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

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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 (m₃^{2,2,7}GpppN) (TMG) that replaces the typical eukaryotic monomethylguanosine (m⁷GpppN)(m⁷G) cap. Both trans-spliced (m₃^{2,2,7}GpppN-SL-RNA) and not trans-spliced (m⁷GpppN-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 m₃^{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 m⁷G-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 m⁷G-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 (Sarco-

mastigophora), 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 (m⁷G-ppp-N⁶,N⁶,2'-O-trimethyladenosine*p*-2'-O-methyladenosine*p*-2'-O-methyl-uridine) to the RNAs [19]. Polycistronic transcripts are also pro-

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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 (\sim 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 mRNAs coexist in these organisms: (1) non-trans-spliced mRNAs with the m⁷GpppN 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 transspliced elements to an RNA does not significantly enhance the translation or stability of the mRNAs compared with non-transspliced 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^7 GpppG, 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 gel electrophoresis. Determination of capping efficiency and cap orientation on transcripts was carried out as described [37]. These analyses indicated that similar capping efficiencies were obtained for m⁷GpppG and m^{2,2,7}GpppG caps and that 90% of m⁷GpppG and 80% of m^{2,2,7}GpppG caps were added in the correct orientation. These analyses demonstrated that differences in translation or stability observed were not a function of differences in capping efficiency or orientation on the transcripts.

2.4. Luciferase assays

Following introduction of the RNA by particle bombardment, embryos were incubated in 35-mm Petri dishes and processed as previously described using Promega Renilla, Luciferase, or Dual Assay Systems with a Sirius Luminometer (Berthold Detection Systems, Germany) [39]. Although experimental data were similar with or without normalization to protein and RNA concentrations in the samples as previously described [39], in a number of experiments normalization was carried out by cotransfection of a firefly reporter RNA with either Renilla or Gaussia RNA and the use of the Dual-Luciferase assay.

2.5. Kinetic determination of RNA translation efficiency and functional half-life

The kinetics of luciferase activity over time in the embryos was used to determine both in vivo mRNA translational efficiency and functional half-life [42]. Translational efficiency was determined from the slope of the rising curve derived from luciferase activity over time using the equation: $Y = Y_{\text{max}} \times (1 - \exp[-K \times X])$. Functional half-life, or the length of time over which the mRNA is actively engaged in translation, was determined and defined as the time required following mRNA delivery to reach 50% of the final level of protein produced and calculated using the equation $t_{1/2} = 0.69/K$. Functional half-life measures the length of over time in which the mRNA is actively engaged in translation. In comparison, physical half-life only measures the physical longevity of the RNA without regard to translational competence and may not readily reflect changes in mRNA poly(A) tail length or mRNA cap removal which play important roles in mRNA translation.

2.6. Analysis of physical RNA half-life

RNA transcripts were biolistically introduced into Ascaris embryos and total RNA isolated from the embryos over time using Trizol [39]. For PCR analyses of transfected RNAs in the embryos, cDNA was synthesized using random hexamers and Superscript III as recommended by the manufacturer (Invitrogen) and semi-quantitative PCR carried out as described (9) or by Real-Time PCR using SYBR Green and a Roche LightCycler 480. For analysis of the decay of ³²P-labeled transcripts, ³²P-GTP labeled transcripts were synthesized using Promega's Riboprobe in vitro transcription system. Decay of labeled RNAs introduced into embryos was analyzed and quantified by phosphorimager analysis of denaturing gel electrophoresis separations using Molecular Dynamics STORM 860 and Image-Quant software.

3. Results

3.1. Nematode translation is highly m^7G cap-dependent

Three different luciferase reporters (Firefly, Renilla, and Gaussia) were used to evaluate the contribution of the TMG cap and SL to RNA translation and stability in vivo. Reporter RNAs were synthesized in vitro with different caps (or no cap), 5' UTRs, luciferase reporter open reading frames, 3' UTRs, and poly(A) tails (or no tail) (see Fig. 1 in Supplementary data for reporter characteristics). These RNAs were then biolistically introduced into \sim 800,000, 32–64 cell Ascaris embryos and the embryos assayed for luciferase activity (Davis et al. [39]). The relative activities, time courses and dose responses were examined for all three reporters (Fig. 1; Fig. 2 in Supplementary data; some data not shown). All three reporters demonstrated a linear dose response and were used at the lower end of the linear range (2.5 µg Firefly; 1 µg Renilla; 0.5 µg Gaussia). Maximal luciferase activity following introduction of the reporter RNA was typically reached at 60 min (Gaussia) or 90 min (Firefly and Renilla).

To provide a framework for the cap-dependence of nematode translation, we initially compared the translation of uncapped (pppG), GpppG-, and m⁷GpppG-capped RNAs. Luciferase activity derived from uncapped (pppG) RNAs was not detectable whereas significant levels of luciferase activity were derived from m⁷GpppG-capped RNAs (Fig. 2A; Fig. 3 in Supplementary data). Translation of GpppG-capped RNAs was also observed (Fig. 3 in Supplementary data). However, GpppG-capped RNAs introduced into Ascaris cellfree extracts or embryos are rapidly N7-methylated [37] (data not shown). Addition of the methyl donor inhibitor SAH (*S*-adenosyl-homocysteine) prevents N7-methylation and eliminates translation of GpppG-capped RNA in the cell-free extracts. Overall, these data indicate that the GpppG-capped RNAs are not the direct substrates for translation.

In vivo methylation of GpppG-capped RNAs precluded our use of this cap as a baseline for guanine methylation-dependent translation. As an alternative, we examined ApppG-capped reporter RNAs, reasoning that the 5' end of the mRNA would likely be more stable than pppG, as its 5'-ppp-5' linkage would protect the 5' end of the mRNA from degradation (Fig. 2A). Translation of ApppG capped RNAs was detectable at a low level and therefore we used this capped RNA as a baseline for comparisons with other methylated, guanosine capped RNAs in subsequent experiments (Fig. 2B–D).

