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In vivo translation and stability of trans-spliced mRNAs in nematode embryos

Guofeng Cheng [a,1], Leah Cohen [b,1], Claudette Mikhli [b], Marzena Jankowska-Anyszka [c], Janusz Stepinski [d], Edward Darzynkiewicz [d], Richard E. Davis [a,b,*]

[a] Departments of Pediatrics and Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, CO 80045, United States
[b] Department of Biology, City University of New York Graduate Center, CSI, 2800 Victory Boulevard, Staten Island, NY 10314, United States
[c] Department of Chemistry, Warsaw University, 02-093 Warsaw, Poland
[d] Department of Biophysics, Institute of Experimental Physics, Warsaw University, 02-089 Warsaw, Poland

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Abstract

Spliced leader trans-splicing adds a short exon, the spliced leader (SL), to pre-mRNAs to generate 5′ ends of mRNAs. Addition of the SL in metazoa also adds a new cap to the mRNA, a trimethylguanosine ($m_3{}^{2,2,7}$GpppN) (TMG) that replaces the typical eukaryotic monomethylguanosine (m^7GpppN)(m^7G) cap. Both trans-spliced ($m_3{}^{2,2,7}$GpppN-SL-RNA) and not trans-spliced (m^7GpppN-RNA) mRNAs are present in the same cells. Previous studies using cell-free systems to compare the overall translation of trans-spliced versus non-trans-spliced RNAs led to different conclusions. Here, we examine the contribution of $m_3{}^{2,2,7}$GpppG-cap and SL sequence and other RNA elements to in vivo mRNA translation and stability in nematode embryos. Although 70–90% of all nematode mRNAs have a TMG-cap, the TMG cap does not support translation as well as an m^7G-cap. However, when the TMG cap and SL are present together, they synergistically interact and translation is enhanced, indicating both trans-spliced elements are necessary to promote efficient translation. The SL by itself does not act as a cap-independent enhancer of translation. The poly(A)-tail synergistically interacts with the mRNA cap enhancing translation and plays a greater role in facilitating translation of TMG-SL mRNAs. In general, recipient mRNA sequences between the SL and AUG and the 3′ UTR do not significantly contribute to the translation of trans-spliced mRNAs. Overall, the combination of TMG cap and SL contribute to mRNA translation and stability in a manner typical of a eukaryotic m^7G-cap and 5′ UTRs, but they do not differentially enhance mRNA translation or stability compared to RNAs without the trans-spliced elements.
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1. Introduction

Spliced leader (SL) trans-spliced mRNAs are formed by splicing a small exon, the spliced leader (16–52 nt depending on the species), to a splice acceptor site in an independently transcribed pre-mRNA [1–5]. SL addition thereby generates the mature 5′ end of the mRNA. This form of gene expression is present in diverse eukaryotes including some protozoa (Sarcomastigophora), several major invertebrate lineages (cnidarians, flatworms, rotifers, and nematodes), and in primitive chordates (tunicates) [6–12].

Trans-splicing in the Kinetoplastid protozoans serves to resolve polycistronic transcripts into mature monocistronic mRNAs by trans-splicing at the 5′ end of each protein coding cistron [13–16, for review, see 17]. Analysis of a subset of these polycistronic transcripts indicates, that unlike protein coding genes in most eukaryotes, these primary transcripts are uncapped [18]. Thus, trans-splicing, in addition to resolving these polycistronic transcripts into monocistronic mRNAs, also serves as a capping mechanism for some transcripts, and adds a novel cap 4 structure (m^7G-ppp-*N*^{6},*N*^{6},2′-*O*-trimethyladenosine*p*-2′-*O*-methyladenosine-*p*-2′-*O*-methylcytosine-*p*-N^3,2′-*O*-methyluridine) to the RNAs [19]. Polycistronic transcripts are also pro-

* Corresponding author at: Departments of Pediatrics and Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Mail Stop 8101, Box 6511, RC-1 South, 12801 East 17th Avenue, Aurora, CO 80045, United States. Tel.: +1 303 724 3226; fax: +1 303 724 3215.
E-mail address: Richard.Davis@uchsc.edu (R.E. Davis).
[1] These authors contributed equally to this work.

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duced in *Caenorhabditis elegans* [1,20,21] and the organization of a number of trans-spliced genes in other nematodes, flatworms and tunicates suggests they are likely to be polycistronic [22–25]. Thus, a major function of trans-splicing in diverse eukaryotes is the resolution of polycistronic transcription units to form monocistronic, capped mRNAs.

The majority of trans-spliced transcripts (∼50–80%) in nematodes are not derived from polycistronic transcripts. While the SL RNA substrate and a spliced leader are necessary for *C. elegans* embryogenesis [26,27], the function of most trans-splicing in nematodes (and other metazoa) remains unknown. It has been proposed that trans-splicing in metazoa might be functionally correlated with unusual characteristics of the promoters, transcription, capping, or the 5′ untranslated regions (UTR) of trans-spliced genes or that it plays a role in mRNA export, localization, translation, or stability [1–3,5,28,29].

