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# Exon junction complex enhances translation of spliced mRNAs at multiple steps

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ABSTRACT

Translation of spliced mRNAs is enhanced by exon junction complex (EJC), which is deposited on mRNAs as a result of splicing. Although this phenomenon itself is well known, the underlying molecular mechanism remains poorly understood. Here we show, using siRNAs against Y14 and eIF4AIII and spliced or intronless constructs that contain different types of internal ribosome entry sites (IRESes), that Y14 and eIF4AIII increase translation of spliced mRNAs before and after formation of the 80S ribosome complex, respectively. These results suggest that EJC modulates translation of spliced mRNA at multiple steps.


## Introduction

In mammalian cells, translation of intron-containing transcripts or spliced mRNAs occurs more efficiently than that of identical but intronless mRNAs [1–7]. Increased translational efficiency of spliced mRNAs is mediated by exon junction complex (EJC), which is deposited 20–24 nucleotides upstream of each exon–exon junction as a result of splicing [8]. EJC is a multi-functional protein complex that is involved in various cellular processes [1,7] including splicing, mRNA export, nonsense-mediated mRNA decay (NMD; [9–11]), subcellular localization [12,13], and translation [7,14,15]. In particular, enhanced translation of spliced mRNA was demonstrated by tethering EJC components or NMD factors to an intronless reporter mRNA [5].

Recently, PYM, a component of the EJC was shown to interact with the Y14-magoh complex, 40S ribosome, and 48S pre-initiation complex [2]. Thus, PYM links EJC-bound spliced mRNAs and the ribosomal 48S pre-initiation complex, promoting translation of spliced mRNAs [2]. In addition, EJC links mammalian target of rapamycin (mTOR) signaling to the pioneer round of translation initiation complex, such that S6K1 Aly/REF-like substrate (SKAR), a recently identified component of the EJC, enhances the pioneer round of translation of spliced mRNAs [4]. However, the precise molecular mechanism for the translational increase of spliced mRNAs by EJC remains to be elucidated.

Here, we show that downregulation of Y14 or eIF4AIII, which is a component of EJC, inhibits the increased translation of spliced mRNAs over intronless mRNAs. Furthermore, using spliced or intronless constructs that contain different types of internal ribosome entry sites (IRESes), we show that eIF4AIII increases translation of spliced mRNAs at the step after formation of the 80S ribosome complex. On the other hand, Y14 is involved in increasing the translation of spliced mRNAs at the step before formation of the 80S ribosome complex. Considering our results and the previous report showing that Y14/magoh complex recruits ribosome to mRNA via its interaction with PYM [2], our findings suggest that EJC modulates translation of spliced mRNA at multiple steps.

## Materials and methods

*Plasmid constructions.* To construct pcDNA3-HA-eIF4AIII-M, site-specific mutations were introduced using the Quick Change® Site-Directed Mutagenesis Kit (Stratagene). PCR was performed using pcDNA3-HA-eIF4AIII (a gift from Dr. Nahum Sonenberg; [16]) as a template and two oligonucleotides: 5′-GCTTTGATCTTGGCTCCCA *GGAGAGTTGCT*GCTGTGCAGATCCAGAAG-3′ (sense) and 5′-CTTCTG GATCTGCACAGC*AGCAA*CTCT*CC*TGGGAGCCAAGATCAAAGC-3′ (antisense), in which the mutated nucleotides are italicized. The PCR fragment was self-ligated according to the manufacturer's instructions (Stratagene).

To construct EMCV-No-intron or EMCV-5′-intron plasmid, a Klenow-filled XhoI/XbaI fragment from pR/EMCV/F [17] was ligated to a Klenow-filled HindIII fragment from No-intron plasmid or 5′-intron plasmid [6].

To construct CrPV-No-intron or CrPV-5′-intron plasmid, a PCR-amplified CrPV fragment was ligated to a HindIII fragment of No-intron plasmid or 5′-intron plasmid. The CrPV IRES was amplified using pR/CrPV/F [18,19] and two oligonucleotides: 5′-

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CCC*AAGCTT*GAGTTCTCAAAAATGAACAATAATTCTAGGGG-3′ (sense) and 5′-CCC*AAGCTT*CATGGTATCTTGAAATGTAGCAGGTAAATTTC-3′ (antisense), where the italicized nucleotides specify the HindIII site.

*Cell culture, transfections, and protein and RNA purification*. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) containing 10% fetal bovine serum (Lonza), and 1% penicillin/streptomycin (Lonza).

For the plasmid transfections, HeLa cells ($2 \times 10^6$) cultured in 60-mm dishes were transiently transfected with (i) 0.2 μg of a reporter plasmid, either intronless or intron-containing and (ii) 1 μg of reference plasmid pGL3-Control (Promega). Where indicated, HeLa cells were co-transfected with 0.5 μg of effector plasmid, pcDNA3-HA, pcDNA3-HA-eIF4AIII-WT, pcDNA3-HA-eIF4AIII-M, pCMV-Myc, pCMV-Myc-Upf1-WT, or pCMV-Myc-Upf1-R844C (gifts from Dr. Maquat; [20]). Two days after transfection, protein was purified from half of the cells using passive lysis buffer (Promega) and total RNA was purified from the other half using TRIzol Reagent (Invitrogen).

For the siRNA transfections, HeLa cells were transiently transfected with 100 nM of siRNA (Invitrogen) using Oligofectamine (Invitrogen). Endogenous Upf1 [20], Upf2 [20], Upf3X [20], Y14 [21], and eIF4AIII [13] were downregulated using 5′-r(GAUGC AGUUCCGCUCCAUU)d(TT)-3′, 5′-r(GGCUUUUGUCCCAGCCAUC) d(TT)-3′, 5′-r(GGAGAAGCGAGUAACCCUG)d(TT)-3′, 5′-r(UCCAGCCU UCAACAGAGCG)d(TT)-3′, and 5′-r(CGAGCAAUCAAGCAGAUCA) d(TT)-3′, respectively. Two days after siRNA transfection, cells were retransfected with reporter and reference plasmids. After an additional day, protein and total RNA were purified as described above.

*Semi-quantitative RT-PCR*. Semi-quantitative RT-PCRs were performed as described previously [20,22–24]. In brief, total RNAs were purified from cells using TRIzol Reagent (Invitrogen). RT-PCRs were performed using specific oligonucleotides and α-[$^{32}$P]-dATP (Perkin-Elmer NEN). Radioactive PCR products were separated in 5% polyacrylamide gel, visualized by PhosphorImaging (BAS-2500; Fuji Photo Film Co.), and then quantitated by Multigauge (Fuji Photo Film Co.). A standard curve of intensity versus RNA amount was prepared using 2-fold serial dilutions of purified RNAs and the relative amounts of PCR products were then determined from the curve.

RLuc mRNAs were amplified using oligonucleotides 5′-TGATCCA GAACAAAGGAAAC-3′ (sense) and 5′-CTTATCTTGATGCTCATAGC-3′ (antisense). FLuc mRNAs were amplified using oligonucleotides 5′-CAACACCCCAACATCTTCG-3′ (sense) and 5′-CTTTCCGCCCTTCT TGGCC-3′ (antisense).

*Dual luciferase assays*. Luciferase activities of RLuc and FLuc were measured using the Dual Luciferase assay kit (Promega) according to the manufacturer's instructions.

*Western blotting*. Protein was electrophoresed in SDS–polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes (Amersham). Membranes were incubated with primary antibodies overnight at 4 °C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham) for 1 h at room temperature. The following antibodies were used: Upf1, Upf2, Upf3/3X (gifts from Dr. Maquat; [20]), eIF4AIII (a gift from Dr. Reed; [25]), Y14 (a gift from Dr. Dreyfuss; [26]), Myc (Calbiochem), HA (Roche), GAPDH (Ab Frontier), and β-actin (Sigma).

*Statistics*. Experiments were performed at least three times, and two-tailed, equal-sample variance Student's *t*-tests were used to determine statistical significance of differences between the data sets. Differences of $P < 0.01$ were considered significant.

## Results and discussion

### *A subset of EJC constituents is involved in translational enhancement of spliced mRNAs*

To test whether each component of EJC has a distinct role in the translation of spliced mRNAs, we first analyzed the translational efficiency of spliced mRNAs upon downregulation of EJC constituents (Fig. 1). To this end, HeLa cells were transiently transfected with Upf1 siRNA, Upf2 siRNA, Upf3X siRNA, Y14 siRNA, eIF4AIII siRNA, or a nonspecific Control siRNA. All tested proteins except Upf1 are components of EJC [8–13]; Upf1 is a key NMD factor [9–11]. Two days after transfection, cells were retransfected with (i) a reporter plasmid encoding a *Renilla* luciferase cDNA (RLuc) that does (5′-intron plasmid) or does not (No-intron plasmid) contain intron 6 and flanking exons from the human triose phosphate isomerase gene (TPI) at the 5′-end [6] and (ii) a reference plasmid pGL3-Control encoding firefly luciferase cDNA (FLuc) without intron sequence to control for variations in the efficiencies of transfection and RNA recovery (Fig. 1A).

Western blotting showed that the levels of endogenous Upf1, Upf2, Upf3X, Y14, and eIF4AIII were downregulated, respectively, to 2%, 2%, 8%, 9%, and 7% of normal, where normal is defined as the level in the presence of the nonspecific control siRNA (Fig. 1B). Semi-quantitative RT-PCR revealed that the levels of spliced RLuc mRNAs (5′-intron) were higher than those of intronless RLuc mRNAs (No-intron) by 3- to 4-fold (Fig. 1C), which was consistent with previous findings [1,6,7]. This increase could be due to enhanced transcription, efficient 3′-end formation of pre-mRNA, or stabilization of pre-mRNA or mRNA [1,6,7]. Notably, these increases in the abundance of spliced RLuc mRNAs over intronless RLuc mRNAs were not affected by downregulation of any tested proteins (Fig. 1C). Dual luciferase assays were performed and translational efficiency (the amount of protein produced per mRNA) was calculated (Fig. 1D). The results showed that the translational efficiencies of spliced RLuc mRNAs (5′-intron) were approximately 7-fold higher than those of intronless RLuc mRNAs (No-intron). Intriguingly, these increased translation efficiencies were inhibited by approximately 2-fold by downregulation of Y14 or eIF4AIII, but not of Upf1, Upf2, or Upf3X (Fig. 1D).

### *An increase in translational efficiency of spliced RLuc mRNA over intronless RLuc mRNA is significantly blocked by overexpression of dominant-negative eIF4AIII, but not Upf1-R844C*

The roles of eIF4AIII and Upf1 in the preferential increase of translation of spliced mRNAs were further demonstrated by overexpression of dominant-negative eIF4AIII (eIF4AIII-M) or Upf1-R844C (Fig. 2). HeLa cells were transiently transfected with three plasmids (i) an effector plasmid expressing HA tag, HA-eIF4AIII-wild type (WT), or HA-eIF4AIII-M, (ii) a reporter plasmid, either 5′-intron plasmid or No-intron plasmid, and (iii) a reference plasmid pGL3-Control (Fig. 2A–C). eIF4AIII-M has three amino acid substitutions in motif Ia, in which PTRELA was mutated to PRRVAA. eIF4AIII-M was shown to block the EJC formation and interaction with Magoh but not with MLN51/Barentsz [27], suggesting that this mutant functions as a dominant-negative protein. Comparable expressions of exogenously transfected HA-eIF4AIII-WT and HA-eIF4AIII-M, and endogenous eIF4AIII were demonstrated by Western blotting (Fig. 2A). The results revealed that the increase in translational efficiency (Fig. 2C), but not an increase in abundance (Fig. 2B), of spliced RLuc mRNA (5′-intron) over intronless RLuc mRNA (No-intron) was significantly blocked by overexpression of HA-eIF4AIII-M, which was consistent with the results obtained using siRNA (Fig. 1). In parallel, HeLa cells were transiently transfected with plasmid expressing either wild-type or the helicase mutant version of Upf1 (Upf1-R844C), which contains a single amino acid substitution of arginine to cysteine at amino acid-844 within the helicase domain of Upf1 and functions as a dominant-negative protein in NMD (Fig. 2D–F; [28]). Neither an increase in translational efficiency (Fig. 2E) nor an increase in abundance (Fig. 2F) of spliced RLuc mRNA over intronless RLuc mRNA was significantly affected by overexpression of Upf1-R844C (Fig. 2D–F),