3.2. TMG cap does not support translation as efficiently as an m^7 GpppG cap

We next examined the individual contributions of N7- and N2-methylation of the guanosine cap to translation. Nematode embryo translation is highly m⁷G-cap-dependent, as illustrated by the comparison between identical RNAs that differ only in



Fig. 1. Luciferase RNA reporter dose response, activity over time, and comparison of reporter activities in Ascaris embryos. (A) Renilla (Ren) RNA reporter dose response. Varying amounts of the illustrated RNAs (0.25-5 µg) were precipitated onto gold particles, the particles introduced into 32-64 cell Ascaris embryos, the embryos incubated at 30C for 90 min, and the embryos collected and assayed for luciferase activity. (B) Renilla luciferase activity over time following introduction into Ascaris embryos. In A and B, the 30 nt 5' UTR for m⁷G-Ren is GGUCAUUCCGGUACUGUUAGGCUAGCCACCAUG and the 33 nt 5' UTR for m^{2,2,7}G-SL-Ren is GGUUUAAUUACCCAAGU-UUGAGGGCUAGCCACCAUG, where bold type is the SL sequence, plain type Renilla sequence, the underlined ACC the -1 to -3 AUG context, and the underlined and bold type the initiator methionine AUG. (C) Comparison of relative reporter luciferase activity after 90 min following introduction of 1 µg of reporter RNA. The reporter RNAs contained an m⁷GpppG cap and 75-85 nt poly(A) tail and are described in Fig. 1 in Supplementary data. All data illustrate representative experiments and show the mean and standard error derived from triplicate transfections for A and C and the mean for triplicate transfections in B.

an ApppG-cap versus m^7 GpppG-cap (Fig. 2A and B; Table 1). Overall, translation of TMG-capped RNA (2 additional methyl groups at N2) was 2–3 fold lower than m^7 G-capped RNA. The m^7 GpppG-cap dependence and lower overall translation of TMG-capped RNA were observed with all three luciferase reporters and different 5' and 3' UTRs (see Fig. 3A and B; Fig. 3 in Supplementary data; data not shown).

3.3. Poly(A) tail synergizes with methylated cap to enhance translation

Addition of a 85 nt poly(A) tail greatly increased translation of either m⁷GpppG- or m₃^{2,2,7}GpppG-capped RNAs, whereas the tail had only a small effect on ApppG-capped RNA (Fig. 2B; Table 1). The effect of the methylated, guanosine cap and poly(A) tail together on translation are synergistic (Table 2). Interestingly, this synergistic effect is greater for RNAs that have both the TMG cap and SL (the trans-spliced elements) compared to m⁷GpppG-capped RNAs.

3.4. SL facilitates translation of TMG-capped RNA

In order to determine the effect of the SL sequence itself on translation in vivo, we substituted the 5' terminal 22 nt of the Renilla 5' UTR with the SL sequence. The effect of this substitution was an $\sim 50\%$ decrease in overall translation of a m⁷GpppGcapped RNA (Fig. 2C; Tables 1 and 2). However, translation of $m_3^{2,2,7}$ GpppG-capped RNAs increased ~2-fold when the spliced leader was in the 5' UTR. This 2-fold enhancement requires a poly(A) tail, as the same SL substitution in the 5' UTR of a non-polyadenylated, m₃^{2,2,7}GpppG-capped RNA had little effect on translation (Compare Fig. 2C and D; Tables 1 and 2). These data suggest the spliced leader has a positive, synergistic effect on the translation of TMG-capped RNAs, whereas when it is substituted into the 5' UTR of m⁷GpppG-capped RNAs, the SL has a negative effect on translation (Fig. 2D; Tables 1 and 2). Importantly, optimal translation of TMG-capped RNAs requires a downstream SL and a poly(A) tail. The data also indicate that the SL by itself does not act as a cap-independent enhancer of translation (Fig. 2C and D). Similar results were observed using Firefly reporter RNAs (data not shown).

3.5. Sequences between the SL and AUG in recipient mRNAs do not enhance translation of TMG-SL RNAs

The 22 nt nematode SL is typically added 0–10 nucleotides upstream of the AUG in the recipient RNA [37]. To further examine the contribution of the SL and other downstream sequences between the SL and AUG in the 5' UTR on translation, we evaluated native Ascaris 5' UTRs of three trans-spliced mRNAs. RNA transfection efficiencies in these experiments were normalized by co-transfection of a control Firefly luciferase RNA. Consistent with the results in Fig. 2C and D, overall translation of TMG-capped reporter RNAs is considerably less compared to identical RNAs with an m⁷G-cap unless the SL sequence is present at the 5' end (Fig. 3A and B). Furthermore, sequences located between the SL and the initiation codon in native transspliced 5' UTRs (SL-cDNA 12 and SL-cDNA 4) do not appear to play a large role in influencing translation of TMG-capped RNAs as illustrated by the comparison of the level of translation of the SL alone compared to SL-cDNA 12 or SL-cDNA 4 (Fig. 3A).



Fig. 2. Translation in Ascaris embryos is methyl-guanosine cap-dependent, enhanced by a poly(A) tail, and both a spliced leader and poly(A) tail are required for efficient translation of TMG-capped RNAs. (A) Methyl-guanosine cap dependence of translation. The reporter RNA was Gaussia (Sh Gluc)(5' UTR = GGUCAUUCCGGUACUGUUAGGCUAGCCACCAUG) with a 75 nt poly(A) tail (see Fig. 1 in Supplementary data). (B) Poly(A) tail enhances translation of methyl-guanosine capped RNAs. The reporter RNA was Ren (5' UTR = GGUCAUUCCGGUACUGUUAGGCUAGCCACCAUG) (see Fig. 1 in Supplementary data) with and without an 85 nt poly(A) tail. (C) Effect of spliced leader on translation of different capped RNAs. The reporter RNAs were Ren (no SL) or SL Ren (+ SL) without a poly(A) tail (see Fig. 1 in Supplementary data). (D) Effect of spliced leader and poly(A) tail on translation of different capped RNAs. The reporter RNAs were Ren (no SL) (5' UTR = GGUCAUUCCGGUACUGUUAGGCUAGCCACCAUG) or SL Ren (+SL) (5' UTR = GGUUUAAUUACCCAAGUUUGAGGCUAGCCACCAUG) with an 85 nt poly(A) tail (see Fig. 1 in Supplementary data). (D) Effect of spliced leader and poly(A) tail on translation of different capped RNAs. The reporter RNAs were Ren (no SL) (5' UTR = GGUCAUUCCGGUACUGUUAGGCUAGCCACCAUG) or SL Ren (+SL) (5' UTR = GGUUUAAUUACCCAAGUUUGAGGCUAGCCACCAUG) with an 85 nt poly(A) tail (see Fig. 1 in Supplementary data). Gaussia (A) or Renilla (B–D) reporter RNAs were biolistically introduced into Ascaris embryos and the luciferase activity measured after 90 min. Data illustrate representative experiments and show the mean and standard error derived from triplicate transfections. The Gaussia activity was normalized to a co-transfected Firefly RNA using the Dual-Luciferase assay.

To more closely examine the specificity of SL enhancement of the translation of TMG-capped RNAs, we compared the effect of the SL with non-trans-spliced 5' UTRs (L37, Lg, and Sh) (Fig. 3B) and random sequences (N₁₈-GAG and N₂₁-ACC) (Fig. 3C) on the translation of TMG-capped RNA. Enhanced translation of TMG-capped RNA is observed only in the presence of the SL in the 5' UTR. Overall, these data and those using three different luciferase reporter RNA 5' UTRs (Firefly, Renilla, and Gaussia) demonstrate that the SL enhancement of TMG-capped RNA translation is a specific property of the SL sequence.

