Efficient trans-Splicing of Mutated Spliced Leader Exons in Leishmania tarentolae*

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Every kinetoplastid mRNA receives a common, conserved leader sequence via the process of trans-splicing. In Leishmania tarentolae the precursor spliced leader RNA is 96 nucleotides, with a 39-nucleotide exon that is 7meG-capped and methylated on the first 4 nucleotides. trans-Splicing was inferred from the presence of tagged leader in the high molecular weight RNA population and confirmed for accuracy by cDNA cloning. Linker scan substitutions within the exon between positions 10 and 39 did not affect trans-splicing. The trans-splicing efficiency for three of the scan exons was proportional to the tagged/wild type ratio in the spliced leader precursor population. Two scan leader RNAs that were efficiently spliced showed reduced methylation. Longer exons showed reduced splicing, whereas 10- or 20-base pair deletions abolished splicing. These results indicate that size, but not content, of the exon is a constraint on the splicing process. These results, in combination with previous data eliminating a role in transcription initiation, suggest that translation may be the selective pressure on the leader content.

** The abbreviations used are: nt, nucleotide(s); IT, intron tag; PCR, polymerase chain reaction; RT, reverse transcriptase; SL, spliced leader; tSL, tagged SL; WT, wild type; bp, base pair(s).

1 The 39-nnt1 spliced leader (SL) in the kinetoplastids is extensively conserved (1–3), such that PCR amplification of SL RNA genes from diverse kinetoplastids can be performed with a single set of oligonucleotide primers (4, 5). These conserved sequences are presumed to be important for one or more basic biological processes, which could include transcription, trans-splicing, and translation. In the nematode Ascaris, part of the 22-nt SL sequence functions as a promoter (6); however, a major role for the SL in transcription initiation has been eliminated in Leishmania tarentolae and Trypanosoma brucei, which have upstream promoters (7, 8). An Ascaris SL of two nucleotides is accurately trans-spliced in vitro (9), indicating that information for splicing is not contained within the SL. By contrast, the SL sequence is important for both transcription (10) and trans-splicing (11) in some kinetoplastids.

Although the U5 small nuclear RNA may be cross-linked to exons near the splice junction (12), the roles of exons in cis-splicing care generally constrained by their protein-coding capacities. There are no such constraints apparent in trans-splicing. The kinetoplastid SL functions as a cap 4 donor (13) and as a 5’-untranslated region, allowing the possibility for an active role in trans-splicing. Several inter- and intramolecular interactions have been predicted to occur within the SL. Two small RNAs, SL-associated 1 and U5, have been demonstrated in vivo cross-linking to interact with the SL (14, 15). Intramolecular interactions of the SL include alternation between two structural forms involving SL-SL (Form I) and SL-intron (Form II) base pairing in vitro (16) and in vivo (17) and proposed U1 and U5 functions (18).

To determine the importance of the conserved kinetoplastid SL in trans-splicing we evaluated several SL mutants for trans-splicing in vivo. We show that SLs containing 10-bp replacement mutations between positions 10 and 39 are efficiently and accurately trans-spliced. In addition, some spliced mutants are undermethylated at their 5’ ends. These results indicate that neither the primary sequence of a 39-nt SL nor cap 4 methylation are critical for trans-splicing; however, there may be constraints on the absolute size of the SL.

EXPERIMENTAL PROCEDURES

Generation of Mutations and Transfectants—The intron tag (IT) containing SL mutations (1/9, 10/19, 20/29, and 30/39, which introduced an XhoI site) were generated originally for promoter localization studies (7). An SL reporter lacking the IT was created by removal of the IT from 30/39 and called tagged SL (tSL). Control plasmids that lack active promoters were constructed by PCR (7), resulting in the versions of 1/9, 10/19, 20/29, 30/39, and tSL. The shortened SLs, 1–29 and 1–10, were created by subcloning the XhoI-KpnI fragments from 10/19 or from 20/29 into the tSL vector. SLs of increased size were generated by digestion of tSL with XhoI followed by Klenow fill-in and religation (+1) or insertion of a double-stranded oligonucleotide possessing XhoI-compatible ends (+241).

