The Leishmania tarentolae Spliced Leader Contains Determinants for Association with Polyomes*

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In kinetoplastids, every nuclear-derived mRNA contains an identical 39-nucleotide (nt) spliced leader at its 5′-terminus. The spliced leader is derived from substrate spliced leader RNA and joined to pre-mRNA by trans-spooling, thus providing mature mRNAs with an m7G cap and additional methylations referred to as cap 4. It was shown previously that mutations spanning nucleotides 10–39 of the spliced leader did not affect substrate spliced leader RNA transcription or trans-splicing in Leishmania tarentolae (Saito, R. M., Elgort, M. G., and Campbell, D. A. (1994) EMBO J. 13, 5460–5469). In this study we examined these sequences for a possible role in translation by assaying the association of mRNAs, which possess mutated spliced leaders, with polysomes. For the nt 28–39 mutated spliced leaders, both the substrate spliced leader RNA and the spliced leader demonstrated a wild-type methylation pattern; spliced nt 28–39 mRNA was found in polysomes. Thus, the nt 28–39 region conserved primary sequence is not a determinant of polysome association. An undermethylated cap 4 structure was present on substrate and mRNA spliced leaders in nt 20–29 mutated exons; nt 20–29 mRNA was not present in polysomes. A differential pattern of cap 4 methylation was seen between the nt 10–19 substrate spliced leader RNA and the nt 10–19 spliced leaders found in the poly(A)⁺ population of RNA; the nt 10–19 mRNA was not seen in polysomes. Undermethylated spliced leaders did not associate efficiently with polysomes, suggesting a requirement for the cap 4 and/or primary sequence of the spliced leader in translation. This is the first report demonstrating that the spliced leader contains critical structural or sequence determinants for association with polysomes and, hence, translation.

The 39-nucleotide exon of the spliced leader (SL)¹ RNA is trans-spliced onto every nuclear messenger RNA (mRNA) in the kinetoplastid cell. Trans-splicing defines the 5′-end of each mRNA by providing polycistronically transcribed pre-mRNAs with an identical SL (1). The SL is conserved throughout the order Kinetoplastida (2) and contains a number of post-transcriptional modifications (3). There is a specialized 5′-cap structure comprised of m7G (4, 5), a series of 2′-O-methylations on nt 1–4 (AACC) and three base methylations on nt 1 and 4 that are collectively known as cap 4 (6–8), a likely base modification at A⁶ (adenosine at position 6) (6), and a pseudouridine at nt 28 (ψ₂₈) (9). Although m7G is likely required for mRNA stability, intracellular transport, and translation by analogy with other eukaryotic systems (10), the exact functions of the extensive and unique cap 4 methylations and ψ₂₈ are unknown.

A possible role for the cap 4 structure may be in the interaction or recognition of the mRNA with the ribosome. Studies with mammalian Mononegavirales showed that cap 1 structures are better templates for translation than cap 0 structures in vitro (11). In vivo studies in Xenopus have implicated the 2′-O-methyl cap 1 structure on mRNAs in translation of maternally inherited mRNA (12). Base methylations and ψ in tRNAs, small nuclear RNAs, and ribosomal RNA are common in active sites and are thought to enhance the RNA-RNA interactions required of these molecules (13, 14), although the absence of a single modification does not generally have an adverse effect on cell viability (15).

We have established a system with which to study the expression and function of SL RNA genes in vivo using episomal expression of mutated genes in the background of the complete chromosomal complement of wild-type SL RNA genes in Leishmania tarentolae (16). Transcription occurs efficiently when the conserved sequence elements in the nt 10–39 region of the SL exon are mutated, indicating that they are not necessary for transcription (16). The resultant mG-cap capped mutated SL RNA substrate products show variable cap 4 methylation, an absence of ψ formation, and disruption of conserved sequence elements but are efficiently trans-spliced (17), implying that none of these features are necessary for trans-splicing. The trans-splicing of undermethylated SLs was also seen in Leptomonas collosoma, where, for example the SL4 mutation was methylated with low efficiency and yet the second step of trans-splicing occurred with 54% efficiency relative to wild type (18), but not in Leptomonas seymouri (19).