3.6. 5' UTR length and context affect translation

Overall translation for several reporter RNAs is also affected by the length of the 5' UTR as illustrated by a comparison of the two Gaussia 5' UTRs (Sh versus Lg = Short versus Long) (Fig. 3B) and random sequence 5' UTRs (data not shown). Reporters with shorter 5' UTRs exhibited higher overall levels of translation compared to those with longer 5' UTRs. The SL sequence immediately adjacent to an initiation codon (see SL in Fig. 3B and C) constitutes a relatively efficient 5' UTR and initiation context for translation when directly compared to a Gaussia 5' UTR of similar length (Sh). By comparison, the first 22 nts of the Firefly and Renilla 5' UTRs provide a more efficient translation context in our experiments, as substitution of the SL for the first 22 nts of these 5' UTRs led to a reduction in overall translation (Figs. 2C and D and 3A). Thus, a Gaussia 5' UTR of the same length as the SL is only $\sim 2/3$ as efficient in translation when the two are directly compared. The lower level of translation of the Gaussia 5' UTR is likely due in part to a combination of both its sequence and the -1 to -3 AUG context

	Renilla Luciferase Activity (RLU/sec)	Effect of Methylated Capª	Effect of Poly(A)- Tail ^b	Effect of Methylated Cap AND Poly(A)-tail ^c	Effect of Spliced Leader ^d	Effect of Methylated Cap AND Spliced Leader ^e	Effect of Spliced Leader AND Poly(A)-tail ^f
ApppG-Ren	3,428 <u>+</u> 47	1	1	1	1	1	1
m ⁷ GpppG-Ren m ^{2.2,7} GpppG-Ren	55,578 <u>+</u> 2,993 30,799 + 850	16 9	1		1 1		1 1
ApppG-Ren-A ₈₅	6,945 <u>+</u> 247	1	2		1	1	·
m ⁷ GpppG-Ren-A ₈₅ m ^{2,2,7} GpppG-Ren-A ₈₅	404,639 <u>+</u> 22,762 137,325 <u>+</u> 9,638	58 20	7 4	118 40	1 1		
ApppG-SL-Ren	3417 <u>+</u> 231	1	1	1	1	1	
m ⁷ GpppG-SL-Ren m ^{2.2.7} GpppG-SL-Ren	31,450 <u>+</u> 3,712 28,581 <u>+</u> 2,223	9 8	1 1		0.6 0.9	9 8	
ApppG-SL-Ren-A ₈₅	4,881 <u>+</u> 527	1	1		0.7	1	1
m ⁷ GpppG-SL-Ren-A ₈₅	203,995 <u>+</u> 8,797	42	6	60	0.5	29 40	3.7
m GpppG-SL-Ren-A ₈₅	202,004 ± 10,003	58	10	83	∠.1	40	9.2

Table 1

Effect of cap, poly(A)-tail, and spliced leader sequence on translation of Renilla RNAs in Ascaris embryos

The illustrated RNAs were biolistically introduced into Ascaris embryos and the luciferase activity measured after 2 h. Relative light units/s represent the mean and standard error of triplicate transfections from the experiments illustrated in Fig. 2. Bold numbers within a column represent the level of increase over a baseline RNA shown as the number 1.

^aEffect of methylated guanosine cap on different RNAs (baseline is the same RNA with an ApppG cap).

^bEffect of poly(A)-tail on different capped RNAs (baseline is the same RNA without a tail).

^cEffect of methylated guanosine cap and poly(A)-tail on different RNAs (baseline is an ApppG capped RNA without a tail).

^dEffect of spliced leader on different RNAs (baseline is the same RNA without the SL).

^eEffect of methylated cap and spliced leader on different RNAs (baseline is an ApppG capped RNA with same 3' end).

^fEffect of spliced leader and poly(A)-tail on different capped RNAs (baseline is same RNA without the SL or poly(A)-tail).

difference (SL = GAG; Gaussia = ACC). As shown in Fig. 3B and C (compare SL ACC, SL, GN_{21} -ACC, and GN_{18} -GAG), the SL GAG provides a more efficient -1 to -3 AUG context than ACC. In addition, the GAG context may contribute to the synergistic interaction between the SL and TMG cap. Thus, our comparisons of several different 5' UTRs to the SL and its context demonstrate that, as observed in other systems, the length and sequence of the 5' UTR contribute to translation efficiency in nematodes. However, in general the SL is not a significantly better component of the 5' UTR than those sequences to which it was compared in the current study.

3.7. Native 3' UTRs of trans-spliced mRNAs do not enhance translation of TMG-SL RNA

In experiments described above, the overall luciferase reporter translation in the context of the TMG cap and SL sequence was similar when different luciferase 3' UTRs were

Table 2

Ascaris embryo translation in vivo exhibits synergistic effects between the cap, poly(A)-tail, and spliced leader sequence

	Synergistic Effect of Cap and Tailª		Synergistic Effect of Cap and SL [♭]		Synergistic Effect of SL and Tail ^e
ApppG-Ren m ⁷ GpppG-Ren ApppG-Ren-A ₈₅ m ⁷ GpppG-Ren-A ₈₅	6	ApppG-Ren-A ₈₅ m ⁷ GpppG-Ren-A ₈₅ ApppG-SL-Ren-A ₈₅ m ⁷ GpppG-SL-Ren-A ₈₅	0.5	m ⁷ GpppG-Ren m ⁷ GpppG-SL-Ren m ⁷ GpppG-Ren-A ₈₅ m ⁷ GpppG-SL-Ren-A ₈₅	0.5
ApppG-SL-Ren m ^{2.2,7} GpppG-SL-Ren ApppG-SL-Ren-A ₈₅ m ^{2.2,7} GpppG-SL-Ren-A ₈₅	9	ApppG-Ren-A ₈₅ m ^{22.7} GpppG-Ren-A ₈₅ ApppG-SL-Ren-A ₈₅ m ^{22.7} GpppG-SL-Ren-A ₈₅	2	m ^{22.7} GpppG-Ren m ^{22.7} GpppG-SL-Ren m ^{22.7} GpppG-Ren-A ₈₅ m ^{22.7} GpppG-SL-Ren-A ₈₅	1.8

Data derived from Table 1 were used to calculate the synergistic effects as illustrated in Tables 1–3 in Supplementary data and the results summarized here. The set of RNAs evaluated to determine the synergism are illustrated.

^aEffect of methylated cap and tail/(effect of methylated cap + effect of A-tail) from Table 1 and Table 1 in Supplementary data.

^bEffect of methylated cap and SL/(effect of methylated cap + effect of SL) from Table 1 and Table 2 in Supplementary data.

^cEffect of SL and tail/(effect of SL + effect of A-tail) from Table 1 and Table 3 in Supplementary data.



