer E supplemented with appropriate nucleotides, as described above. Fractions that corresponded to ribosomal complexes were analyzed by SDS-PAGE with subsequent fluorescent SYPRO (Molecular Probes) staining or western blotting using DHX29 antibodies (Bethyl Laboratories). To investigate the ribosomal association of DHX29 in RRL (Figures 3E, F), 50 μl RRL (Promega) were incubated with 1 mM GMPPNP and 70 μCi [^{35}S]Met at 30^{o}C for 30 minutes and subjected to centrifugation through 10-30% SDGs prepared in buffer E, as described above. In addition to optical density, the ribosomal profile of RRL was also analyzed by scintillation counting to monitor incorporation of [^{35}S]Met-tRNA$^{Met}_{i}$. Gradient fractions were analyzed by western blotting using DHX29 antibodies. Experiments presented in Figures 3A and 3C were done with eIF3 purified from HeLa cells and RRL, respectively, which accounts for the presence of either full-length (Figure 3A) or truncated (Figure 3C) eIF3a subunit (Pisarev et al., 2007).

To compare binding of DHX29 to 43S and 48S complexes (Figure 6G), 5 pmol DHX29 was incubated with a combination of 10 pmol 40S subunits, 100 pmol eIF2, 100 pmol eIF3, 100 pmol eIF1, 100 pmol eIF1A and 50 pmol Met-tRNA$^{Met}_{i}$ (to form 43S complexes), or with a combination of 10 pmol 40S subunits, 100 pmol eIF2, 100 pmol eIF3, 100 pmol eIF1, 100 pmol eIF1A, 50 pmol Met-tRNA$^{Met}_{i}$, 100 pmol eIF4A, 100 pmol eIF4B, 30 pmol eIF4F and 25 pmol native globin mRNA (to form 48S complexes) in 400 μl reaction mixtures containing buffer D with 1 mM ATP and 0.2 mM GTP at 37^{o}C

for 10 minutes, and subjected to centrifugation through 10-30% SDGs, as described above. Fractions that corresponded to 43S/48S complexes were analyzed by western blotting using DHX29 antibodies.

Chemical and enzymatic foot-printing analysis of 43S and 43S/DHX29 complexes

Ribosomal complexes were assembled by incubating 10 pmol 40S subunits, 15 pmol eIF3, 20 pmol eIF2, 75 pmol eIF1, 75 pmol eIF1A, 20 pmol Met-tRNA$^{Met}_i$ with or without 10 pmol DHX29 in 400 µl buffer D for 10 min at 37ºC, and then either enzymatically digested by incubation with RNase V1 ($0.28x10^{-3}$ units/ml) or RNase T1 ($50x10^{-3}$ units/ml) for 10 min at 37^{o}C, or modified by incubation with 126 mg/ml CMCT or 1.5% DMS for 10 min at 30^{o}C. Cleavage/modification sites in 18S rRNA were identified by primer extension using AMV-RT and primers complementary to different regions of rRNA.

NTPase assay

To determine the NTPase specificity of DHX29 (Figure 5A), 1 pmol DHX29 was incubated in a 10 µl reaction mixture containing buffer E with 6.7 µM [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]UTP or [α-^{32}P]CTP in the presence/absence of 1 pmol SDG-purified 43S complexes at 37^{o}C for 40 minutes. 2 µl aliquots were spotted onto polyethyleneimine (PEI) cellulose plates for chromatography done using 0.8 M LiCl/0.8 M acetic acid. The NTPase activity of DHX29 was assayed by formation of [α-^{32}P]NDP. To investigate stimulation of the NTPase activity of DHX29 by $(CUUU)_9$ RNA, 18S rRNA, 43S complexes and 43S/$(CUUU)_9$ complexes (Figure 5B), 0.3 pmol DHX29 were incubated in 10 µl reaction mixtures containing buffer D with 6.7 µM [γ-^{32}P]ATP in the presence/absence of 20 pmol $(CUUU)_9$ RNA, 0.3 pmol 18S rRNA, 0.3 pmol 43S complexes, or 0.3 pmol 43S complexes with 20 pmol $(CUUU)_9$ RNA at 37^{o}C. 1.5 µl aliquots were removed after 2-30 minutes of incubation for PEI cellulose chromatography. The ATPase activity of DHX29 was assayed by formation of [^{32}P]P_i.

Helicase assay

For the helicase assay, 2.5 µM short RNA oligonucleotides (Dharmacon) were ^{32}P-labeled with T4 polynucleotide kinase, annealed with an equimolar amount of complementary RNA oligonucleotides, and the resulting RNA duplexes were purified by gel-filtration on Superdex 75. 1 nM purified duplexes was then incubated in 10µl reaction mixtures containing buffer E with 0.15 µM DHX29, 50 nM 43S complexes, 50 nM 43S/DHX29 complexes or 0.15 µM eIF4A/eIF4F, and 0.2 mM NTPs at 37^{o}C for 40 minutes. The reactions were stopped with 2.5 µl buffer containing 20 mM Tris pH 7.5, 50% glycerol, 0.5% SDS, 5mM EDTA, 0.1% NP-40, 0.01% bromophenol blue, 0.01% xylene cyanol. Aliquots were analyzed in 16% non-denaturing gels and quantified on a Phosphoimager.

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Table S1. (A) Identification of DHX29 and (B) determination of the composition of ΔDHX29 by LC/nanospray tandem mass-spectrometry of tryptic peptides. Amino acid residues are numbered according to the sequence of *H. sapiens* DHX29 (Genbank NP_061903)

A

Deduced sequence	Amino acid residues
SLEEEEKFDPNER	251-263
SPNPSFEK	394-401
DLFIAK	489-494
VVVVAGETGSGK	590-601
ASQTLSFQEIALLK	1204-1217
LACIVETAQGK	1243-1253
VLIDSVLR	1334-1341
ILQIITELIK	1356-1365

B

Deduced sequence	Amino acid residues
IIGVINEHK	98-106
SLEEEEKFDPNER	251-263
VVVVAGETGSGK	590-601
VCDELGCENGPGGR	642-655
NSLCGYQIR	656-664