JUN mRNA Translation Regulation is Mediated by Multiple 5’ UTR and Start Codon Features

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ABSTRACT

Regulation of mRNA translation by eukaryotic initiation factors (eIFs) is crucial for cell survival. In humans, eIF3 stimulates translation of the JUN mRNA which encodes the transcription factor JUN, an oncogenic transcription factor involved in cell cycle progression, apoptosis, and cell proliferation. Previous studies revealed that eIF3 activates translation of the JUN mRNA by interacting with a stem loop in the 5′ untranslated region (5′ UTR) and with the 5′-7-methylguanosine cap structure. In addition to its interaction site with eIF3, the JUN 5′ UTR is nearly one kilobase in length, and has a high degree of secondary structure, high GC content, and an upstream start codon (uAUG). This motivated us to explore the complexity of JUN mRNA translation regulation in human cells. Here we find that JUN translation is regulated in a sequence and structure-dependent manner in regions adjacent to the eIF3-interacting site in the JUN 5′ UTR. Furthermore, we identify contributions of an additional initiation factor, eIF4A, in JUN regulation. We show that enhancing the interaction of eIF4A with JUN by using the compound Rocaglamide A (RocA) represses JUN translation. We also find that both the upstream AUG (uAUG) and the main AUG (mAUG) contribute to JUN translation and that they are conserved throughout vertebrates. Our results reveal additional layers of regulation for JUN translation and show the potential of JUN as a model transcript for understanding multiple interacting modes of translation regulation.
INTRODUCTION

Protein translation is one of the most energetically expensive cellular processes and is highly regulated, especially during translation initiation (Buttgereit and Brand 1995; Sonenberg and Hinnebusch 2009; Topisirovic and Sonenberg 2011; Hershey et al. 2012; Leibovitch and Topisirovic 2018). Translation initiation is a complex process which regulates expression of eukaryotic genes and employs over a dozen eukaryotic translation initiation factors (eIFs) (Sachs and Varani 2000; Jackson et al. 2010; Hinnebusch 2011; Aitken and Lorsch 2012). These include eIF1, eIF1A, eIF3, eIF5, eIF2 and the eIF4F complex, which is composed of eIF4E, eIF4A and eIF4G (Jackson et al. 2010; Aitken and Lorsch 2012). During eukaryotic translation initiation, a ternary complex made up of initiator methionyl-tRNA (Met-tRNA\textsubscript{i}), eIF2, and GTP is formed (Olsen et al. 2003; Hinnebusch 2014). The 43S pre-initiation complex (PIC) then comes together by recruitment of the ternary complex, the 40S ribosomal subunit, and eukaryotic initiation factors 1, 1A, 3 and 5 (Asano et al. 2001; Algire et al. 2002; Majumdar et al. 2003; Kolupaeva et al. 2005; Pestova et al. 1998). After adopting an open conformation, the 43S PIC joins eIF4F at the mRNA 5’ cap in order to recruit the mRNA to form the 48S PIC (Hinnebusch 2014). This newly formed 48S PIC is then capable of scanning the mRNA through its 5’ untranslated region (5’ UTR) until it locates a start codon (Pestova and Kolupaeva 2002). Once the start codon is recognized, several initiation factors are released in order for the ribosome to begin elongation (Aitken and Lorsch 2012; Hinnebusch 2014). During initiation, the roles of several eIFs have been linked to translation regulation of subsets of mRNAs. For example, experiments performed in human cells revealed that eIF3 regulates the translation of specific mRNAs by direct interactions (Lee et al. 2015, 2016; De Silva et al. 2021). These eIF3-mRNA interactions are important for homeostasis but also play essential roles upon nutrient deprivation and drive the integrated stress response, among other functions (Xu et al. 2012; Lee et al. 2015, 2016; Pulos-Holmes et al. 2019; Tacca et al. 2019; Cate 2017; Gomes-Duarte et al. 2017; Lamper et al. 2020; Lin et al. 2020; Wolf et al. 2020; De Silva et al. 2021; Mukhopadhyay et al. 2023; Mestre-Fos et al. 2023). eIF4A, an RNA helicase, has also been associated with translation regulation of a subset of mRNAs in human cells, more specifically by unwinding 5’ UTRs that are highly structured and polypurine rich and many
of which are related to cell-cycle progression and apoptosis (Svitkin et al. 2001; Rubio et al. 2014; Iwasaki et al. 2016, 2019). Moreover, eIF1 and eIF5 play important roles in the selection of translational start sites, depending not only on the AUG translational context, but also on the abundance of these initiation factors and specific cellular conditions (Hann et al. 1992; Fletcher et al. 1999; Sonenberg and Dever 2003; Loughran et al. 2012; Ivanov et al. 2008, 2010, 2022).

Translation initiation factor eIF3 is a crucial player in protein expression regulation through its roles in bridging the 43S PIC and eIF4F complexes, and also by performing specialized regulatory roles (Kolupaeva et al. 2005; Hinnebusch 2006; Valášek et al. 2003). eIF3 specifically binds to and regulates translation of a subset of mRNAs, many of which are involved in cell cycle regulation, cell growth, differentiation, and other crucial cellular functions. The interaction between eIF3 and mRNAs was shown to be mediated by RNA structural elements in the 5’ UTR of specific mRNAs in human embryonic kidney (HEK293T) cells and to cause translational activation or repression of these mRNAs (Lee et al. 2015). eIF3 has also been shown to have cell-specific regulatory roles in T cells, with eIF3 interactions throughout the entire length of the transcript for specific mRNAs, such as the ones encoding the T cell receptor alpha and beta subunits (TCRA and TCRB, respectively), mediating a translational burst essential for T cell activation (De Silva et al. 2021). In yeast, eIF3 has also been linked to mRNA recruitment and scanning as a mediator of mRNA-PIC interactions (Jivotovskaya et al. 2006; Chiu et al. 2010; Mitchell et al. 2010). Furthermore, in zebrafish eIF3 subunit H (EIF3H) was shown to regulate translation of mRNAs encoding the eye lens protein crystallin during embryogenesis (Choudhuri et al. 2013). These examples demonstrate that eIF3 plays a variety of mRNA-specific regulatory roles.