Fig. 3. Contribution of m^7 GpppG vs. $m^{2.2.7}$ GpppG cap, SL, and different 5' and 3' UTRs to translation in Ascaris embryos. The illustrated RNAs (see Fig. 1 in Supplementary data for additional information on the RNAs) and a Firefly control reporter RNA were biolistically co-transfected into 32–64 cell Ascaris embryos and luciferase activity measured after 60–90 min using a Dual-Luciferase assay. Activity was normalized to the co-transfected Firefly RNA activity. Data illustrate representative experiments and show the mean and standard error derived from a minimum of triplicate transfections. (A) Comparison of Renilla, SL-Renilla and native trans-spliced mRNA 5' UTRs on the translation of a Renilla RNA reporter. Normalized luciferase activity derived from the m^7 GpppG-Ren RNA was set at 100%. (B) Effect of 5' UTR length and native sequence on the translation of a Gaussia RNA reporter. Normalized luciferase activity derived from the m^7 GpppG-Lg Gaussia RNA was set at 100%. (C) Effect of a Gaussia, SL, and random nucleotide 5' UTRs and their initiation context on translation of a Gaussia RNA reporter. (D) Effect of the native trans-spliced mRNA 5' UTRs and their homologous 3' UTRs on the translation of a Gaussia RNA reporter. Note that values represent the percentage of translation obtained the identical RNA except with an m^7 GpppG cap. The sequences for the 5' UTRs are illustrated in A–C and the 3' UTR sequences are in Fig. 1 in Supplementary data. Sequences in the 5' UTR are designated orange for the SL sequence, grey for Renilla sequence, the -1 to -3 AUG context is underlined, and green illustrates the AUG initiator methionine.

present (Firefly, Renilla, and Gaussia 3' UTRs). To determine whether the 3' UTR of a trans-spliced mRNA contributes to the translation of TMG-SL mRNAs, we examined reporter RNAs with both the native 5' and 3' UTRs of two trans-spliced Ascaris mRNAs (cDNA 12, encoding vacuolar H+ ATPase 16 kDa proteolipid subunit, and cDNA 4, encoding ribosomal protein L23) (Fig. 3D). Reporter RNA activity was not significantly affected when both the native 5' and 3' UTR of these trans-spliced messages were present compared to only the trans-spliced 5' UTR. Similar data were obtained with a Renilla and Firefly reporter (data not shown). These data demonstrate, that in the context of a luciferase reporter, the native 3' UTRs did not cooperate with the SL or directly contribute to the in vivo translation of trans-spliced mRNAs.

Table 3	
Poly(A)-tail affects both translation and stability in Ascaris embryos	s

RNA	Translational efficiency (RLU/s/min)	Relative rate of translation	Functional half-life (min)	Relative mRNA stability	Maximum accumulation (RLU/s)	Relative level of expression
m ^{2,2,7} GpppG-SL-Ren-A ₀	341	1.0	19	1.0	17,487	1.0
m ^{2,2,7} GpppG-SL-Ren-A ₃₀	998	2.9	39	2.1	65,360	3.7
m ^{2,2,7} GpppG-SL-Ren-A ₈₅	1291	3.8	57	3.0	120,630	6.9

Data from the time course illustrated in Fig. 5 in Supplementary data were used to calculate translation efficiency (relative luciferase light units/s/min), functional half-life (min), and maximum accumulation of luciferase (relative luciferase light units/s) as described in Section 2. Note that increasing poly(A)-tail length on the $m_3^{2,2,7}$ GpppG-SL-Renilla RNA effects both the translation efficiency and half-life which together contribute to the overall level of relative expression. Similar results were obtained with an m⁷GpppG-Renilla RNA and Firefly RNAs. Data illustrate the mean of triplicate transfections.

3.8. TMG and SL do not play a differential role in 1–2 cell embryos

In the above studies, we examined the role of the TMGcap and SL on translation in 32–64 cell embryos. To determine whether translation of trans-spliced mRNAs was the same in earlier embryos and/or in a discrete subset of embryo cells, we examined the translation of several mRNAs in 1–2 cell embryos. Translation of RNAs with TMG-cap alone compared to RNAs with an m⁷GpppG-cap was even lower in early embryos. Overall, similar data were obtained for 1–2 cell embryos as observed for the 32–64 cell embryos described above (Fig. 4 in Supplementary data).

3.9. TMG cap and SL together do not differentially affect RNA stability

The luciferase activity data presented above represent the sum of both RNA translation efficiency and stability. To differentiate the contributions of the TMG cap and SL on translation efficiency and functional RNA half-life, the kinetics of luciferase activity over time following RNA introduction into the embryos were determined (see Section 2) [42]. This methodology facilitates analysis of the independent contributions of RNA elements to translation and stability. This point is illustrated for three different poly(A) tail lengths in Table 3 and Fig. 5 in Supplementary data. Increasing poly(A) tail length acts to enhance translation and to increase functional RNA half-life. Thus, the increased overall level of translation as a function of poly(A) tail length is a function of both an increase in mRNA translation efficiency and functional RNA half-life.

As illustrated in previous experiments (Fig. 2B), the SL or TMG cap added to a Renilla test RNA results in a reduction in translational efficiency (Table 4). The SL sequence substituted for sequences in the 5' UTR of a Renilla test RNA causes a small decrease in mRNA stability. The reduction in both translation and stability by substitution of the SL by itself leads to an overall decrease in relative luciferase expression. Substitution of the m⁷G-cap with a TMG cap results in a significant decrease in translation efficiency, but does not affect functional mRNA half-life. Importantly, as shown in Table 4, the overall translational efficiencies and mRNA stabilities of non-trans-spliced (m⁷GpppG-Ren-A₈₅) and trans-spliced test mRNAs (m^{2,2,7}GpppG-SL-Ren-A₈₅) are similar. Similar data were obtained with a Firefly reporter RNA and the functional half-lives of Gaussia reporter RNAs were not different (data not shown). In summary, the (1) luciferase activity data in previous sections are largely a function of overall translation efficiency and (2) RNAs that have both the TMG-cap and SL (representing trans-spliced RNA) do not have significantly different functional half-lives compared to similar RNAs with the m⁷GpppG cap (representing non-trans-spliced RNA).

To further compare the half-lives of these mRNAs, we also examined the physical stability of m⁷G-capped (non-trans-spliced) and TMG-capped SL (representing trans-spliced) reporter RNAs in Ascaris embryos using PCR (semi-quantitative PCR and real-time PCR of Renilla and Gaussia RNAs) and the decay of ³²P-labeled Gaussia transcripts. The decay slopes for the two RNAs were similar (Fig. 4 and data not shown). As shown in Fig. 4, levels of RNA drop dramatically within the first 10 min following RNA transfection. Following biolistic introduction of the RNAs, media added to the embryos contained RNase A to limit evaluation of RNA present in the media or

Table 4

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RNA	Translational efficiency (RLU/s/min)	Relative rate of translation	Functional half-life (min)	Relative mRNA stability	Maximum accumulation (RLU/s)	Relative level of expression
m ⁷ GpppG-Ren-A ₈₅	979	1.0	56	1.0	158,479	1.0
m ⁷ GpppG-SL-Ren-A ₈₅	820	0.8	43	0.8	62,420	0.4
m ^{2,2,7} GpppG-Ren-A ₈₅	396	0.4	57	1.0	49,036	0.3
m ^{2,2,7} GpppG-SL-Ren-A ₈₅	850	0.9	54	1.0	137,175	0.9

The illustrated RNAs were introduced into Ascaris embryos and samples of embryos collected at time intervals and assayed for luciferase activity. The time course of luciferase activity was used to calculate translation efficiency (relative luciferase light units/s/min), functional half-life (min), and maximum accumulation of luciferase (relative luciferase light units/s) as described in Section 2. Data illustrate a representative experiment and represent the mean of triplicate transfections.



Fig. 4. Physical half-life of trans-spliced vs. non-trans-spliced test mRNA in Ascaris embryos. (A) Decay of labeled Gaussia luciferase RNAs in embryos. ³²P-labeled RNAs were introduced into embryos (see Fig. 1 in Supplementary data for additional RNA information; orange shaded sequence is the SL), RNA extracted at intervals, the RNA resolved by electrophoresis, and visualized by phosphoimager analysis. (B) Decay curves of ³²P-labeled Gaussia RNAs in 'A'. RNA levels illustrated in 'A' were quantitated by phosphoimager analysis and plotted using levels observed at time zero as 100%. Similar data were obtained using transfection of unlabeled RNAs and analysis by real-time PCR (data not shown).

adhering to the surface of the embryos from our analysis. We attribute the dramatic drop in RNA in the first 10 min to the decay or degradation of RNAs that are not incorporated into Ascaris embryo cells. Similar data were obtained using real-time PCR (data not shown). In addition, differences in the half-lives of RNAs determined using the kinetics of Firefly luciferase in Table 4 and Gaussia physical half-life in Fig. 4 are attributable to different luciferase RNAs (Renilla in Table 4 and Gaussia in Fig. 4) and the methods used to measure RNA half-lives. The important observation derived from these experiments is that trans-spliced and non-trans-spliced RNAs have similar half-lives in vivo demonstrating that the mRNA stabilities of trans-spliced and non-trans-spliced reporter RNAs are similar.

Real-time PCR analysis of luciferase RNAs over time following transfection enabled us to normalize luciferase activity to RNA levels in the embryos. These analyses (data not shown) led to similar data as those illustrated in Figs. 2 and 3 and Tables 1 and 4. Thus, overall translation differences observed were not a function of different levels of RNA introduced or present in the embryos.

4. Discussion

Spliced leader trans-splicing adds a trimethylguanosine cap and conserved 5' terminal sequence to metazoan mRNAs. Given the importance of the cap and 5' UTR on mRNA translation, it has been proposed that spliced leader addition might function to enhance translation of recipient mRNAs. In the current in vivo study, we determined whether the TMG cap and SL acquired by SL trans-splicing differentially affected RNA translation and stability compared to m⁷GpppG-capped RNAs with several different 5' UTRs. We also examined the contributions of different caps (ApppG, m⁷GpppG, and m^{2,2,7}GpppG), the SL, trans-spliced RNA sequences between the SL and AUG, transspliced mRNA 3' UTRs, and a poly(A) tail on in vivo mRNA translation in Ascaris embryos. Uncapped or ApppG-capped RNAs are very poorly translated (Fig. 2A; Fig. 3 in Supplementary data). As observed for other eukaryotes, nematode mRNA translation in vivo is highly m⁷G-cap dependent (Fig. 2; Table 1; Fig. 3 in Supplementary data), the cap synergizes with the poly(A) tail to enhance translation (Fig. 2; Tables 1 and 2), and the poly(A) tail is also a determinant of mRNA stability (Table 3). The TMG cap by itself does not support translation as well as an m⁷G-cap even though it is present on the majority of nematode mRNAs (Figs. 2 and 3; Tables 1 and 4). However, when the TMG cap and SL are present together as is the case for trans-spliced RNAs, the SL synergistically enhances translation of TMG-capped, polyadenylated RNA (Figs. 2 and 3; Fig. 4 in Supplementary data; Tables 1 and 4). These in vivo studies extend previous studies carried out using Ascaris embryo cell-free systems [37,38]. In addition, our in vivo studies demonstrate that several key characteristics of nematode translation are faithfully reproduced in the Ascaris cell-free system. These studies demonstrate the nematode translational machinery has evolved to translate the m3^{2,2,7}GpppG cap in the context of the SL, and both these trans-spliced elements are required to promote efficient translation of a recipient polyadenylated RNA.

In nematode cells, as well as those of other trans-splicing metazoa, trans-spliced and non-trans-spliced mRNAs are both present. Our in vivo data suggest the TMG cap and SL sequence play a similar role in mRNA translation and stability as the m⁷GpppG cap and 5' UTR of non-trans-spliced mRNAs. On average, RNAs with the TMG-cap and SL are translated at similar levels as non-trans-spliced m⁷G-capped RNAs (Figs. 2 and 3). Thus, the trans-spliced elements themselves do not appear to confer any particular translational advantage to RNAs. However, both $m_3^{2,2,7}$ GpppG-cap and the spliced leader sequence must be present together to obtain translational efficiencies similar to those observed for m⁷GpppG-capped RNAs (Figs. 2 and 3). Our data further suggest that RNAs with the TMG-cap and SL do not have different functional or physical half-lives compared to non-trans-spliced m⁷G-capped RNAs (Table 4; Fig. 4). Translation of mRNAs with the TMG-cap and SL is more dependent on the poly(A) tail (Fig. 2C and D; Tables 1 and 2), and consequently, trans-spliced RNAs may be more subject to translational regulation by poly(A) tail length alterations.

The effect on translation of the SL in the 5' UTR was examined in a variety of contexts and compared to the 5' UTRs from Firefly, Renilla, Gaussia, and a native, non-trans-spliced Ascaris RNA (Figs. 2 and 3). The SL was less efficient in translation than the first 22 nts of the Firefly and Renilla 5' UTRs, whereas the SL was equal to or 30–50% more efficient than a Gaussia (Sh) or non-trans-spliced native Ascaris 5' UTR (L37) when the SL is placed immediately adjacent to the reporter AUG. The greater efficiency for the SL with the Gaussia reporter is attributable in part to 5' UTR length (shorter 5' UTRs are more efficient for Gaussia reporters and the L37 is longer) and to the different -1to -3 AUG sequence. The -1 to -3 GAG of the SL is more efficient than the ACC present in several RNAs (see Fig. 3B and C). Our interpretation of these data is that the spliced leader contributes a reasonably efficient 5' UTR and initiation context for translation of mRNAs. Our data also suggest that the spliced leader sequence by itself does not enhance cap-independent translation. The SL therefore does not likely enhance translation by directly interacting with components of the translation machinery other than eIF4E binding to the cap [37].

