The Role of Intron Structures in trans-Splicing and Cap 4 Formation for the Leishmania Spliced Leader RNA*

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A 39-nucleotide leader is trans-spliced onto all trypanosomal nuclear mRNAs. The precursor spliced leader RNA was tested for trans-splicing function in vivo by mutating the intron. We report that in Leishmania tarentolae spliced leader RNA 5′ modification is influenced by the primary sequence of stem-loop II, the Sm-binding site, and the secondary structure of stem-loop III. The sequence of stem-loop II was found to be important for cap 4 formation and splicing. As in Ascaris, mutagenesis of the bulge nucleotide in stem-loop II was detrimental to trans-splicing. Because restoration of the L. tarentolae stem-loop II structure was not sufficient to restore splicing, this result contrasts the findings in the kinetoplastid Leptomonas, where mutations that restored stem-loop II structure supported splicing. Methylation of the cap 4 structure and splicing was also dependent on both the Sm-binding site and the structure of stem-loop III and was inhibited by incomplete 3′ end processing. The critical nature of the L. tarentolae Sm-binding site is consistent with its essential role in the Ascaris spliced leader RNA, whereas in Leptomonas mutation of the Sm-binding site and deletion of stem-loop III did not affect trans-splicing. A pathway for Leishmania spliced leader RNA processing and maturation is proposed.

Kineto plastid nuclear gene expression is dependent on the trans-splicing process. The common substrate for all trans-splicing reactions is the spliced leader (SL) RNA, also known as the mini-exon derived RNA, whose first 39 nt constitute the 5′ ends of both mono- and polycistrionically synthesized mRNAs (1). The polycistrionic pre-mRNAs require trans-splicing to acquire the specialized “cap 4” structure on the SL RNA. The cap 4 consists of a 7mG attached to the first nucleotide (2), in addition to methylation of the first four and sixth nucleotides of the SL RNA (3–5). These modifications are made to the primary SL RNA and spliced onto the mRNA as part of the SL RNA (3–5). These modifications are made to the primary SL RNA and spliced onto the mRNA as part of the 39-nt exon. The cap 4 may have roles in mRNA trans-splicing, transport, stability and translation.

The SL RNA contains two functional domains as follows: the exon and the intron or snRNA-like domain (6). The exon sequence is conserved among 38 different members of the order Kinetoplastida (7). Positions 1–9 and 20–39 of the exon are nearly identical, whereas positions 10–19 are relatively heterogeneous and characteristically A/T-rich. This conservation cannot be ascribed to internal promoter location in Leishmania (8, 9), as found in Ascaris (10). It was surprising that mutations within positions 20–39 permitted accurate trans-splicing in Leishmania tarentolae and did not lower splicing efficiency (11) because these results contrasted with findings in Leptomonas (12). Thus, the results in L. tarentolae more closely resemble the findings in worms as follows: in Ascaris, exon sequences are not necessary for trans-splicing in vitro (13); in Caenorhabditis elegans, length, primary sequence, and composition of the SL are not critical parameters for essential embryonic function, although certain nucleotides may be essential for in vivo splicing of the SL1 RNA (14, 15).

The primary sequence of the SL RNA intron is not conserved among the trypanosomatids (7); however, the secondary structure is consistent (16). This structure has been confirmed by physical-chemical and enzymatic studies (17, 18) and examined by mutagenesis (12). An equivalent structure is also conserved in the nematode SL RNAs (16, 19). The intron contains a putative Sm-binding site (16), an element found in the small nuclear RNAs of higher eukaryotes but apparently lacking in all U-RNAs of kinetoplastids (20) except U5 RNA (21, 22). The Sm-binding element is required for SL RNA trans-splicing in Ascaris (23) but not in Leptomonas (12).

We demonstrated recently that the T tract downstream of the SL RNA gene is a transcription termination element and that staggered T tract termination products are processed via nucleolytic cleavage to the base of stem-loop III (24). The signals for 3′-processing begin in the Sm-binding site at position 76 and include the structure, but not content, of stem-loop III. Studies in Leptomonas seymouri demonstrated that mutation of a variety of elements in the intron was acceptable for trans-splicing (12), whereas in Leptomonas collosoma the loop portions of stem-loops II and III were tolerant to insertions but not to replacement with the Trypanosoma brucei intron (25). By contrast, the bulge of stem-loop II was critical for trans-splicing in Ascaris (26).