One of the reported eIF3-target mRNAs in human cells, JUN, encodes the transcription factor JUN, also known as c-Jun, which regulates gene expression in response to different stimuli (Wisdom et al. 1999; Meng and Xia 2011). As a component of the activator protein-1 (AP-1) complex, JUN regulates transcription of a large number of genes and acts mainly as a transcriptional activator (Smeal et al. 1991). JUN is therefore highly involved in various cellular processes including cell proliferation, apoptosis, tumorigenesis, and it was the first oncogenic transcription factor discovered
Regulation of JUN expression is particularly important because its downregulation can lead to cell cycle defects and its upregulation can lead to accelerated cell proliferation, which occurs in some cancers (Blau et al. 2012). Therefore, it is not surprising for JUN expression regulation to be complex and to occur at both the transcriptional and translational levels. 

At the transcriptional level, JUN mRNA expression is regulated by its own protein product, which binds a high-affinity AP-1 binding site in the JUN promoter region and in turn induces its transcription (Nakamura et al. 1991; Angel et al. 1988; Lamph et al. 1988). JUN expression regulation at the translational level is mediated by its mRNA interaction with eIF3. Binding of eIF3 subunits EIF3A, EIF3B, EIF3D, and EIF3G to a stem loop in the JUN 5′ UTR results in activation of translation (Lee et al. 2015). Moreover, eIF3 subunit D (EIF3D) acts as a 5′ cap-binding protein on the JUN mRNA, mediated by a cis-acting RNA element located in the 153 nucleotides immediately downstream of the JUN 5′-7-methylguanosine cap structure (Lee et al. 2016). This RNA element is also thought to block recruitment of the eIF4F complex (Lee et al. 2016). JUN expression regulation at the translational level has also been shown to be affected by m6A methylation by METTL3 in its 3′ UTR and by contributions of an RNA structural element which activates its translation in glioblastoma (Blau et al. 2012; Suphakhong et al. 2022). JUN possesses a longer than average 977-nucleotide 5′ UTR that is highly GC rich. Due to its length and complexity, JUN's 5′ UTR might present additional layers of translational regulation of its mRNA through novel structural and/or sequence elements. Previously reported involvement of several initiation factors, including eIF3 and eIF4A, in the recruitment of mRNAs with long and structurally complex 5′ UTRs further supports a 5′ UTR-mediated mechanism for JUN translation regulation and suggests that additional factors may be involved in JUN regulation (Stanciu et al. 2022). For example, JUN was recently shown to be sensitive to RocA, an anti-cancer drug that clamps eIF4A onto specific polypurine sequences in the 5′ UTRs of a subset of mRNAs (Iwasaki et al. 2016, 2019). However, the implications of this interaction on JUN translation have not been previously evaluated. JUN also possesses two potential translational start sites, an upstream start codon (uAUG) located 4 codons upstream of the main start codon (mAUG). However, translational start site selection for the JUN mRNA has not been
previously explored. Therefore, we further investigated \textit{JUN} translation regulation in human cells by exploring different regions of the \textit{JUN} 5’ UTR and how mRNA features and the interaction of initiation factors in these regions contribute to \textit{JUN} translation. Firstly, we applied mutagenesis to the \textit{JUN} 5’ UTR near the eIF3 binding site. We also investigated the contributions of eIF4A to \textit{JUN} translation both by mRNA mutagenesis and through cellular treatment with RocA. Finally, we explored how the translational context of both of the \textit{JUN} start codons affect start site selection. Our results demonstrate that \textit{JUN} translation regulation is a complex multilayered process that involves various initiation factors, including eIF3 and eIF4A, and mRNA features such as secondary structures in the 5’ UTR.

\section*{RESULTS}

\textit{JUN} translation is regulated by 5’ UTR sequence and structural elements

Binding of eIF3 to a stem-loop in the 5’ UTR of the \textit{JUN} mRNA leads to its translational activation (Lee et al. 2015). Mutations in this stem loop have been shown to disrupt the interaction with eIF3 and to repress \textit{JUN} translation (Lee et al. 2015). However, the effects of other mutations in the \textit{JUN} 5’ UTR remain to be explored. We first tested whether mutations in other regions within and near the \textit{JUN}-eIF3 interacting stem loop (SL) affect \textit{JUN} translation. We generated mRNA reporter constructs containing the full-length \textit{JUN} 5’ UTR and Nanoluciferase (Nluc) coding sequence (CDS) that included mutations in a 208 nucleotide (nt) SL proximal region whose secondary structure was previously determined (Lee et al. 2015) by selective 2’-hydroxyl acylation analyzed by primer extension, also known as SHAPE (Figure 1A, SHAPE). All of the mutations disrupt either the secondary structure or the sequence of highly structured regions within the SL proximal region (Figure 1B). We transfected these constructs into HEK293T cells, together with an mRNA reporter with the Hemoglobin Beta Subunit (\textit{HBB}) 5’ UTR and a Firefly luciferase (Fluc) CDS as an internal control, and assessed translation using luciferase assays.

As expected, deletion of the \textit{JUN}-eIF3 interacting stem loop (Figure 1C, mutant ΔSL) significantly represses \textit{JUN} reporter translation when compared to the WT construct. Mutations to SL loop nucleotides C128-U129, previously shown to be
unreactive by SHAPE mapping in vitro and therefore likely to be involved in RNA-RNA contacts, also significantly affected JUN reporter translation, with U129G dramatically increasing translation (Figure 1C, mutant A) (Lee et al. 2015). Interestingly, replacing the SL loop with a much smaller and possibly more stable UUCG tetraloop substantially increased JUN reporter translation (Figure 1C, mutant E) (Antao et al. 1991). However, replacing all of the U’s with A’s in the loop sequence had little effect on JUN translation (Figure 1C, mutant D). As a whole, these findings support the importance of the SL loop in JUN translation regulation, yet reveal a complexity in its role maintaining and stabilizing the secondary structure of the SL region. Mutations in other structured regions of the JUN 5’ UTR near the eIF3 binding site also significantly affected JUN translation. For example, disrupting the stem loop between nucleotides 23 and 33 with point mutations in nucleotides 24 and 30 repressed JUN reporter translation (Figure 1C, mutant F). By contrast, deleting the bulge loop formed by nucleotides 42 to 47 increased JUN reporter translation (Figure 1C, mutant G). These findings suggest that these secondary structure features in the JUN 5’ UTR outside the originally identified eIF3 binding site play opposing roles in regulating JUN translation. However, mutations to two other loop and bulge regions near the SL (nts 160-166 and 184-187) had little or no effect on JUN reporter translation (Figure 1C, mutants H-J).

**JUN is highly sensitive to RocA treatment**

Rocaglamide A (RocA) is an anti-cancer compound that specifically clamps eIF4A onto polypurine sequences in a subset of mRNAs, in an ATP-independent manner. This clamping of eIF4A blocks 43S scanning, leading to premature, upstream translation initiation and reducing protein expression from transcripts containing RocA–eIF4A target sequences (Iwasaki et al. 2016, 2019). Interestingly, JUN is one of the mRNAs identified as highly sensitive to RocA treatment (Iwasaki et al. 2016). However, little is known about how promoting or disrupting the JUN interaction with eIF4A affects JUN translation. To this end, we first transfected JUN 5’ UTR and Nluc mRNA reporter constructs designed above (Figure 1B) together with the HBB 5’ UTR and Fluc CDS control mRNA, into HEK293T cells and treated these with increasing concentrations of RocA or DMSO (as a negative control). In all the cases we tested, including the WT, ΔSL, and the UUCG
tetraloop mutation in the SL loop, treatment with RocA strongly suppressed \textit{JUN} reporter translation (Figure 2A). This effect was not observed for the control Nluc reporter mRNA harboring the \textit{HBB} 5′ UTR, which has not been reported as RocA sensitive. The fact that constructs with mutations that affect the eIF3-interacting stem loop in the \textit{JUN} 5′ UTR were still highly sensitive to RocA treatment suggests that the RocA-mediated effects on the \textit{JUN} 5′ UTR are independent of eIF3 regulation. The persistent repressive trend of RocA treatment on \textit{JUN} translation also suggests that eIF4A serves an important role in \textit{JUN} translation regulation.

RocA-sensitive mRNAs are enriched in the polypurine sequence GAA(G/A) (Iwasaki et al. 2016). As shown in Figure 2B, \textit{JUN} possesses 11 of these polypurine sequences across the entire length of its 5′ UTR, with none present in the eIF3-interacting stem loop. In order to evaluate the effect of disrupting these sequences in the \textit{JUN} 5′ UTR, we mutated these polypurine (GAA(G/A)) sequences to the mixed purine/pyrimidine sequence CAAC, previously reported to disrupt RocA-mediated eIF4A binding to mRNAs (Iwasaki et al. 2019). Interestingly, the \textit{JUN} reporter mRNAs with these mutations (mutants CAAC or CAAC + ΔSL) remained highly sensitive to RocA (Figure 2C). This indicates that there are additional eIF4A target sequences in the \textit{JUN} 5′ UTR that are not necessarily equivalent to the reported predominant GAA(G/A) motif. Moreover, deleting the eIF3 interacting stem loop, together with the GAA(G/A) mutations (mutant CAAC + ΔSL), had no further effect on translation. We observed similar effects with the \textit{JUN} mRNA reporters \textit{in vitro} using HEK293T cell extracts (Figure 2D). Taken together, these results support a model in which eIF4A regulates \textit{JUN} translation in an eIF3 independent manner, pointing to further layers of regulation for \textit{JUN} translation mediated by additional initiation factors.

Two start codons contribute to \textit{JUN} translation in cells

Start codon selection regulates the translation of many transcripts (Ivanov et al. 2008, 2010, 2017, 2022; Loughran et al. 2012; Kozak 1986). Recently, it was reported that the translational context of start codons on transcripts with an upstream open reading frame (uORF) and a main open reading frame (mORF) affects which of these is preferentially selected for translation, mediated by eukaryotic initiation factor 1 (eIF1) and
eukaryotic initiation factor 5 (eIF5) (Ivanov et al. 2022). While eIF1 promotes skipping of weak translational start sites, eIF5 increases initiation at these sites. The relative abundance of these two initiation factors determines which start codon is used. The strongest translational context, also known as the ideal Kozak sequence context, contains a purine at the -3 position, preferably an adenosine (A), and a guanosine (G) at the +4 position, relative to the AUG start codon (Figure 3A). A weak translational context results when either of these purines at the -3 and +4 positions is substituted by a pyrimidine. The JUN mRNA possesses two AUG start codons, an in-frame upstream AUG (uAUG) four codons before a main AUG (mAUG), with different translational contexts (Figure 3A). The JUN uAUG possesses a weak translational context, with a uridine (U) at the -3 position and an adenosine (A) at the +4 position. By contrast, the JUN mAUG has a strong translational context, with an adenosine (A) at the -3 position and a guanosine (G) at the +4 position. It is not known which of these JUN AUGs is preferentially selected for translation and there currently is no evidence of JUN peptides that initiate at the uAUG.

To investigate whether JUN translation can initiate at either AUG or whether one is preferentially selected, we designed mRNA reporter constructs containing the JUN 5' UTR and the first 51 nucleotides of the JUN CDS (corresponding to 17 amino acids), followed by the full Nluc CDS (Figure 3B). The WT version of this construct therefore contains both JUN AUG start codons and their intact translational contexts. We then mutated start codons individually or their translational context to test their roles in JUN translation. We transfected these mRNA reporters into HEK293T cells, together with the HBB 5' UTR and Fluc CDS control, and monitored translation using luciferase assays. In general, disrupting either AUG or changing their translational context significantly represses JUN translation, which in turn suggests that translation can initiate at both AUGs (Figure 3C). We found that disrupting either AUG by mutation to AAG repressed JUN reporter translation, consistent with both AUGs contributing to JUN translation (Figures 3B and 3C). The more substantial decrease in JUN reporter translation due to mutation of the mAUG (JUNΔmAUG, 95% reduction) compared to mutation of the uAUG (JUNΔuAUG, 75% reduction) suggests the mAUG start codon may be preferred in our experimental conditions.
Changing the translational context of either AUG also repressed *JUN* translation. Interestingly, making the sequence context for the uAUG stronger – either by introducing an A in the -3 position of the upstream AUG (Figure 3B) or by also including a G mutation in the +4 position to make it an ideal Kozak sequence – resulted in a 50% decrease in translation (Figure 3C). Moreover, using the uAUG in a strong Kozak context while weakening the translational context of the mAUG further represses *JUN* translation, to about 10% of the WT levels (Figure 3C, mutant S-uAUG W-mAUG). Taken together, these results strongly support the hypothesis that both AUGs are used for translation, and that the preference for which AUG is selected for initiation depends partly on its translational context.

*JUN* uAUG and mAUG are conserved in vertebrates

To further investigate whether both *JUN* AUGs contribute to its translation, we examined sequence conservation of the *JUN* 5’ UTR and early CDS region that contains both AUG start codons and their translational context. We searched the 19-nucleotide region spanning the Kozak contexts of both AUGs in 100 species using the Genome Data Viewer (NLM-NCBI) and Ensembl for sequence confirmation (Supplementary Table 1). Remarkably, sequences in this region are conserved both at the nucleotide and at the amino acid level in the species examined (Figure 3D and 3E). Conservation of both *JUN* AUGs is present in all vertebrates (Schoch et al. 2020), whereas in the invertebrates we investigated only the mAUG is present (Figure 3F). This conservation of both of *JUN*'s AUGs and their translational context suggests an ancient mechanism for *JUN* translation regulation and highlights the importance of both *JUN* AUGs.

DISCUSSION

Given that *JUN* was the first oncogenic transcription factor identified (Bohmann et al. 1987; Ryder et al. 1988) it is notable how little is known mechanistically about how *JUN* expression is controlled at the translational level. In this work we probed the contributions of mRNA features and initiation factors to *JUN* translation regulation in human cells. Our study reveals that *JUN* translation regulation is a complex process that is mediated by mRNA target sequences and structural elements spanning the entire *JUN*...
5′ UTR (Figure 4A). Moreover, we provide evidence that initiation factors in addition to eIF3 (Lee et al. 2015) contribute to JUN translation regulation (Figure 4B). We also found that both the uAUG and mAUG contribute to JUN translation. Given that the JUN 5′ UTR has a length that exceeds the average 218 nt human 5′ UTR (Lepekk et al. 2018), a high level of secondary structure (Lee et al. 2015), and a GC rich sequence, many features within its 5′ UTR that participate in JUN translation regulation likely remain to be discovered.

Previous results found that eIF3 can directly bind structures in the 5′ UTR of specific mRNA transcripts to regulate their translation, with JUN serving as a prototypical example (Lee et al. 2015). Here we explored the regulatory roles of RNA structural elements within or near the eIF3-interacting stem loop (SL) region of the JUN 5′ UTR (Figure 1). In addition to the importance of this SL for enhancing JUN translation in cells, we found that replacing the SL loop by a highly-stable UUCG tetraloop (Antao et al. 1991) increases JUN translation. It is possible that significant local rearrangements may be required for the canonical JUN SL loop sequence to bind eIF3 or that the canonical SL loop is highly dynamic, and insertion of the UUCG tetraloop locks this structure in the most favorable conformation for eIF3 binding. Additionally, there may be some sequence specificity in the SL loop, as mutation of two nucleotides in the context of the wild-type loop at positions 128 and 129 also affect JUN translation levels (Figure 1). Interestingly, the ability of this eIF3-interacting SL structure to promote translation is shown by the fact that it can be inserted in a modular way into the 3′ UTR of reporter mRNAs to promote translation, as shown in activated T cells (De Silva et al. 2021). We also found additional structural elements besides the eIF3-interacting SL that contribute to JUN translation. Most notably, these are a stem loop between nucleotides 23 and 33 of the JUN 5′ UTR and a bulge loop between nucleotides 42 and 47, which enhance or repress translation, respectively (Figure 1). These secondary structure elements may serve potential regulatory roles, similar to the one shown for the eIF3-interacting SL. These results are consistent with previous findings which have correlated long and highly structured 5′ UTRs with complex regulation mediated by eIF3 (Stanciu et al. 2022). However, it remains to be determined whether eIF3 interacts directly with these regions. It is also possible these secondary structure elements mediate additional regulatory interactions.
Further evidence will be required to determine whether additional initiation factors interact with the structural elements studied in this SL-proximal region.

We also found evidence for a role for eIF4A in JUN translation regulation. We demonstrated that JUN is highly sensitive to RocA, consistent with prior transcriptome-wide experiments (Iwasaki et al. 2016) and with JUN being a target of eIF4A regulation (Figure 2). Interestingly, RocA sensitivity is independent of JUN interactions with eIF3, since mutations in the JUN eIF3-interacting SL did not affect its sensitivity to RocA (Figure 2). RocA was shown to clamp eIF4A onto GAA(G/A) polypurine sequences in a subset of RocA sensitive mRNAs and these mRNAs are in fact rich in these tetramer motifs (Iwasaki et al. 2016). Notably, JUN possesses 11 of these GAA(G/A) motifs in its 5' UTR. However, mutating these sequences to CAAC did not overcome JUN sensitivity to RocA, suggesting that RocA may clamp eIF4A onto additional polypurine sequences in the JUN 5’ UTR different from the predominant motif previously identified (Iwasaki et al. 2016). A potential polypurine sequence present in the JUN 5’ UTR and to which RocA might clamp eIF4A is AGAG (Iwasaki et al. 2019). Within eIF3, subunit EIF3D can bind to the JUN mRNA 5’-7-methylguanosine cap structure, while an RNA structural element adjacent to the cap blocks recruitment of the eIF4F complex (Lee et al. 2016). However, our results with RocA treatment suggest that at least some of the eIF4F components may contribute to JUN mRNA recruitment and scanning. This suggests that there may be a novel mRNA recruitment complex for JUN, in which eIF4A is present despite the absence of eIF4E, with EIF3D possibly acting as the cap-binding protein in this context.

Although JUN possesses a 5’ UTR nearly 1 kb in length, it also has two closely-spaced potential start codons, an upstream start codon (uAUG) 4 codons away from a downstream “main” AUG (mAUG). However, which of these start codons is preferentially selected and whether they both contribute to JUN translation is currently unknown. Notably, experimental evidence for usage of the uAUG would be missed in published mass spectrometry experiments due to presence of a lysine at codon -1 relative to the mAUG, which would lead to removal of the leading peptide in commonly-used trypsin digests. Using reporters with the full-length JUN 5’ UTR and both AUGs, we find that both AUGs likely contribute to JUN translation, albeit in a complex way (Figure 3). For example, deleting each AUG individually repressed JUN translation significantly, with deletion of
the mAUG causing a more severe reduction. However, the contexts of the uAUG and mAUG do not always correlate with translational output. For example, changing the weak context of the uAUG seen in WT JUN into a strong context decreased translation by 50% rather than increasing it. In this case, the mAUG is also likely used, as weakening the translational context of the mAUG in the strong uAUG context background further repressed translation to about 10% of WT levels. These results suggest that while both AUGs contribute to JUN translation, perhaps the mAUG plays a major role. These results also raise the possibility that translational efficiency of the first 4 codons including the uAUG may be lower than that of the mAUG, which would result in a lower translational output from the uAUG when it is used.

The fact that both AUGs may contribute to JUN translation suggests they may be part of a regulatory switch in varying cellular conditions. For example, unwinding of an RNA secondary structure downstream of an uAUG in an immune response promotes translation initiation at the mAUG of specific mRNAs in Arabidopsis thaliana (Xiang et al. 2023). Other cellular conditions, such as stress, starvation, or polyamine abundance could influence start codon selection (Hinnebusch 2005; Starck et al. 2016)(Hann et al. 1992; Ivanov et al. 2008). Finally, the relative abundance of eIF1 and eIF5 – which regulate the stringency of start codon selection (Loughran et al. 2012; Ivanov et al. 2010, 2022) – could influence which JUN start codon is used, and thus the translational output of the JUN mRNA. Further experiments will be needed to test this hypothesis.

When exploring the evolution of JUN’s AUGs we found that both are conserved in vertebrates, which suggests an ancient mechanism of regulation for JUN by means of translational start site selection. Importantly, the translational context is also conserved for most of the examined species (Figures 3D and 3E, Supplementary Table 1), suggesting that the translational context plays a significant role in determining which start codon is selected. Our observations align with previous reports which showed that uAUGs are highly conserved in higher eukaryotes due to their roles in regulating translation initiation under regulatory circumstances (Chew et al. 2016; Zhang et al. 2021). In addition, the evolutionary conservation suggests that more than one JUN polypeptide may be expressed by initiation of translation at both the uAUG and the mAUG. This type of alternative initiation has been shown previously by leaky scanning of uAUGs in a weak
translational context, especially of those that are close to their downstream mAUG which allows for backward oscillation of the ribosome (Smith et al. 2005; Matsuda and Dreher 2006). Further studies are needed to test whether JUN leads to expression of more than one polypeptide, depending on the start codon selected. For example, this would require using a different protease for mass spectrometry besides trypsin to avoid cleavage after the lysine at position -1 relative to the mAUG, to retain N-terminal peptides originating at the uAUG.