Previous studies using lethal *C. elegans* mutants where the entire SL1 locus is deleted indicated that the reintroduction of mutant SLs could rescue lethality whereas several mutant SLs could not [26,27]. The mutant SLs that could not rescue lethality may affect the synergistic interaction between the TMG cap and SL resulting in lower levels of trans-spliced RNA translation thus leading to lethality.

4.1. In vitro versus in vivo analyses

Previously, Maroney et al. [38] noted that the $m_3^{2,2,7}$ GpppGcap and spliced leader sequence interact cooperatively to increase translational efficiency in a cell-free system. We recently further characterized this interaction and demonstrated it was synergistic using a modified, competitive Ascaris cell-free translation system [37]. We have shown here that this interaction is physiologically relevant in vivo, that the synergism is dependent on the presence of a poly(A) tail, and that sequences between the SL and AUG and in the 3' UTR of the trans-spliced mRNAs examined do not significantly contribute to translation of mRNAs with a TMG-cap and SL. Maroney et al. also observed that translation of the native trans-spliced vacuolar H+ ATPase mRNA (cDNA 12) with an m₃^{2,2,7}GpppG-cap, SL, and poly(A) tail was \sim 2.5-fold more efficient than the same RNA with an m⁷GpppG-cap and no spliced leader sequence in the 5' UTR. Both our current in vivo and previous in vitro data [37] differ from the data of Maroney et al. We did not observe a large enhancement in translation from addition of a m₃^{2,2,7}GpppG cap and spliced leader to several luciferase reporters. Our experiments were also carried out with the complete native 5' and 3' UTRs of the same trans-spliced RNA (cDNA 12) studied by Maroney et al. on a luciferase reporter. Similarly, addition of the complete native 5' and 3' UTRs of a second trans-spliced cDNA (cDNA 4) to a reporter RNA did not enhance translation compared to a non-trans-spliced reporter RNA (m⁷GpppG capped luciferase with various 5' UTRs). The RNAs examined in vivo in the current study were also evaluated in our cell-free system [37], and produced results similar to that observed in vivo (data not shown). The simplest explanations for the differences observed are: (1) the different translation conditions present in vivo and in our modified cell-free system [37] and (2) possible elements within the open reading frame of vacuolar ATP synthase 16 kDa proteolipid subunit RNA (cDNA 12) that contribute to translation [38].

4.2. Function of trans-splicing

The TMG cap and in some contexts the SL have negative effects on translation, while together they function synergistically to support translation. We interpret this result to suggest that the nematode translation machinery evolved to translate mRNAs with the TMG cap specifically in the context of the SL. If the TMG cap and SL do not confer any discrete properties to their recipient mRNAs such as enhanced translation or stability, why then is there SL trans-splicing of mRNAs that are not derived from polycistronic RNAs? One possible answer might be related to the primary transcripts of trans-spliced genes. We have recently shown that the spliced leader is typically added to nematode mRNAs at a relatively conserved distance from the mRNA AUG (usually less than 10 nucleotides). Notably, this conserved distance coincides with the optimal spacing for translation in a cell-free Ascaris system [37]. Thus, spliced leader addition in nematodes may serve to improve the translation initiation context for the primary transcripts of trans-spliced genes. The majority of trans-spliced test RNAs examined in the current study have this optimal spacing of SL to AUG. What function does trans-splicing serve in creating this spacing or improving the initiation context? If the initiation sites for primary transcripts of trans-spliced genes were heterogeneous, this might result in mRNAs with heterogeneous 5' ends that could have upstream AUGs or short open reading frames in the 5' UTR. These mRNAs would likely be poorly translated. SL addition might then function to trim these mRNAs to a uniform length

that would be more optimal for translation initiation [37]. Alternatively, since trans-splicing trims a pre-mRNA to generate the mature 5' UTR, there would be no selection pressure to eliminate mutations that could lead to upstream AUGs or open reading frames that would be deleterious to translation. Thus, once spliced leader trans-splicing is present it might need to be maintained to override deleterious effects of sequences in the 5' UTR of pre-mRNAs [43].

Another potential function for nematode SL trans-splicing, albeit perhaps less likely, might be that primary transcripts for these genes are not efficiently capped. At this point, there is no available information on the capping efficiency of primary transcripts for trans-spliced genes in nematodes. If capping efficiency were low, trans-splicing might serve a capping function as observed from some transcripts in the Kinetoplastida. Finally, it remains possible that the function(s) that SL trans-splicing originally served and/or evolved to address in nematodes other than processing of polycistronic transcripts is no longer required or needed. In this view, it could be that some trans-splicing remains, but no longer serves a discrete function.

4.3. SL and RNA decapping

We recently observed that addition of the SL sequence to test mRNAs leads to a \sim 10-fold reduction in nematode Dcp2 RNA decapping in vitro [44]. In addition, we previously observed that 5' to 3' RNA decay appears significantly less active than 3' to 5' RNA decay in an Ascaris embryo in vitro decay system [45]. In the current study, addition of the SL sequence to RNAs did not lead to significant increases in mRNA stability in vivo. One interpretation of these data is that Dcp2 decapping and 5' to 3' decay plays a minor role in general decay both in vivo and in vitro in nematode embryos. Dcp2 decapping of trans-spliced mRNAs might be a potential mechanism for regulated mRNA decay in nematodes.

4.4. Contribution of nuclear history

In the current studies, we introduced RNA directly into embryo cells bypassing the typical maturation of an mRNA in the nucleus and its export to the cytoplasm. This approach was necessary since it is currently not possible to design DNA constructs that can directly synthesize m3^{2,2,7}GpppG-capped mRNAs in vivo. Furthermore, the current strategy circumvents potential differences in promoter activity, splicing and polyadenylation (including tail length), and mRNA export that could compromise our ability to analyze the effect of individual RNA elements on translation and stability. However, we note that several lines of recent evidence suggests that the transcription and nuclear history of an mRNA can have a significant effect on splicing, export, and the cytoplasmic metabolism of the mRNA, including its translation [17,46–55]. Thus, it remains possible that during cellular maturation of trans-spliced mRNAs the m₃^{2,2,7}GpppG-cap and spliced leader may play a greater role in mRNA translation and stability than observed here. Our introduction of the reporter RNAs into a variety of cells in the 32-64 cell embryos and subsequent assay of luciferase activity provides an average of the translation and stability of the introduced RNAs in a variety of different cell types. We have observed similar results in 1-2 and 2-4 cell embryos (Fig. 4; data not shown). However, it remains possible that the m₃^{2,2,7}GpppG-cap and spliced leader could play a discrete role in mRNA translation and/or stability in a subset of embryo cells. Furthermore, the presence of a number of eIF4E isoforms (5) expressed in different tissues in some nematodes such as *C. elegans* may play a role in the regulation of translation of trans-spliced mRNAs [56–59].

5. Conclusion

Overall, trans-splicing addition of the $m_3^{2,2,7}$ GpppG-cap and SL sequence to mRNAs does not result in mRNAs with significantly enhanced translation or stability in vivo when compared to non-trans-spliced test RNAs. The $m_3^{2,2,7}$ GpppG-cap and spliced leader sequence function best together and synergistically interact to increase translation in the presence of a poly(A) tail. It remains to be determined whether the nuclear maturation and export of trans-spliced mRNAs enables the $m_3^{2,2,7}$ GpppG-cap and spliced leader sequence to differentially affect mRNA translation and stability or if these elements play differential roles in a subset of nematode cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.02.003.

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Supplementary Table 1. Ascaris embryo translation in vivo is dependent on methylated guanosine cap and poly(A)-tail and exhibits synergism between the cap and tail. The illustrated RNAs were biolistically introduced into Ascaris embryos and the luciferase activity measured after 2 hr. Relative light units/sec represent the mean of three replicates. Luciferase activity standard errors are provided in Table 1.

	Renilla Luciferase Activity (RLU/sec)	Effect of Methylated Cap on mRNA Translation	Eff poly(A ml Tran	ect of)-Tail on RNA Islation	Effect of Methylated Cap AND Poly(A)-Tail on mRNA Translation	Synergistic Effect Cap and Tail
ApppG-Ren	3,428	1	1		1	
m ⁷ GpppG-Ren	55,578	16 ^a		1		6 ^e
ApppG-Ren-A85	6,945	1	2 ^b			U
m ⁷ GpppG-Ren-A85	404,639	58		7 ^c	118 ^d	
ApppG-SLRen	3,417	1	1		1	
m ^{2,2,7} GpppG-SLRen	26,417	8		1		
ApppG-SLRen-A85	4,881	1	1			9
m ^{2,2,7} GpppG-SLRen-A85	288,798	59		11	85	

^a Comparison of Cap Effect on RNAs of similar tail length

^b Comparison of Tail Effect on ApppG capped RNAs

^c Comparison of Tail effect on methylated capped RNAs

^d Comparison of RNA with methylated cap and poly(A)-tail to RNA with ApppG cap and no tail

^e Synergistic effect = Effect of d/(Effect of a + Effect of b)

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Supplementary Table 2. *Ascaris* **embryo translation in vivo exhibitss synergism between the cap and SL.** The illustrated RNAs were biolistically introduced into Ascaris embryos and the luciferase activity measured after 2 hr. Relative light units/sec represent the mean of three replicates. Luciferase activity standard errors are provided in Table 1.

	Renilla Luciferase Activity (RLU/sec)	Effect of Methylated Cap on mRNA Translation	Eff Spliced on r Tran	ect of d Leader nRNA islation	Effect of Methylated Cap AND Spliced Leader on mRNA Translation	Synergistic Effect Cap and SL
ApppG-Ren-A85 m ⁷ GpppG-Ren-A85 ApppG-SLRen-A85 m ⁷ GpppG-SLRen-A85	6,945 404,639 4,881 203,995	1 58 ^{°a} 1 42	1 0.7 ^b	1 0.5 [°]	1 29 ^d	0.5 ^e
ApppG-Ren-A85 m ^{2,2,7} GpppG-Ren-A85 ApppG-SLRen-A85 m ^{2,2,7} GpppG-SLRen-A85	6,945 137,325 4,881 288,798	1 20 1 59	1 0.7	1 2.1	1 42	2.0

^a Comparison of Cap Effect on RNAs containing similar 5'UTRs (non-SL vs. SL)

^b Comparison of SL Effect on ApppG capped RNAs

^c Comparison of SL Effect on methylated capped RNAs

 $^{\rm d}$ Comparison of RNA with methylated cap and SL to RNA with ApppG cap and no SL

^e Synergistic effect = Effect of d/(Effect of a + Effect of b)

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Supplementary Table 3. *Ascaris* **embryo translation in vivo exhibits synergism between SL and poly(A)-tail.** The illustrated RNAs were biolistically introduced into Ascaris embryos and the luciferase activity measured after 2 hr. Relative light units/sec represent the mean of three replicates. Luciferase activity standard errors are provided in Table 1.

	Renilla Luciferase Activity (RLU/sec)	Effect of Spliced Leader on mRNA Translation	Eff Poly(on 1 Tran	ect of (A)-Tail nRNA uslation	Effect of Spliced LeaderAND Poly(A)-Tail on mRNA Translation	Synergistic Effect SL and poly(A)-tail
m ⁷ GpppG-Ren m ⁷ GpppG-SLRen m ⁷ pppG-Ren-A85 m ⁷ GpppG-SLRen-A85	55,578 35,127 404,639 203,995	1 0.6 ^a 1 0.5	1 7 ^b	1 6 ^c	1 4 ^d	0.5 ^e
m ^{2,2,7} GpppG-Ren m ^{2,2,7} GpppG-SLRen m ^{2,2,7} GpppG-Ren-A85 m ^{2,2,7} GpppG-SLRen-A85	30,799 26,417 137,325 288,798	1 0.9 1 2.1	1	1	1 9	1.8

^a Comparison of Spliced Leader Effect on RNAs of similar tail length

^b Comparison of Poly(A)-Tail Effect on non-SL RNAs

^c Comparison of Poly(A)-Tail Effect on SL RNAs

^d Comparison of RNA with SL and Poly(A)-tail to RNA without SL and no tail

^e Synergistic effect = Effect of d/(Effect of a + Effect of b)

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Suppl. Figure 1. Luciferase Reporters and their 5' and 3' UTRs. Length of open reading frames and the 5' and 3' UTRs of the luciferase reporters used in the current study. N represents equal representation of all 4 nucleotides in transcribed RNA 5' UTR derived from degenerate PCR primers used for template preparation. Poly(A) tails of known length were added to the last nucleotide of the illustrated 3' UTRs. Both short and long 3' UTRs derived from the Firefly and Renilla reporters were used which gave similar results (data not shown).

Suppl. Figure 2. Gaussia luciferase RNA reporter dose response and activity over time. A). Gaussia (Gluc) RNA reporter dose response. Varying amounts of the illustrated RNA (0.1 to 2 μg) were precipitated onto gold particles, the particles introduced into 32-64 cell *Ascaris* embryos, the embryos incubated at 30 C for 90 minutes, and the embryos collected and assayed for luciferase activity. The Gaussia activity was normalized to a co-transfected Firefly RNA using the Dual-Luciferase Assay. **B**). Guassia reporter activity over time following introduction into Ascaris embryos. All data illustrate representative experiments and show the mean and standard error derived from triplicate transfections for A and the mean for triplicate transfections in B. The Sh Gaussia reporter is described in Suppl. Figure 1.