Transfections were performed as described (7), except that cells were cultured in brain heart infusion (Difco) supplemented with 10 μg/ml hemin and selected initially with 200 μg/ml paromomycin (Humatin; Parke-Davis). Subsequent drug selection was performed with either paromomycin or 100 μg/ml G418 (Life Technologies, Inc.) in brain heart infusion/gemini Science).

RNA Analysis—RNA was purified using Trizol reagent (Life Technologies, Inc.) and was electrophoresed through 1.1% agarose-formaldehyde or 8% acrylamide/8M urea gels, transferred, and hybridized as described previously (7, 19). Quantitation was performed using a PhosphorImager (Molecular Dynamics).

Oligonucleotide probes used for SL RNA detection were: 10/19 tag, 5'-TGGAGTTCCTG AGGGCGCCGGCGG; 2/29 tag, 5'-ATTTCTTCGTAGGCTCCAG; 3/29 tag, 5'-ACTTCCTCCTTGGCCAGTGA; LtSLintron, 5'-TGCGGATCGCCTTCTGGCCACCC; arl(16) and arl(17) were hybridized to the 96-nt SL, 5'-TGCGGATCGCCTTCTGGCCACCC; and to the tagged:wild type ratio in the spliced leader precursor population. Two scan leader RNAs that were efficiently spliced showed reduced methylation. Longer exons showed reduced splicing, whereas 10- or 20-base pair deletions abolished splicing. These results indicate that size, but not content, of the exon is a constraint on the splicing process. These results, in combination with previous data eliminating a role in transcription initiation, suggest that translation may be the selective pressure on the leader content.
**RESULTS AND DISCUSSION**

The importance in *trans*-splicing of particular SL sequence elements was examined for a series of SL mutations generated following a linker scan approach in conjunction with an IT (7) and derivative mutations lacking the IT and varying in SL size (summarized in Fig. 1A). The predicted size differences because of the presence of IT sequences or exon mutations are indicated (Fig. 1A) and shown in total RNA from transfectants electrophoresed through an acrylamide-urea gel (Fig. 1B). The +4 and +24 SL RNAs each required specific exon probes and migrated as predicted on comparable 8% gels (data not shown). These data confirmed the transcript size predictions of the various constructs and demonstrated that transcription was not affected by mutation of the SL RNA gene sequences.

To assay for *trans*-splicing, total RNAs from mutants were hybridized with the exon tag oligonucleotides (Fig. 2A; data not shown for SL size mutants and not determined for 1/9). The unspliced, mutated SL RNAs were visible at the bottom of the blots. Hybridization of tag-specific oligonucleotides to higher molecular weight RNA species in 10/19, 20/29, 30/39, and tSL but not in their respective promoter knockout (7) mutants indicated that active *trans*-splicing of the mutated SLs occurred. The −1.4-kilobase bands and faint background present in RNA from the four promoter knockout mutants were anticipated artifacts, reflecting transcripts from the randomly initiated run-around transcription that drives the neomycin resistance gene (23, 24). The −10, −20, +4, and +24 SLs did not appear to be efficient substrates for *trans*-splicing by this assay (data not shown).

The transfectant mRNAs were examined in a more sensitive manner and for accuracy of splicing by RT-PCR, blot and nucleotide sequence analysis for the presence of mutated SL on *arl* or *ubi* mRNAs; the cDNA products were subsequently used as templates in PCR reactions with the general LtSL5 primer (all SL) corresponding to the first 12 nucleotides of the SL RNA such that both WT and mutated products would be amplified, as shown in the schematic of the assay. The products were electrophoresed, transferred to nylon membranes, and hybridized with the 20/29 tag and 30/39 tag oligonucleotides. The tSL, −67/−58 products were amplified in the same manner using the SL primer 30/39−5′ (mutant SL or m) in the PCR reactions, and the spliced products were detected by hybridization using the 30/39 tag, +4 tag, or +24 tag oligonucleotides. The *arl* product size of 300 bp is indicated. The absence or presence of specific *arl* amplification products is shown for 1/9 and 10/19 in an ethidium bromide-stained agarose gel.

**Fig. 1. Intron and SL-tagged SLs are expressed.** A, mutations introduced into the SL. The WT SL RNA sequence (accession number X73121) is shown at the top, followed by a box representing the splice donor GT site and a line representing the intron. The WT SL + IT (mutant IT) and linker scan mutants 1/9, 10/19, 20/29, and 30/39 contain the 40-bp IT shown as a hatched box and previously described in Ref. 7. Nucleotides that are identical to the WT are shown by dots, and specific changes are indicated by the particular nucleotide letter. The tSL mutation is the same as 30/39 but without the IT. The size variation mutations −20, −10, +4, and +24 also lack the IT. B, the size differences of the mutated SL products are shown in 8% acrylamide/8 M urea gels. The 20/29 and −10, 10/19 and −20, and 30/39 and tSL RNAs are recognized by the 20/29 tag, 10/19 tag, and 30/39 tag probes, respectively. The tSL mutant migrated at the expected WT size of 96 nt, mutant −20 migrated at 76, and −10 migrated at 86 nt. The IT-containing mutants migrated at 148 nt, with an additional band (*) above the SL RNA that was an artifact of the expression system (7).

**Fig. 2. Splicing of substitution mutated SLs.** A, the mutated SL signals are found in high molecular weight RNA. A 1.1% agarose-formaldehyde gel was used to resolve 5 μg of total cell RNA from WT and transfected *L. tarentolae* cell lines. Specific probes were used to visualize mutated SL sequences in both the free SL RNA population (96 and 148 nt) as well as in the high molecular weight *trans*-spliced mRNA population. The background hybridization resulting from randomly initiated run-around transcription is shown in the promoter knockout (−67/−58) controls. Duplicated lanes represent different RNA preparations. The size markers refer to two of the three ribosomal bands (2.2 and 1.5 kilobases, visible by their "exclusion shadows") and the free SL RNAs. B, exon tags are detected in the population of spliced mRNA amplification products. Total RNAs from transfected clones expressing WT, 20/29, 30/39, tSL, +4, +24, and −67/−58 +1.5 kilobases, visible by their "exclusion shadows") and the free SL RNAs. B, exon tags are detected in the population of spliced mRNA amplification products. Total RNAs from transfected clones expressing WT, 20/29, 30/39, tSL, +4, +24, and −67/−58 +1.5 kilobases, visible by their "exclusion shadows") and the free SL RNAs.
that indicated splicing of 10/19 but not of 1/9 (Fig. 2B). When subjected to the RT-PCR assay, the −20 and −10 SLs did not yield trans-splicing products even with mutation-specific 5′ primers (data not shown). +4 and +24 were inefficient substrates for trans-splicing, because products were generated with the mutant-specific 5′ primer but not the general primer (Fig. 2B).

The resulting RT-PCR products for arl and ubi mRNAs were cloned and sequenced to determine the accuracy of trans-splicing for 10/19, 20/29, 30/39, and tSL transfectants. In each case, trans-splicing with the mutated SL was mapped to the site previously identified as WT (25),2 with an additional, alternative, splice acceptor site mapped for the arl mRNA with one of the 20/29 clones (data not shown). The sequence data confirm that the RT-PCR reactions were not producing artificial products and that these scan SLs were trans-spliced accurately.

Lack of detectable spliced products containing the 1/9 SL may be a result of the low abundance of mutated precursor SL RNA, which was previously shown to be efficiently transcribed but unstable (7). Changing the initiation nucleotide from an A to a C residue will prevent addition of the 7meG cap to the SL RNA, thus reducing the stability of the 1/9 SL RNA and any mRNAs receiving this mutated SL. The inefficient splicing of the 1/9 SL was 3%, and the 30/39 SL was 6% (Fig. 3A) revealed that the 10/19 SL RNA was 2% of the total unspliced SL population, the 20/29 SL was 3%, and the 30/39 SL was 6%. WT SL was approximately 2% in the RNA samples used for the PCR assays, as determined by normalization of duplicate blots with the SL-associated 1 RNA (Ref. 26 and data not shown). The ratio of WT:mutated SL trans-spliced products gave internally consistent results, indicating that the proportion of trans-spliced mutated:WT SLs was equivalent to the proportion of substrate mutant:WT SL in total RNA. If the mutant SL was PCR-amplified proportionally to the WT SL RNAs, we define this as efficient trans-splicing. The proportional nature of the amplification was demonstrated by amplifying varying amounts of the cloned WT and tSL spliced arl cDNAs and comparing the signals of the resulting products to the signal of the input plasmids (data not shown). Thus, the mutations within the SL had a minimal effect on the efficiency of trans-splicing.

Primer extension analysis was performed to examine the methylation status of the cap 4 at the 5′ end of the mutated SLs (Fig. 3B). The IT mutant was used for determination of WT methylation levels; this construction has no exon mutation and showed comparable methylation to WT SL (data not shown). 30/39 SL methylation varied with RNA preparations between 33 (Fig. 3B) and 75% (data not shown) as seen in IT (Fig. 3B). Surprisingly, the unstable SL 1/9 was methylated at 75% (extended exposure, Fig. 3B), indicating that methylation and capping are not linked. 10/19 and 20/29 showed sharply reduced methylation (<5%; extended exposure, Fig. 3B), allowing the RT to progress 3 or 4 nucleotides farther along the transcript and resulting in a larger product. The majority of 10/19 products extended to +1; 20/29 extended predominantly to +2. SL tag-specific oligonucleotides were used to confirm the methylation results for the 10/19 and 20/29 transfectants (data not shown) and to examine the −10 and −20 SL RNAs. The tSL mutant is consistently methylated at 75% in the WT pattern, but −10 and −20 showed reduced methylation (data not

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**Table I**

| Mutant | Splicing general | RT-PCR Cap 4 methyl %
|--------|-----------------|------------------------|
|        | Non-specific     | Mutant-specific         |
| IT     | NA              | NA                     | 75
| 1/9    | −−−             | −−−                    | 75
| 10/19  | −−−             | −−−                    | <5
| 20/29  | +++             | +++                    | <5
| 30/39  | +++             | +++                    | 33−75
| WT     | ND              | +++                    | 75
| tSL    | +++             | +++                    | 75
| −20    | −               | −                      | <5
| −10    | −               | −                      | <5
| +4     | +               | +                      | ND
| +24    | +               | +                      | ND

a. Agarose gel assay (Fig. 2A and data not shown).
shown). The mutant phenotypes are summarized in Table I.

Efficient splicing of under-methylated SL contrasts with findings in whole and permeabilized cells (27–29). Inhibitor studies using S-adenosyl-l-homocysteine (27) and sinefungin (28) are likely to have had a global effect on cellular methylation processes and not inhibited SL methylation exclusively. Thus, these results are reflective of the importance of methylation in the trans-splicing pathway as a whole rather than methylation of the SL in particular. The antisense oligonucleotide studies may have been confounded by the presence of the oligonucleotide itself forming a duplex structure that could affect other SL interactions in addition to disrupting the cap 4 formation process (29).

The results presented here indicate that the SL sequence between positions 10 and 39 is not important for trans-splicing and suggest that the secondary structure of stem-loop I (16) is not necessary for accurate trans-splicing. However, there appears to be an optimal size for the SL for splicing in vivo, unlike the situation in Ascaris where the exon size can vary from 2–246 nt and maintain efficient splicing in vitro (9).

Our results contrast with those of Lücke et al. (11), whose studies conclude that much of the SL sequence is important for splicing; they also conclude that size is not limiting based on a single efficiently spliced mutant with a 45-nt exon (+6 nt). The results of our two studies are largely in agreement regarding the areas of the exon important for methylation of the cap 4 (11); however, the role of the cap 4 methylations in the trans-splicing process is challenged by the efficient splicing in Leishmania of the 20/29 mutation and, based on the total RNA analysis, the 10/19 mutation. The interpretation of negative trans-splicing results must be approached with caution, because the result may be due to secondary effects of the mutation; a positive result provokes further study.

Because our studies reveal that contingencies for splicing do not exist within the primary sequence of the kinetoplastid SL, we are left to hypothesize that the conservation is necessary for proper function of the translation machinery in these organisms. In future experiments we will attempt to address this possibility directly.

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