Given that JUN was the first oncogenic transcription factor identified (Bohmann et al. 1987; Ryder et al. 1988) it is notable that many different mechanisms regulate JUN expression at the translational level. Our study demonstrates the potential of the JUN mRNA as a model transcript for understanding new mechanisms of mRNA translation regulation. It opens the doors for further exploration of the regulatory roles of long and highly structured 5’ UTRs and the initiation factors that participate in translation regulation. It also points to possible new roles for JUN mRNA translation levels in mediating cellular response to a wide array of physiological conditions.
Figure 1. *JUN* translation is regulated by 5' UTR sequence and structural elements.

(A) Depiction of the full *JUN* 5' UTR. The locations of the 208-nt region studied by SHAPE (SHAPE) and the eIF3-interacting stem loop (SL) are marked, along with the nucleotides involved in each region. (B) The secondary structure of the 208-nt region in the *JUN* 5' UTR mapped by SHAPE is shown. Nucleotides are numbered according to their position in the 5’ UTR. Mutant *JUN* 5’ UTR mRNA constructs and their corresponding mutations are described in the associated tables. (C) Luminescence measured from HEK293T cells transfected with the *JUN* 5’ UTR reporter mRNAs expressing Nanoluciferase (Nluc). Translation was assessed using a dual-luciferase assay and normalized to a control mRNA harboring an *HBB* 5’ UTR and a Firefly luciferase (Fluc) CDS. Nluc/Fluc ratios were normalized to the WT *JUN* 5’ UTR, set as 100%. Technical triplicates for each biological replicate, and a total of at least three biological replicates were taken for each measurement. P values determined using a one-sample t test versus a hypothetical value of 100 are shown as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The mean value of the replicates and standard error of the mean are shown.
Figure 2. JUN is highly sensitive to RocA treatment. (A) HEK293T cells co-transfected with JUN 5′ UTR and Nluc CDS reporter mRNAs (WT, ΔSL or mutant G, Figure 1) and with an HBB 5′ UTR and Fluc mRNA as an internal control, were treated with increasing concentrations of RocA (+RocA) or DMSO control (+DMSO) 3 hours post-transfection, as previously reported (Iwasaki et al. 2016). An mRNA with the HBB 5′ UTR and Nluc CDS mRNA was also used as a RocA-insensitive control. Translation was assessed using a dual-luciferase assay as in Figure 1. Nluc/Fluc measurements were normalized to the corresponding untreated condition (0 nM RocA) and reported as a percentage of this measurement. (B) The location of polypurine (GAA(G/A)) sequences in the JUN 5′ UTR are indicated with yellow lines. Each of these 11 sequences was mutated to CAAC. (C) Luminescence of HEK293T cells transfected with JUN 5′ UTR and Nluc CDS reporter mRNAs (WT, ΔSL, CAAC or CAAC + ΔSL), together with the HBB 5′ UTR and Fluc CDS mRNA control. Transfected cells were treated with 300 nM RocA (+RocA) or DMSO (+DMSO) 3 hours post-transfection. Translation was assessed using a dual-luciferase assay as in Figure 1, and Nluc/Fluc measurements were normalized to the WT JUN 5′ UTR and Nluc CDS +DMSO measurements, reported as percentages. (D) Luminescence from in vitro translation reactions using the JUN 5′ UTR and Nluc CDS reporter mRNAs (WT, ΔSL, CAAC or CAAC + ΔSL). Reactions were treated with 300 nM RocA (+RocA) or DMSO (+DMSO). Luminescence values of each mutant were normalized to the WT JUN 5′ UTR and Nluc CDS +DMSO measurements and reported as percentages. In panels A, C, and D, technical triplicates for each biological replicate, and a total of at least three biological replicates were taken for each measurement. P values determined using a one-sample t test versus a hypothetical value of 100 are shown as follows: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. The mean value of the replicates and standard error of the mean are shown.
**Figure 3. Two start codons contribute to JUN translation.** (A) Diagram depicting the ideal Kozak context for a generic open reading frame (A/GnnAUGG). Below, diagrams depicting each of the JUN start codons (AUG) and their translational contexts. (B) Diagram depicting JUN mRNA reporter constructs, with their corresponding mutations in each of the JUN start codons and their translational contexts. The constructs contained the full JUN 5’ UTR sequence along with the first 51 nucleotides of the JUN CDS, upstream of the full Nluc CDS. (C) Luminescence from HEK293T cells transfected with JUN 5’ UTR and 51nt JUN CDS/Nluc CDS reporter mRNAs (WT, ΔuAUG, ΔmAUG, s-uAUG, S-uAUG or S-uAUG W-mAUG), together with an HBB 5’ UTR and Fluc CDS control, assessed using a dual-luciferase assay as in Figure 1. Nluc/Fluc measurements of each mutant were normalized to the WT JUN 5’ UTR and 51nt JUN CDS and Nluc CDS measurements and reported as percentages. Technical triplicates for each biological replicate, and a total of at least three biological replicates were taken for each measurement. P values determined using a one-sample t test versus a hypothetical value of 100 are shown as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The mean value of the replicates and standard error of the mean are shown. (D) Sequence logo depicting the conservation of the 19 nucleotide JUN sequence spanning both start codons and their translational context amongst 100 species. (E) Sequence logo depicting the conservation of the 5 amino acid JUN sequence containing both start codon methionines amongst 100 species. (F) Phylogenetic tree depicting the conservation of both JUN AUGs amongst 100 species. Species with both the JUN uAUG and the JUN mAUG are depicted with blue branches, while species with only one JUN mAUG are depicted in orange.
Figure 4. **JUN translation regulation is mediated by 5' UTR features and multiple translation initiation factors.** (A) Diagram showing all JUN regions investigated in this study. (B) Diagram depicting different contributors to JUN translation regulation. These factors include: JUN mRNA secondary structure (depicted as a stem loop in the 5' UTR), JUN sequences (such as the GAA(A/G) polypurine sequences depicted in yellow), JUN AUGs (depicted in orange), and initiation factors (such as eIF3, eIF4A, and potentially eIF1 and eIF5). EIF3 (depicted in pink) interacts with structured regions of the JUN 5' UTR. EIF4A (depicted in yellow) interacts with GAA(A/G) sequences (black arrow) and with other unknown sequences (gray arrows) in the JUN 5' UTR. EIF1 (depicted in brown) and eIF5 (depicted in orange) may also play roles in JUN start codon selection.
MATERIALS AND METHODS