Suppl. Figure 3. Translation in nematode embryos is methyl-guanosine cap-dependent. A). Firefly reporter RNAs. **B).** Renilla reporter RNAs. **C).** Gaussia reporter RNAs. The luciferase RNAs are described in Suppl. Figure 1. Reporter RNAs were biolistically introduced into Ascaris embryos and the luciferase activity measured after 90 minutes. Data illustrate representative experiments and show the mean and standard error derived from triplicate transfections.

Suppl. Figure 4. Translation in 1-2 cell embryos is similar to 32-64 cell Ascaris embryos. The illustrated RNAs (see Suppl. Figure 1 for additional information on the RNAs) and a Firefly control reporter RNA were biolistically co-transfected into 1-2 cell Ascaris embryos and luciferase activity measured after 90 minutes using a Dual-Luciferase assay. Activity was normalized to the co-transfected Firefly RNA activity. Data illustrate representative experiments and show the mean and standard error derived from triplicate transfections.

Suppl. Figure 5. Luciferase activity time course as a function of poly(A) tail length. TMG-SL Renilla RNAs (5' UTR = GGUUUAAUUACCCAAGUUUGAGGGCUAGCCACCAUG; see Suppl. Figure 1 for additional information) with 0, 30, and 85 nucleotide poly(A) tails were introduced into 32-64 cell Ascaris embryos, samples of embryos collected at time

intervals, and assayed for luciferase activity. Data illustrate a representative experiment with the mean and standard error derived from triplicate transfections.

Renilla (Ren) (ORF = 933 nts)

	5' UTRs	
Ren	GGUCAUUCCGGUACUGUUA <mark>GGCUAGCC<mark>ACC</mark>AUG</mark>	(30 nt)
SL Ren	<mark>GGUUUAAUUACCCAAGUUUGAG</mark> GGCUAGCC <u>ACC<mark>AUG</mark></u>	(33 nt)
SL cDNA 12 Ren (Vacuolar H ATPase	<mark>GGUUUAAUUACCCAAGUUUGAG</mark> UAUAUUGA <u>AGG<mark>AUG</mark> 16 kd proteolipid subunit)</u>	(33 nt)
SL cDNA 4 Ren (Ribosomal protein	<mark>gguuuaauuacccaaguuugag</mark> g <u>gaa</u> aug L23)	(26 nt)
GGN ₂₈ ACC Ren	GGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN <u>ACC<mark>AUG</mark></u>	(33 nt)
GGN ₂₂ ACC Ren	GGNNNNNNNNNNNNNNNNNNNNNN <u>ACC<mark>AUG</mark></u>	(27 nt)

3' UTR

Ren short (53 nt) = UUCUAGAGCGGCCGCUUCGAGCAGACAUGAUAAGAUACAUUGAUGAGUUUGGA

Ren long (172 nt) =

UUCUAGAGCGGCCGCUUCGAGCAGACAUGAUAAGAUACAUUGAUGAGUUUGGACAAACCACAACUAGAAUGCAGUGAAAAAAAUGCUUUAUUUGUGAAAUUUGUGAUGCUAU UGCUUUAUUUGUAACCAUUAUAAGCUGCAAUAAACAAGUUAACAACAACAACUAGAUUGCAUUCA

Gaussia (Gluc) (ORF = 555 nts)

Lg Gluc	GGACAAGCUUGGUACCGAGCUCGGAUCCAGCC <u>ACC<mark>AUG</mark></u>	(35 nt)
Sh Gluc	GGUACCGAGCUCGGAUCCAGCC <mark>ACC</mark> AUG	(25 nt)
GGN ₂₁ ACC Gluc	GGNNNNNNNNNNNNNNNNNNNNNN <u>ACC<mark>AUG</mark></u>	(26 nt)
GGN ₁₈ GAG Gluc	GGNNNNNNNNNNNNNNNN <u>GAG<mark>AUG</mark></u>	(22 nt)
SL Gluc	<mark>GGUUUAAUUACCCAAGUUU<u>GAG</u>AUG</mark>	(22 nt)
SL ACC Gluc	<mark>GGUUUAAUUACCCAAGUUUGAG</mark> ACC <mark>AUG</mark>	(25 nt)
SL cDNA 4 Ren (Ribosomal protein L23)	<mark>GGUUUAAUUACCCAAGUUUGAG</mark> G <u>GAA<mark>AUG</mark></u>	(26 nt)
SL cDNA 12 Gluc (Vacuolar H ATPase 16 kd pr	GGUUUAAUUACCCAAGUUUGAGUAUAUUGAAGG <mark>AUG</mark> oteolipid subunit)	(33 nt)
L37 Gluc (Ribosomal protein L37a)	GGAUUAUUGCUGUGAUUGACGAUCCUUCU <u>GCG<mark>AUG</mark></u>	(32 nt)

3' UTRs

Gaussia (41 nt) = GCGGCCGCUCGAGCAUGCAUCUAGAAAUAAUUCUUACUGUC

cDNA 4 (88 nt) = GGAGTTATTCTCGTTGAGGGAATGCGATTTGTTGTTGTGTTATCAACTATGTTGAATTTATCCTCTCCAAATTTCAATAAAATATTTCCGTT

cDNA 12 (213 nt) =

Firefly (Luc) (ORF = 1649 nts)

	5' UTRS	
Luc	GCAUUCCGGUACUGUUGGUAAAGCCACCAUG (28	nt)
SL Luc	GGUUUAAUUACCCAAGUUUGAGGCCACC <mark>AUG</mark> (28	nt)
SL cDNA 12 LucGGUU(Vacuolar H ATPase 16 kd prote	<mark>/UAAUUACCCAAGUUUGAG</mark> UAUAUUGA <u>AGGAUG</u> (33 .olipid subunit)	nt)

3' UTRs

Luc short (63 nt) = UUCUAGAGUCGGGGCGGCCGGCCGCCUUCGAGCAGACAUGAUAAGAUACAUUGAUGAGUUUGGA

Luc long (182 nt) =

Spliced Leader =	GGUUUAAUUACCCAAGUUUGAG
Renilla sequence =	GGCUAGCCACC
Luciferase sequence =	GCCACC
Initiator Methionine =	AUG
Underlined triplet =	-1 to -3 AUG context
(nt) =	length of 5 or 3' UTR

Cheng et al Suppl. Figure 1



Cheng et al Suppl. Figure 2

A





Cheng et al Suppl. Figure 4



Cheng et al Suppl. Figure 5