The SL RNA in metazoa has a trimethylated ($m_3{}^{2,2,7}$GpppN or trimethylguanosine = TMG) cap in which methyl groups are also present at the N2 position of guanine [9,10,24,30,31]. This cap is transferred to mRNAs with the spliced leader sequence producing $m_3{}^{2,2,7}$GpppN – SL – mRNAs [32,33]. Although 100% of the mRNA are trans-spliced in the Kinetoplastida, this is not the case in metazoa where ∼10 to 80% of mRNA are trans-spliced. Consequently, two populations of mRNAs coexist in these organisms: (1) non-trans-spliced mRNAs with the m^7GpppN cap structure and different sequences for the 5′ UTR of each mRNA and (2) trans-spliced mRNAs with the TMG cap and conserved spliced leader.

mRNA cap structure and the 5′ UTR play important roles in gene expression contributing to efficient mRNA translation and stability [34–36]. In previous studies using Ascaris cell-free translation systems [37,38], one study concluded that mRNAs with trans-spliced elements were most efficiently translated whereas a second study observed non-trans-spliced mRNAs were slightly more efficiently translated. Here, we describe in vivo studies extending our understanding of nematode mRNA translation and further examine the hypothesis that TMG cap and conserved spliced leader sequence differentially contribute to mRNA translation and stability in early embryos of the nematode *Ascaris*. The current data indicate that the addition of trans-spliced elements to an RNA does not significantly enhance the translation or stability of the mRNAs compared with non-trans-spliced 5′ UTRs. Furthermore, these in vivo studies demonstrate that a previously described Ascaris embryo cell-free translation system [37] faithfully reproduces many aspects of in vivo translation.

2. Materials and methods

2.1. Isolation and preparation of Ascaris embryos

Adult female *Ascaris* were obtained from Carolina Biological Supply, the eggs isolated from uteri, stored, embryonated, and prepared for particle bombardment essentially as described previously [39].

2.2. Biolistics

Preparation of gold microcarriers and biolistics were performed as previously described [39] with the following modifications: (1) gold particles were spherical ∼2.2 μm gold from Degussa (10KM) (dmc2 Metals Group, South Plainfield, NJ) and (2) instead of lyophilization of the RNA onto the gold carriers, an alcohol precipitation was used. In vitro transcribed RNAs were precipitated onto gold particles using 2.5 M ammonium acetate/ethanol precipitation using 0.1–5 μg of reporter RNA per 1 mg of gold particles. The RNA/gold pellet was washed with 200 μl of ice-cold ethanol, resuspended in ice-cold 100% ethanol (18 μl/mg gold) and spread onto macrocarriers and processed as previously described [39].

Binding of RNA onto the gold particles was evaluated by formaldehyde agarose gel analysis to determine the integrity of RNA bound to the beads, and RiboGreen (Molecular Probes, Carlsbad, CA) [40] was used to quantitate RNA loaded onto the particles for data normalization. Analysis of RNA bound to beads indicated little to no degradation of the RNA occurred prior to particle bombardment and that typically 80–85% of the precipitated RNA was bound onto the gold particles. Approximately 800,000 *Ascaris* embryos were evenly spread onto 35-mm Petri dishes and bombarded in a Bio-Rad Biolistic PDS-1000/HE Particle Delivery System at 15-in Hg of chamber vacuum, target distance of 3 cm (stage 1), and 1350-psi particle acceleration pressure. After bombardment, 2–3 ml of nematode blastomere media [41] was added, and the embryos were incubated in an air shaker at 30 °C at 110 rpm (to keep the embryos in suspension). Following incubation, embryos were collected, pelleted, and assayed for luciferase activity. Evaluation of gold particle delivery was carried out as previously described [39].

2.3. Transcription template and RNA preparation

PCR templates for in vitro transcription reactions were derived from constructs made in Promega plasmids pGL3 Basic and pRLnull or pGLUC Basic-1 (Targeting Systems, Santee, CA). Using PCR primers that added a 5′ T7 promoter and additional sequences to the 5′ or 3′ end of the template, transcription templates were generated that contained different 5′ UTRs, a luciferase open reading frame (Firefly, Renilla, or Gaussia) and 3′UTRs (derived from pGL3, pRLnull, pGluc-1, the Ascaris vacuolar ATP synthase 16 kDa proteolipid subunit RNA, and the Ascaris ribosomal protein L23 RNA) with or without a poly(A) tail of various lengths. RNAs were generated by in vitro transcription from these templates using a T7 MegaScript kit as described by the manufacturer (Ambion, Austin, TX) with either no cap or ApppG, m^7GpppG, or $m_3{}^{2,2,7}$GpppG cap at a ratio of 4:1 or 10:1 cap analog to GTP. The 5′ and 3′ UTRs of the synthesized RNAs are provided in Fig. 1 in Supplementary data. Transcription reactions were DNase I-treated, extracted with TRIzol (Invitrogen, Carlsbad, CA), and the RNAs precipitated twice, once with isopropanol and then with ammonium acetate/ethanol. Precipitated RNAs were further washed with 70% ethanol, dissolved in water, quantitated spectrophotometrically, and examined by agarose-formaldehyde denaturing

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