Given the importance of the 5′-end of mRNAs in translation (19, 20), it is possible that the primary sequence of the SL may play a role in the association with ribosomes and translation initiation machinery. There is good evidence that mRNAs with SL are found on polysomes in Trypanosoma brucei (21, 22) and Caenorhabditis elegans (23); however, the nature of this association has not been addressed experimentally. It is not known whether this association is a direct or indirect effect, or whether it is dependent upon the primary sequence of the SL or its cap 4 structure. To query the role of the SL primary sequence and nucleotide modifications in translation, we examined mRNAs containing mutated SL sequences in the nt 10–19, 20–29, and 28–39 regions created as part of a linker scan series ordered as SL, spliced leader; nt, nucleotide; mut, mutant; WT, wild-type.

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§ The abbreviations used are: SL, spliced leader; nt, nucleotide; mut, mutant; WT, wild-type.

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The nt 28–39 mutated SL RNAs are detectable in poly(A) mRNA. Previously, we assayed three SL exon mutations, nt 10–19, 20–29, and 28–39 (Fig. 1A) for effects on cap 4 formation and trans-splicing (17). All studies were performed in vivo with stable transfectant L. tarentolae cell lines containing episomal copies of a mutated SL RNA gene (16). All epismally derived mutated SL RNAs in these cell lines are in a background of endogenous SL RNA and can be readily distinguished from endogenous SL RNA by their individual mutation-specific sequence tags. Whereas all three mutated SL RNAs were stable (16) and were trans-spliced as assessed by formaldehyde-agarose RNA blots (MicroPolyAPurist, Ambion), ribosomal RNA populations using γ-32P-labeled SL RNA-specific oligonucleotides. Extension products were resolved in 6% or 20% sequencing gels with a cognate sequencing ladder. Oligonucleotides used for primer extension are as follows: nt 10–19 mut, 5′-AAAATGACAGA AACTG ATTCC-3′; nt 20–29 mut, 5′-CAATA AAGTA TCTTT CGGAGG-3′; nt 28–39 mut, 5′-AAGCTTC CTCCA GGGT AA-3′; WT exon, 5′-CAATA AAGTA CAGAA AACTG ATTCC-3′; and SL intron, 5′-GTTTCC GGAAG TTCGT CATAC-3′.

RESULTS

Mutated SL Is Detectable in Poly(A)+ mRNA—Previously, we assayed three SL exon mutations, nt 10–19, 20–29, and 28–39 (Fig. 1A) for effects on cap 4 formation and trans-splicing (17). All studies were performed in vivo with stable transfectant L. tarentolae cell lines containing episomal copies of a mutated SL RNA gene (16). All epismally derived mutated SL RNAs in these cell lines are in a background of endogenous SL RNA and can be readily distinguished from endogenous SL RNA by their individual mutation-specific sequence tags. Whereas all three mutated SL RNAs were stable (16) and were trans-spliced as assessed by formaldehyde-agarose RNA blotting and reverse transcription PCR (17), only the nt 28–39 mutated SL was cap 4 methylated at levels indistinguishable from wild-type as determined by primer extension from whole cell RNA (17). In these earlier experiments, the detection primer hybridized to a tag within the intron of SL RNA; thus, the primer extension assayed specifically the SL cap 4 status of substrate (i.e. unspliced) SL RNA and did not assay the cap 4 status of mRNAs containing mutated SL exons (i.e. spliced). Thus, it was possible that the undermethylated cap 4 status observed for the nt 10–19 and 20–29 mutated SL RNAs represented a pool of SL RNA molecules that could not be trans-spliced and that mutated SL on mRNAs may have different cap 4 structures. To address this possibility, primer extension was performed on mutated SL RNA from whole cell RNA and poly(A)+ purified RNA using oligonucleotides specific to the mutations in the

exons (shown schematically in Fig. 1B). As the extension oligos were placed within the SL itself, we controlled for both mRNA degradation and substrate SL RNA contamination. Thus, the
stability of the mRNAs containing mutated spliced leaders was examined using an exon-specific primer, whereas the quality of the poly(A)\(^+\) preparation was controlled using the intron-specific primer to detect any unspliced substrate SL RNA contamination (Fig. 1F). The poly(A)\(^+\) extension profiles were compared with whole cell RNA extension profiles.

A shift in the relative amount of mature cap 4 methylation products was detected between the SL RNA substrate population and the mRNA population in the nt 10–19 SL mutant (Fig. 1C). Control (dNTP) titration primer extensions eliminate cap 4 read-through as an explanation for this observed shift in the substrate and poly(A)\(^+\) mRNA SL populations (3). An extension product corresponding to the cap 2 position was observed in whole cell RNA, whereas the most abundant stop in mRNA corresponded to the cap 1 position. The cap 4 product was present in both samples at 20–30%. This result differs with the profile previously observed for the unspliced substrate nt 10–19 mutated SL RNA (17). The nt 10–19 SL RNAs used in these two studies differ in the inclusion of an intron tag (17); analysis of the intron tag-containing mutant showed that the majority species was unmethylated at cap 0 (>90%), and a minor proportion (<5%) of the population was modified to cap 4. Acquisition of cap 4 methylations in intron tag-containing mutants was later determined to be slowed by the presence of the intron tag (26), but this retardation alone is not enough to account for the difference in methylation patterns between the differentially tagged SL RNAs.

In contrast to the nt 10–19 results, the nt 20–29 mutated SL poly(A)\(^+\) mRNA population displayed the same cap 1/cap 2 methylation primer extension stops as the substrate molecule (Fig. 1D). The nt 20–29 poly(A)\(^+\) purified mRNA sample was also extended with the WT exon primer, which recognizes only endogenous SL RNA, to demonstrate that cap 4-containing mRNAs are present in this poly(A)\(^+\) purified sample. Because of sequence differences in the two primers used in Fig. 1D, the WT exon extension migrates slightly faster than the nt 20–29 mutant extension and, therefore, cannot be directly compared with the sequence ladder generated with the nt 20–29 mutant primer.

Spliced and substrate nt 28–39 mutated SL (Fig. 1E) showed a minor relative increase of the cap 3 form in the poly(A)\(^+\) mRNA population. Wild-type SL showed a similar shift between the poly(A)\(^+\) mRNA and whole cell profiles (Fig. 1F, WT exon). This preparation was examined for the depletion of substrate SL RNA (SL intron) in the poly(A)\(^+\) mRNA preparation (Fig. 1F). The presence of some substrate SL RNA in the poly(A)\(^+\) fraction could be due to contamination of the preparation or the polyadenylation of substrate molecules (27, 28).

Thus, the nt 10–19, 28–39, and 20–29 SL RNAs possessed substantial, minor, and no differences, respectively, in the cap 4 status of substrate SL and spliced SL populations. These results confirm, by a third assay in addition to RNA blotting and RT-PCR, that exon-mutated SL RNAs are trans-spliced (17). The detection of an exon-specific signal with minimal substrate background in a population of mRNAs that possess intact 3’-ends demonstrates that these mRNAs are full-length products that are detectable in total steady-state mRNA.

Polysome Analysis of mRNA Containing Mutated SL—To ascertain the translatability of our SL mutated mRNAs, we adapted a polysome fractionation protocol used successfully in T. brucei (24). The presence of multiple ribosomes on mRNAs is highly suggestive of active translation, although polysome association is not a direct demonstration of translation. Cleared cell lysates generated in the presence of the ribosome-arresting drug cycloheximide were centrifuged through a sucrose gradient. An A\(_{254}\) trace of the gradient demonstrated the presence of 80 S subunits (29) just above the center of the gradient and also multiple (8) smaller peaks, suggestive of polysomes, in the lower half of the gradient (Fig. 2A). A gradient from the same experiment was fractionated into nine 1.2-ml aliquots, and the RNA was purified for electrophoresis through a formaldehyde-agarose gel and blotting to a nylon membrane. Hybridization of the blot with a probe that covers the small subunit (SSU) and large subunit 1 (LSU1) portions of the ribosomal RNA gene (Fig. 2B) revealed the following: 1) no hybridization at the top of the gradient (fraction 1); 2) strong hybridization to fractions 3 and 4, which correspond to the 80 S peak; and 3) hybridization to fractions 6–9 in the lower half of the gradient, consistent with the presence of polysomes. When 20 mm EDTA was included in the gradient to disrupt the ribosomes (24), hybridization of the resulting blot revealed the presence of small subunit and large subunit 1 ribosomal RNAs predominantly in the top four fractions of the gradient; substantially reduced or no hybridization was observed in the bottom four fractions of the gradient (Fig. 2B). Thus, the presence of multiple A\(_{254}\) peaks and ribosomal RNA in fractions 6–9 of the gradient support the identification of polysomes in these denser fractions, as reported by other groups (24, 29, 30). In these gradients, small RNAs (e.g. the SSU ribonucleoprotein) remain at the top of the gradient (see Fig. 3C).

L. tarentolae transfectant lines for nt 10–19, 20–29, and 28–39 SL mutations were subjected to similar polysome analysis (Figs. 3–5). The sucrose gradients were collected in 12 fractions, and extracted RNAs were subjected to primer extension with the corresponding mutation-specific oligonucleotides to query the presence of mutated SL mRNAs in the lower half of the gradient and the cap 4 status of these spliced SLs (see Fig. 1F for primer locations). The primer extension analysis for each mutant included a lane of whole cell RNA for comparison. The gradient fractions were controlled with a primer extension of additional primers, including oligonucleotides targeting the endogenous total SL (SL exon) as a positive control for the integrity of the gradient and the SL intron as a negative control.
for the detection of the SL RNA substrate (SL intron), which does not penetrate into the polysome fractions (see Fig. 5C). The interpretation of the primer extension analysis carries a caveat, i.e. termination of extension at 5′ is not necessarily indicative of mature cap 4, because base or ribose methylation events produce the same primer extension product (31, 32).

In the gradient fractions from the nt 28–39 SL mutant, the presence of trans-spliced mRNAs loaded onto polysomes was revealed by control (WT exon) primer extension products in lanes distributed throughout gradient fractions 1–11 (Fig. 3A, top panel). Substrate SL RNA was only present in fractions 1–3 at the top of the gradient as demonstrated by primer extension profiles using an SL intron primer (middle panel). Extension products with the nt 28–39 mut primer were obtained throughout the 11 fractions (bottom panel), mirroring the distribution of mRNA with wild-type SL. The presence of mutated SL in the lower half of the gradient suggests that it, like wild-type SL, is associated with polysomes. The presence of mRNA at the top of the gradient may be due to differential loading of mRNA with ribosomes as observed in *Trypanosoma cruzi* (33).

To confirm that the SL being measured in the primer extension assay represents intact mRNA, we also subjected gradient-fractionated nt 28–39 mutated SL RNA to size analysis by agarose gel electrophoresis and blotting (Fig. 3B). Hybridization with the nt 28–39 mut oligonucleotide revealed the 96-nt SL RNA only in the first fraction at the top of the gradient. A smear of longer RNA (> 0.5 kb) was present in total cell RNA (Fig. 3B, lane marked WC, and Ref.17) and found in all eight fractions of the gradient. This result suggests that SL-containing RNA being detected by the primer extension assay is intact mRNA. A control gradient of fractions from the nt 28–39 SL mutant was performed in the presence of 50 mM EDTA. All primer extension signals were limited predominantly to the top four (Fig. 3C; WT exon, top panel) and two (SL intron, bottom panel) fractions of the gradient, consistent with the disassembly of polysomes under conditions of depleted Mg2+. Degradation product migration would not be affected by the EDTA treatment.

The primer extension assay with the nt 10–19 mut oligonucleotide on RNA extracted from sucrose gradient-fractionated nt 10–19 SL extracts (Fig. 4) yielded products only at the top of the gradient (fractions 1–3); no evidence of polysome association was seen with overexposure of the gel (Fig. 4, top two panels). This result mirrored the distribution of the substrate SL RNA (SL intron panel) in the same sample. As the control wild-type SL RNA was present in the bottom half of the gradient (fractions 7–12) in the same tube, we concluded that the nt 10–19 SL mRNA, which is stable and detectable (Fig. 1C), is not associated with polysomes.

Similar to the results from nt 10–19, the primer extension assay with the nt 20–29 mut oligonucleotide on nt 20–29 mutant fractionated RNA revealed the spliced exon only in the top three fractions (Fig. 5); no evidence of polysome association was seen with overexposure of the gel (Fig. 5, top two panels). Control extensions with the SL RNA primer demonstrated again that the SL RNP is predominantly in the top three fractions of the same samples (Fig. 5, panel marked SL intron), and the wild-type spliced SL is throughout the gradient, including fractions 8–11 (Fig. 5, panel marked WT exon). Thus the differential association of mutated SL (nt 28–39 versus 10–19 and 20–29) indicates that the SL contains determinants for the association of mRNA with polysomes.

**DISCUSSION**

This is the first report of trans-spliced mRNAs with mutated SL and incomplete cap 4 formation that do not associate with polysomes, implying a role for one or both of these features of the SL in translation. Splicing of mutated SLs, including those with undermethylated cap 4 structures, occurred efficiently and did not destabilize the recipient mRNA. One of the mutated SLs, the cap 4-methylated nt 28–39, was present in polysomes, whereas the undermethylated nt 10–19 and 20–29 mutant mRNAs were poor substrates for ribosome association (summarized in Fig. 6A). Because of the differential presence of these mRNAs in polysome fractions, we have identified a specific role for the cap 4 methylations and/or the primary sequence of the SL in the translation of kinetoplastid mRNAs. Three major observations have been made. First, undermethylated mRNAs are detectable. Second, there is heterogeneity between the cap 4 status of substrate and poly(A)+ mRNA populations for two of SL mutants and for wild-type SL. Third, the SL contains determinants of mRNA translation.

There are a number of mechanisms by which translation initiation may occur in eukaryotes and prokaryotes (34), including recognition of the cap structure by an initiation com-
plex and potential base pairing between the ribosome and the mRNA, including 5′-untranslated regions (35−37), downstream boxes (38−40), ribosome-shunting models (41), and internal ribosome entry sites (IRES) (42). Because of the presence of the identical 39-nt sequence at the 5′-end of all nuclear mRNAs, trypanosomatids have the potential to use a unique mechanism for translation initiation, which may explain the level of conservation in the SL sequence seen throughout the order Kinetoplastida.

The significance of cap 4 heterogeneity between the substrate and poly(A)+ mRNA populations is not clear. These results may reflect differences in stability between undermethylated and completely cap 4 methylated populations, specifically in the nt 10−19 SL. The minor increase in cap 3 abundance observed for the nt 28−39 mRNAs was also seen in for wild-type SL mRNAs, indicating that this mutated mRNA is behaving in a manner consistent with endogenous mRNA. mRNAs containing cap 3 and cap 4 do not differentially associate with polysomes over the length of the gradient, linking these changes in methylation status with the ability to be translated and possibly degraded by a translation-dependant pathway, as seen in other eukaryotes (20, 43). The possibility of a causal relationship between translation and the increase in cap 3 cannot be discounted.

Because of the presence of nt 28−39 mRNAs in the polysome fractions, several conclusions can be drawn regarding the function of SL sequence and structural elements and their roles in polysome association. The primary sequence of the SL nt 20−39 region is 95% similar throughout the order Kinetoplastida (n > 40 SL RNAs sequenced to date; Refs. 2 and 44), yet a biological function for this region of the SL exon has not been elucidated. We have shown previously that this region is not involved in transcription (16) or trans-splicing (17). Based on the polysome association of the nt 28−39 mRNA, it is evident that at least part of this region does not play a role in translation of mRNA. Furthermore, by serendipity in the synthesis of the mutagenic oligonucleotides, the nt 10−19, 20−29, and 28−39 SL RNAs all have a 28U→A mutation (uridine at position 28 mutating to adenosine). U28 has recently been shown to be modified to a pseudouridine in wild-type SL specified by an interaction between the 21−32 region of the SL and the spliced leader-associated 1 RNA in L. tarentolae (9); the base pairing interaction in this key region may account for the level of conservation seen in the SL. Because an adenosine cannot be pseudouridinylated, it is reasonable to infer that our mutants do not contain ψ28. Thus, it follows that ψ28 is not necessary for polysome loading or mRNA stability. However, translational efficiency may be enhanced by the presence of the ψ28, a possibility that would be best evaluated in a purely mutated SL mRNA system.

In contrast with the nt 28−39 region, it is clear that the nt 10−27 region of the L. tarentolae SL carries determinant(s) of mRNA translation. There are two likely possibilities. First, the primary sequence of SL exon nt 10−27 may be recognized directly by the translation initiation machinery, analogous to one of models in which mRNA element(s) bind ribosomes directly to initiate translation. A model for interaction between
the \textit{L. tarentolae} SL and small subunit rRNA (Fig. 6B), based on a proposed similar interaction in \textit{T. brucei} (45), is not supported by the data presented here. The nt 28–39 mutation weakens the proposed base pairing in the stronger stem such that it is unlikely to form. Because the nt 28–39 mutated SL was found on polysomes, we infer that the interaction is not necessary for translation.

A second possibility is that the two SL mutants covering this region, nt 10–19 and 20–39 SL, are m7G-capped but undermethylated (nt 10–19 SL is only 30% cap 4; nt 20–39 SL is exclusively cap 1 and 2). By analogy to the mechanism of mRNA recognition by the translation initiation machinery, cap 4 may be part of the translation substrate recognition signal for the kinetoplastid homologues of the translation initiation factor eIF4E. Thus, even though nt 10–19 mRNAs appear to be cap 4, it is possible that this primer extension stop is due to only the ribose or base methylation and does not represent \textit{bona fide} cap 4.

SL-independent translation can occur in the cytosol of kinetoplastids. Several classes of retroposon have been characterized in \textit{T. brucei} and \textit{T. cruzi} that produce transcripts that are not \textit{trans}-spliced; however, translation of these transcripts has not been demonstrated (46, 47). In another example, cap-independent internal ribosome entry site-mediated translation occurs for \textit{Leishmania} virus 1 transcripts (48) that do not contain the SL (49). There have been no reports of endogenous nuclear mRNAs that are translated in the absence of \textit{trans}-splicing.

The recognition of different cap structures by the translation initiation machinery is a possibility in the kinetoplastids, wherein there are at least two isoforms of the cytoplasmic m7G cap-binding subunit eIF4E (GenBank\textsuperscript{TM} accession numbers AL493211 and AQ944612). Consistent with this hypothesis, the nematode \textit{C. elegans}, which matures the 5'/H11032-end of mRNA with and without \textit{trans}-splicing, possesses multiple isoforms of eIF4E (50) that can discriminate between the standard m7G cap 0 and the \textit{trans}-spliced trimethylated m2,2,7G cap (51, 52). Future studies will be aimed at determining the precise structure of the cap in the mutated SLs and demonstrating translation of mRNAs carrying mutated SL sequences using a direct reporter.

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\textbf{FIG. 6. The role of SL in mRNA translation.} \textit{A}, summary of the \textit{L. tarentolae} SL domains that may affect translation. Of the 39 nucleotides of the SL exon, nt 10–19 comprises the "AU-rich" region, whereas nt 1–9 and 20–39 are conserved throughout the order Kinetoplastida. A summary of the phenotypes associated with this study is provided. \textit{Plus (+), efficient; plus minus (±), partial; and minus (−), inefficient. B}, model for potential base pairing between nt positions 21–39 of the \textit{L. tarentolae} SL (53) and 3'-end (nt positions 2164–2195) of the \textit{L. tarentolae} small subunit (SSU) rRNA (54) based on the proposed interaction of these regions in \textit{T. brucei} (45). A \textit{vertical bar (|)} indicates regular base pairing, and a \textit{dot (·)} indicates G-U base pairing. ORF, open reading frame.
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