Reporter plasmids

To generate the JUN 5’ UTR and the HBB 5’ UTR Nluc reporter plasmids, the JUN 5’ UTR (ENST00000371222.4) previously generated by amplification from human cDNA (Lee et al. 2015) and the HBB 5’ UTR (ENST00000335295.4) commercially generated (IDT) sequences were each inserted into the pNL1.1 NanoLuc luciferase reporter plasmid (Promega, GenBank Accession Number JQ437370) downstream of a T7 promoter using overlap-extension PCR with Q5 High-Fidelity DNA Polymerase (NEB) and InFusion cloning (Takara Bio). For the JUN AUG mutants, the first 51 nucleotides of the JUN CDS were inserted downstream of the full JUN 5’ UTR sequence and upstream of the full Nluc CDS in the pNL1.1 plasmid. For the Fluc reporter plasmid, the HBB 5’ UTR Nluc reporter plasmid was amplified and the NanoLuc luciferase sequence was replaced by a commercially generated Firefly luciferase sequence (IDT) (Giacomelli et al. 2018). Subsequent mutant versions of the JUN reporter plasmids were made by amplifying the plasmid using overlap-extension PCR with Q5 High-Fidelity DNA Polymerase (NEB) and primers containing the corresponding mutations, insertions, or deletions, followed by InFusion cloning (Takara). All primers used for amplification can be found in Supplementary Table 2. All sequences were verified by Sanger sequencing.

In vitro transcription

All RNA reporters were made by in vitro transcription with a standard T7 RNA polymerase protocol using DNA template gel extracted using the Zymoclean Gel DNA Recovery Kit (Zymo), 1x T7 RNA Polymerase buffer (NEB), 5 mM ATP (Thermo Fisher Scientific), 5 mM CTP (Thermo Fisher Scientific), 5 mM GTP (Thermo Fisher Scientific), 5mM UTP (Thermo Fisher Scientific), 5 µg BSA (NEB), 9 mM DTT, 25 mM MgCl₂, 200U T7 RNA polymerase (NEB), 50U Murine RNAse inhibitor (NEB) and incubating for 4 hours at 37 °C. The DNA template used for in vitro transcription was generated by PCR amplification from the corresponding reporter plasmid using the Q5 High-Fidelity DNA Polymerase (NEB) with a reaction including a forward primer containing the T7 promoter sequence and a 60T reverse primer for polyadenylation. Primers used for each transcript can be found in Supplementary Table 2. After in vitro transcription, RNAs were treated with RQ1
DNAse (Promega) following the manufacturer’s protocol and precipitated with 7.5 M lithium chloride. RNAs were then capped using Vaccinia D1/D2 (Capping enzyme) (NEB) and 2’ O-methylated using Vaccinia VP39 (2’ O Methyltransferase) (NEB) in a reaction that also included 1X capping buffer (NEB), 10 mM GTP (Thermo Fisher Scientific) and 4 mM SAM (NEB). RNAs were then purified with the RNA Clean and Concentrator-5 Kit (Zymo). In order to verify the integrity of the in vitro transcribed mRNAs, 6% polyacrylamide TBE-Urea denaturing gels were run using 1X TBE (Invitrogen), a ssRNA ladder (NEB) and SYBR safe stain (see representative gel in Supplementary Figure 1).

HEK293T cells and mRNA transfections

HEK293T cells were maintained in DMEM (Gibco) supplemented with 10% FBS (VWR) and 1% Pen/Strep (Gibco). Cells were grown at 37 °C in 5% carbon dioxide and 100% humidity. Luciferase reporter mRNAs were transfected into these cells using the TransIT-mRNA Transfection Kit (Mirus), with the following protocol modifications. HEK293T cells were seeded into opaque 96-well plates (Thermo Fisher Scientific) about 16 hours prior to transfections. The next day, once the cells reached 80% confluency, transfections were performed by adding the following to each well: 7 µL of pre-warmed OptiMEM media (Invitrogen), 500 ng of 5’-capped and 3’-polyadenylated Nluc reporter mRNA, 150 ng of 5’-capped and 3’-polyadenylated Fluc reporter mRNA, 2 µL of Boost reagent (Mirus Bio) and 2 µL of TransIT mRNA reagent (Mirus Bio). Transfection reactions were incubated at room temperature for 3 minutes prior to drop-wise addition into each well. Transfected cells were incubated at 37 °C for 8 hours, after which luciferase assays were performed using the NanoGlo Dual-Luciferase Reporter Assay System (Promega) following the manufacturer’s protocol. Luminescence was then measured using a Spark multimode microplate reader (TECAN). Nluc/Fluc ratios were normalized to the corresponding control condition, set as 100%. Technical triplicates for each biological replicate, and a total of at least three biological replicates were taken for each measurement. P values were determined using a one-sample t test versus a hypothetical value of 100. The mean value of the replicates and standard error of the mean were plotted.
HEK293T pSB-HygB-GADD34-K3L cells and extract preparation