In this paper we report that methylation of nucleotides in the cap 4 structure of the Leishmania SL RNA is influenced by formation of stem-loop III, the Sm-binding site, and specific sequences in stem-loop II. The methylation of the cap 4 structure correlates with correct 3′ end formation; defects in 3′ end processing and cap 4 formation result in failure of the mutated SL RNA to undergo trans-splicing. However, correct maturation of the SL RNA is not sufficient to obtain a positive splicing phenotype since mutation of nucleotides in the stem I region of the intron can also result in loss of function. Our data from L. tarentolae broadly reflect the results obtained in vitro in the nematode Ascaris, where nucleotides in stem-loop II and the
FIG. 1. A linker scan series was generated through the intron of the L. tarentolae SL RNA gene. The secondary structure of L. tarentolae SL RNA is based on the L. collosoma structure that predominates in vivo (17, 18); numbering is relative to the start of transcription. The stem-loop structures are labeled I, II, and III, and the exon-intron junction is indicated by the arrow following nucleotide 39. The Sm-binding site sequence AUUUUGG and the mature 3′ end of the SL RNA are indicated. The 7mG cap is shown at the 5′ end, along with the methylated nucleotides (*) that comprise the cap 4 structure (3, 4). All constructs contained an exon tag (tSL) sequence at position 28–39, indicated by lowercase letters. The scan sequence, CTCGAGCTCA, included XhoI and SalI sites; positions within the 10-base pair blocks that are altered are indicated in lowercase letters (3) adjacent to the WT position. Three additional scan mutations, 100/109, 110/119, and 120/129, are not shown but are described elsewhere (24).

Sm-binding site are necessary for splicing. Our data broadly contrast the results obtained in Leptomonas, where the structure and not the primary sequence of stem-loop II was necessary for splicing, and where the Sm-binding site and stem-loop III were not required for splicing. A model summarizing the features of the Leishmania SL RNA involved in maturation and trans-splicing is presented.

EXPERIMENTAL PROCEDURES

Generation of Mutations and Transfectants—Mutagenesis was performed using the Sculptor Mutagenesis kit (Amersham Pharmacia Biotech) or using PCR to generate mutated DNA fragments for subcloning into the transfection vector. Mutated fragments were cloned for transfection into a pX plasmid (27) containing an SL RNA gene (9).

Transfections were performed by electroporation as described (11). Nucleic Acid Isolation and Gel Analysis—RNA was purified using TriZOL reagent (Life Technologies, Inc.) and was electrophoresed through 1.1% agarose-formaldehyde, blotted, and hybridized with an oligonucleotide (28/39 tag) directed against the exon tag. Two of the ribosomal RNA exclusion zones (2.2 and 1.5 kb) and the SL RNA (96 nt) served as size markers (M). B, low levels of trans-splicing detected by RT-PCR. RT-PCR assays were performed on the mutant RNA populations with either nonspecific SL RNA primer (top and middle) or 28/32 tag-specific SL RNA primer (bottom) using the L. tarentolae Arl mRNA (29) as the query template. Control reactions included RNA from a mutant containing tSL coupled with an inactive promoter, –67/–58 + tSL (11), –RT, and -RNA reactions. Additional 5′ and 3′ end phenotypes (24) are summarized as follows: + = WT, = WT, and – = T tract for 3′ end formation, and − = WT, = WT, and – = <5% for cap 4 methylation.

RESULTS

Intron Mutations Affect trans-Splicing—To localize specific elements within the intron of the SL RNA that play a role in the process of trans-splicing, a systematic mutagenesis approach was adopted (Fig. 1). To differentiate mutated, episomally derived SL RNA from the endogenous WT SL RNA population, an exon tagged at positions 28 and 30–39 (28/39), which was previously shown to trans-splice accurately and efficiently (11), was used as a molecular tag (tSL RNA) for detection by hybridization. A series of linker scan (CTCGAGCTCA) mutations in the tSL RNA gene was created for transfection to L. tarentolae. Two mutations in the 40–49 region created as follows: a mutant with alterations in bases 43 and 44 (43/44 = GT versus TG in WT) tested a postulated SL RNA-U6 snRNA interaction (31), and a second mutant, altered at positions 42–48 (42/48), changed all but the splice donor site with the linker scan sequences. Subsequent intron mutations continued from position 50 (52/59) and proceeded through the end of the intron. Three mutations lay downstream of the mature 3′ end of the SL RNA transcript (position 96) and were included to identify potential adjacent expression elements (100/109, 110/119, 120/129; WT sequence not shown).

Analysis of total RNA from the transfectants demonstrated tSL RNA (~96 nt) in all the samples by low resolution formaldehyde-agarose gel blotting (Fig. 2A). A broadened size range from the wild-type (WT) 96 nt to at least 175 nt was noted for the 100/109 tSL RNA, consistent with discrete higher molecular weight bands visible in higher resolution gel analyses (24). The presence of tagged precursor SL RNA indicated that all the mutants have the potential to trans-splice. An artificial tran-
script (~1.45 kb) that accumulated in each sample provided an internal control for transfection, should stability be disrupted. The presence of the exon tag in a range of high molecular weight RNA species (500 nt to 9 kb) in the tSL, 43/44, 100/109, 110/119, and 120/129 samples suggested that active trans-splicing was occurring in these transfectants (11). Splicing of 43/44 is consistent with results from a similar study in Leptomonas (12). Conversely, splicing of the tagged exon was impaired in the 42/48, 52/59, 62/69, 70/79, 80/89, and 90/99 mutants, where only substrate tSL RNA and the artifactual transcripts accumulated. The levels of accumulating tSL RNAs varied relative to the artifactual transcripts and the episomally encoded drug selectable marker mRNA NEO (data not shown). The 52/59 mutant in particular showed an increased accumulation of substrate molecules relative to other non-splicing mutants.

In addition, trans-splicing was assayed by RT-PCR (11, 29) to detect low levels of splicing (Fig. 2B). When “total” SL primer (i.e. will amplify from both WT and tSL exons) was used in the amplification, all samples showed the positive control WT amplification products, but the 28/39-tag oligonucleotide hybridized only to the tSL, 43/44, 100/109, 110/119, and 120/129 products, consistent with the total RNA blot analysis. However, using a tSL-specific primer for amplification, some level of splicing was detected in all but the 70/79 mutant. These experiments included WT L. tarentolae RNA, no reverse transcriptase, and no RNA reactions as negative controls for contamination and dependence on the use of RNA templates. Furthermore, a promoter knockout in combination with tSL (-67/− 58 + tSL (11)) was used as a control for spurious PCR amplification; this cell line resulted in an artifactual ~1.45-kb transcript containing the 28/39-tag sequence but no mature tSL RNA (shown in Fig. 5B) and did not yield a tSL PCR product. Previously determined phenotypes for cap 4 and 3′ end formation (24) are also indicated in Fig. 2. Thus, trans-splicing was adversely affected in mutants 42/48, 52/59, 62/69, 80/89, and 90/99 and appeared to be abolished in mutant 70/79.

Structural analyses of the SL RNA predict three stem-loop structures and a single-stranded region containing the Sm-binding site (Ref. 17; Fig. 1). Previously, it was demonstrated that stem-loop I is not required for trans-splicing in L. tarentolae (11). Because trans-splicing was reduced or abolished in mutants 52/59, 62/69, 70/79, 80/89, and 90/99, we considered the effects of mutations in stem-loop II, the Sm-binding site, and stem-loop III on SL RNA trans-splicing with regard to the structural or sequence elements. The mutations described below are organized with regard to both these elements and the linker scan mutation results in the following order: structural features of stem-loop II, fine analysis of the 70–81 region which includes part of stem-loop II and the Sm-binding site, and features of stem-loop III.

### Sequence and Structure of Stem-Loop II Are Necessary for trans-Splicing—Two mutations, 52/59 and 62/69, disrupted stem-loop II (Fig. 3A) and were not efficiently trans-spliced (Fig. 2). To address the importance of stem-loop II, 52/59 was further mutated to restore base pairing (52/59 + 65/72; Fig. 3A); this replaced the stem structure but with a different sequence content than WT. A further mutation was designed (42/48 + 77/80) to restore a possible extension of stem-loop II in the 45–48 region, which was disrupted by mutations 42/48 and 70/79 (Fig. 3A). 77/80 was also tested for independent effects due to its disruption of the conserved Sm-binding site. