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The mammalian orthoreovirus bicistronic M3 mRNA initiates translation using a 5′ end-dependent, scanning mechanism that does not require interaction of 5′–3′ untranslated regions

Vidya Sagar*, Kenneth E. Murray

Department of Biological Sciences, Florida International University, Miami, FL 33199, United States

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ABSTRACT

Mammalian orthoreovirus mRNAs possess short 5′ UTR, lack 3′ poly(A) tails, and may lack 5′ cap structures at late times post-infection. As such, the mechanisms by which these viral mRNAs recruit ribosomes remain completely unknown. Toward addressing this question, we used bicistronic MRV M3 mRNA to analyze the role of 5′ and 3′ UTRs during MRV protein synthesis. The 5′ UTR was found to be dispensable for translation initiation; however, reducing its length promoted increased downstream initiation. Modifying start site Kozak context altered the ratio of upstream to downstream initiation, whereas mutations in the 3′ UTR did not. Moreover, an M3 mRNA lacking a 3′ UTR was able to rescue MRV infection to WT levels in an siRNA trans-complementation assay. Together, these data allow us to propose a model in which the MRV M3 mRNA initiates translation using a 5′ end-dependent, scanning mechanism that does not require the viral mRNA 3′ UTR or 5′–3′ UTRs interaction.

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1. Introduction

Efficient eukaryotic mRNA translation initiation requires the 5′ and 3′ terminus of mRNA to interact and form a circular closed loop. Primarily, this is achieved by the interaction of the 5′ cap binding complex, eukaryotic initiation factor eIF4F, with the 3′ poly (A) tail binding protein, PABP (Hershey and Merrick, 2000). Following mRNA circularization, pre-formed scanning competent 43S pre-initiation complexes (40S ribosomal subunit, eIF3, eIF1, eIF1A, eIF5, and the eIF2–GTP–Met-tRNAi^{Met} ternary complex) are loaded onto the unwound 5′ untranslated region (UTR) (~120 nucleotides long) of the mRNA via an eIF4G–eIF3 interaction. This 43S pre-initiation complex scans the 5′ UTR in a 5′ to 3′ direction until it encounters a start codon (AUG), at which time it forms a 48S initiation complex. Following 48S complex formation, GTP hydrolysis induces eIFs to dissociate from the 40S ribosomes, therein allowing the 60S ribosomal subunit to join and form an elongation competent 80S ribosome (Komarova et al., 2009).

Synthesis of all viral proteins is achieved utilizing host translation factors and ribosomes. As such, many viruses produce 5′ capped, 3′ polyadenylated, monocistronic messages that resemble those produced by the host cell. Many other RNA viruses, however, produce noncanonical mRNAs which frequently lack 5′ cap (m7G(5′)pppG(5′)N cap, where N implies for any nucleotide) structures and/or 3′ poly(A) tails (50–200 residue long poly (A) tail following ~90 nucleotides 3′ UTR). They initiate protein synthesis using alternative mechanisms that necessitate only a subset of host translation factors (Gradi et al., 1998; Balvay et al., 2009; Dutkiewicz et al., 2006; Ilkow et al., 2008; Walsh et al., 2008). Viral mRNAs produced without cap structures may possess highly structured 5′ UTRS that promote direct ribosome loading at internal start sites (Balvay et al., 2009; Niepmann, 2009), or they may be 5′ modified by the covalent linkage of a viral protein (VPg, *v*iral *p*rotein genome-linked) (Pettersson et al., 1978; Richards et al., 1981). VPg proteins vary in size, structure, and function, but do, in some cases, act as protein cap substitutes; mediating both mRNA stability and ribosomal recruitment (Goodfellow et al., 2005; Tacken et al., 2004). Viral mRNAs produced without 3′ poly(A) tails have evolved at least three mechanisms to complement the absence of the poly(A) tail. These mechanisms include: (1) utilizing highly structured 3′ terminal RNA elements to mediate stability and/or facilitate ribosomal recruitment (Miller et al., 2007; Treder et al., 2008; Wang et al., 2009), (2) circularizing and stabilizing mRNAs via the specific binding of cellular proteins to 3′ terminal sequences (Polacek et al., 2009), or (3) circularizing and stabilizing mRNAs via the specific binding of viral proteins to 3′ terminal sequences (Groft and Burley, 2002; Vende et al., 2000).

The mammalian orthoreoviruses, like the closely related rotaviruses, produce nonpolyadenylated messages (Schiff et al., 2007); however, unlike rotaviruses, it has been reported that MRV synthesizes uncapped secondary messages (the bulk of [+] ssRNA

* Corresponding author.
E-mail address: vsaga001@fiu.edu (V. Sagar).

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produced in the MRV infected cell) (Skup and Millward, 1980; Zarbl et al., 1980). It has also been reported that MRV infection alters host translation machinery to favor cap-independent translation (Skup et al., 1981; Sonenberg et al., 1981). Accordingly, during late post-infection MRV-infected cells loss their efficiency to translate capped mRNAs, either cellular or viral. Concomitantly, cell's capability to translate reovirus uncapped mRNA increases. In the same line, ability of eIF4G to interact with eIF4E is decreased during MRV infection; however, the integrity of eIF4G is unchanged (Etchison and Fout, 1985; Mosenkis et al., 1985) suggesting modification in the cap binding complex, eIF4F, to exaggerate the host shut off process (Sonenberg et al., 1981). Together, these observations suggest that MRV mRNAs use a novel mechanism to initiate translation. As such, we hypothesized that MRV mRNAs might: (1) utilize a 5′ UTR sequence to facilitate direct loading of ribosomes at translation (internal initiation), (2) utilize a 3′ terminal structure or sequence to mediate translation initiation, or (3) use a 5′ end-dependent, scanning mechanism to initiate protein synthesis.

In the current study, we investigated the role of 5′ and 3′ UTR sequences during MRV mRNA translation. We engineered mutations into the 5′ and/or 3′ UTRs of the bicistronic M3 gene segment of the type 1 Lang (T1L) strain of MRV. The MRV M3 mRNA encodes two in-frame, carboxy-coterminal proteins, μ-NS and μ-NSC (Fig. 1) (Schiff et al., 2007). We chose to analyze translation of the M3 mRNA for two reasons: (1) the two proteins products (μ-NS and μ-NSC) are easily quantified, and (2) determination of μ-NS/μ-NSC ratios allows direct comparison of WT and mutant RNAs by acting as an internal control for experimental differences in transfection efficiency. Utilizing in vitro and in vivo translation assays, we determined that the 5′ UTR is dispensable for MRV M3 translation. While the upstream reading frame continued to be translated even when the 5′ UTR was reduced to a single nucleotide, we found that shortenings of the 5′ UTR promoted increased translation of the downstream reading frame relative to the upstream (leaky scanning). Independent modification of the Kozak context of each MRV M3 start site altered the ratio of upstream to downstream initiation (μ-NS/μ-NSC ratio); further demonstrating that ribosomes associate the 5′ terminus of RMV M3 mRNA and scan to the first or second AUG to initiate translation. Removal of the 3′ UTR did not alter the μ-NS/μ-NSC ratio, and an M3 mRNA lacking the complete 3′ UTR was able to rescue infection to WT levels in an siRNA trans-complementation assay. These data demonstrate that 3′ UTR is also dispensable for translation, and further demonstrate that 5′–3′ UTR interaction is not necessary for efficient translation of the MRV M3 mRNA. Collectively, these data allow us to propose a model in which the mammalian reovirus M3 mRNA initiates translation using 5′ end-dependent, scanning mechanism that does not require UTRs interaction.

2. Results

2.1. The 5′ untranslated region of MRV M3 mRNA is not necessary for translation initiation

Previous investigation have shown that leaky scanning, the process by which a scanning 43S PIC bypasses the first AUG codon encountered and initiates protein synthesis at a downstream site, can be induced by moving the 5′ proximal AUG codon closer to the 5′ terminus (Kozak, 1991). Thus, in an effort to evaluate 43S PIC scanning on the 5′ UTR of the MRV M3 mRNA, we engineered deletions into the 5′ UTR of a previously described M3 construct (pBOS36). We generated three mutant M3 constructs denoted: M3-9, M3-4, and M3-1. T7 polymerase used for synthesizing mRNAs initiate the transcription from first G base at the 3′ end (5′-TAATACGACTCACTATAG-3′). This resulted in addition of an extra "G" base at the 5′ UTR terminus of all transcripts. Thus, synthesized WT M3 mRNA indicated in manuscript possesses a 5′ UTR that is nineteen nucleotides in length. Transcription of M3-9 produced an mRNA possessing a 5′ UTR nine nucleotides in length (nts 9–18 were deleted). Transcription of M3-4 produced an mRNA possessing a 5′ UTR four nucleotides in lengths (nts 4–18 were deleted), and transcription of M3-1 produced an mRNA with a 5′ UTR comprising a single G residue (nts 2–18 were deleted) (Fig. 1). In addition to our mutants, we also reproduce two previously described control M3 mRNA, ΔAUG1 (produces μNS) and ΔAUG2 (produces μNS) (Arnold et al., 2008; Busch et al., 2011; Kobayashi et al., 2006), to serve as gel markers for each protein (Fig. 1). To evaluate the effect of each mutation on start site selection, we programmed in vitro, micrococcal nuclease treated rabbit reticulocyte lysate (RRL) translation reactions with 2 μg of in vitro transcribed, capped, WT or mutant mRNA, and translated each mRNA in the presence of [^{35}S]methionine at 30 °C for 90 min. Labeled proteins were resolved by electrophoresis in SDS 8% polyacrylamide gels. Gels were dried, and separated proteins were detected and quantified via phosphorimaging (Fig. 2A). Phosphorimage analysis of the radiolabeled gels allowed us to calculate expression ratios (μNS/μNSC ratio) for each construct, which could then be compared directly to the μNS/μNSC ratios of WT constructs (Fig. 2B). These analyses revealed that reducing the length of the 5′ UTR to nine nucleotides resulted in a modest, but statistically significant increase in downstream initiation (reduced μNS/μNSC ratio). These analyses also revealed that further reductions in the length of the 5′ UTR resulted in greater read through of the upstream (μNS) initiation site (Fig. 2A and B). While these results were not entirely unexpected, we were surprised to observe that the reduction of the 5′ UTR to four or even one nucleotide did not prevent recognition of the upstream initiation site. More surprising was the observation that the upstream site was preferentially recognized on the M3-4 and M3-1 mRNA (μNS:μNSC > 1). When the 5′ UTR was reduced to lengths of less than 8 nucleotides we consistently observed the appearance of a third protein product (arrow, Fig. 2A, lanes 3–6). This translation product has been described previously, and is believed to be an in-frame translation product produced when ribosomes scan to the third AUG codon (amino acid position 57) (Schiff et al., 2007). As previously described, the ΔAUG1 mutation abrogated μNS synthesis and promoted increased initiation at both AUG2 and AUG_{57} start sites (Fig. 2A, lane 5), whereas the ΔAUG2 mutation produced WT levels of μNS, trace levels of μNS, and increased levels of the AUG_{57} translation product (Fig. 2A, lane 6). The leaky nature of the ΔAUG2 mutation is consistent with previous reports demonstrating that near cognate initiation sites, i.e. UUG, have been shown to function with 5–8% WT efficiency (Takacs et al., 2011).

Because RRL are known to support the efficient translation of some non-canonical transcripts (uncapped or leaderless mRNA) (Wakiyama et al., 1997), we sought to determine what effect the M3-9, M3-4 and the M3-1 mutations would have on μNS/μNS C ratios in cells. To address this, we transfected WT or mutant M3 mRNA into BsrT7 cells. At 24 h p.t., cells were collected and lysed, and clarified lysates were analyzed by immunoblotting to detect μNS and μNSC proteins. Following detection, individual proteins were quantified using ImageQuant 7.0 software. These analyses revealed that reducing the length of the 5′ UTR to nine nucleotides resulted in a modest, but significant reduction of the μNS/μNSC ratio, and as was described for in vitro RRL reactions, greater reductions in the length of the 5′ UTR (M3-4 and M3-1) further reduced the μNS/μNSC ratio (Fig. 2C and D). The μNS/μNSC ratios observed in transfected BsrT7 cells, however, were more representative of those described for infected cells (Arnold et al., 2008; Miller et al., 2003). Additionally, significant scanning to AUG_{57} was only observed when the upstream start site (AUG1) was

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