HEK293T pSB-HygB-GADD34-K3L cells (Aleksashin et al. 2023) were maintained in DMEM media (Gibco) supplemented with 10% Tet-system approved FBS (Gibco) and 1% Pen/Strep (Gibco). Cells were grown at 37 °C in 5% carbon dioxide and 100% humidity. Cells were grown for extract preparation as follows. The day after plating cells from a frozen stock into a T25 flask (Cell Star), media was exchanged and supplemented with 200 µg/mL Hygromycin B (Invitrogen). The following day, cells were transferred to a T75 flask (Corning) with media supplemented with 200 µg/mL Hygromycin B. Once cells reached 100% confluency, half of the cells were transferred to a T175 flask (Falcon) with media supplemented with 200 µg/mL Hygromycin B. Once cells reached 100% confluency, cells were passaged onto 25 150 mm plates (Corning) at a 1 to 25 ratio. The next day, cells were treated overnight with 20 µg Doxycycline (Takara Bio) per plate. 

In vitro translation extracts were made from HEK293T pSB-HygB-GADD34-K3L cells using a previously described protocol (Aleksashin et al. 2023). Cells were placed on ice, scraped and collected by centrifugation at 1000 xg for 5 minutes at 4 °C. Cells were washed once with ice-cold DPBS (Gibco) and collected once again by centrifugation at 1000 xg for 5 minutes at 4°C. After this, cells were homogenized with an equal volume of freshly made ice-cold hypotonic lysis buffer (10 mM HEPES-KOH pH 7.6, 10 mM KOAc, 0.5 mM Mg(OAc)$_2$, 5 mM dithiothreitol). After hypotonic-induced swelling for 45 min on ice, cells were homogenized using a syringe attached to a 26G needle (BD). Extract was then centrifuged at 15000 xg for 1 minute at 4 °C. The resulting supernatant was aliquoted, frozen with liquid nitrogen, and stored at -80 °C.

In vitro translation

In vitro translation reactions were performed using HEK293T pSB-HygB-GADD34-K3L translation-competent cell extract, as previously described (Aleksashin et al. 2023). Translation reactions contained 50% translation-competent cell extract, 52 mM HEPES pH 7.4 (Takara), 35 mM potassium glutamate (Sigma), 1.75 mM Mg(OAc)$_2$ (Invitrogen), 0.55 mM spermidine (Sigma), 1.5% Glycerol (Fisher Scientific), 0.7 mM putrescine (Sigma), 5 mM DTT (Thermo Scientific), 1.25 mM ATP (Thermo Fisher Scientific), 0.12 mM GTP (Thermo Fisher Scientific), 10 mM L-Arg; 6.7 mM each of L-Gln, L-Ile, L-Leu,
L-Lys, L-Thr, L-Val; 3.3 mM each of L-Ala, L-Asp, L-Asn, L-Glu, Gly, L-His, L-Phe, L-Pro, L-Ser, L-Tyr; 1.7 mM each of L-Cys, L-Met; 0.8 mM L-Trp, 20 mM creatine phosphate (Roche), 60 µg/mL creatine kinase (Roche), 4.65 µg/mL myokinase (Sigma), 0.48 µg/mL nucleoside-diphosphate kinase (Sigma), 0.3 U/mL inorganic pyrophosphatase (Thermo Fisher Scientific), 100 µg/mL total calf tRNA (Sigma), 0.8 U/µL RiboLock RNase inhibitor (Thermo Scientific), and 1000 ng of the corresponding mRNA. Reactions were then incubated for 60 minutes at 32 °C, and Nanoluciferase activity was monitored using the Nano-Glo Luciferase Assay Kit (Promega) using a Spark multimode microplate reader (TECAN). The average of each biological replicate was normalized to the control condition, set as 100%. Technical triplicates for each biological replicate, and a total of at least three biological replicates were taken for each measurement. P values determined using a one-sample t test versus a hypothetical value of 100 are shown as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. The mean value of the replicates and standard error of the mean were plotted.

**Conservation analysis for JUN AUGs**

The 19-nucleotide JUN 5′ UTR and JUN CDS region that spans both AUG start codons and their translational context was searched in 100 species. Species were selected randomly, starting with Homo sapiens and increasing the evolutionary distance throughout the vertebrates up to the invertebrates (Supplementary Table 1). Species sequences were compiled using the Genome Data Viewer (NLM-NCBI) and Ensembl. Sequence logos for the conserved nucleotide and amino acid sequences were created using WebLogo (https://weblogo.berkeley.edu/) (Crooks et al. 2004; Schneider and Stephens 1990). Taxonomy analysis for the species of interest was performed using the NCBI Taxonomy Browser (Schoch et al. 2020; Sayers et al. 2019). Phylogenetic tree was generated using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).
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Common Taxonomy Tree.

FigTree. http://tree.bio.ed.ac.uk/software/figtree/.
Supplementary Figure 1. Representative mRNA TBE-Urea gel.

6% TBE-Urea gel for *in vitro* transcribed mRNA for the WT or ΔSL *JUN* 5’ UTR and Nluc CDS reporter constructs. nt, nucleotide.
Supplementary Table 1. Conservation analysis for JUN uAUG and mAUG.
Compilation of species investigated for JUN AUGs conservation analysis, including the nucleotide and amino acid sequences of the 19-nucleotide JUN 5′ UTR and JUN CDS region that spans both AUG start codons and their translational context for each species, the corresponding Reference Sequence (RefSeq) accession numbers for each sequence, and the percent similarity of each sequence to the human JUN sequence.

Supplementary Table 2. Primers used in this study.
Compilation of primer sequences used for cloning and in vitro transcription amplification of each reporter construct used in this study.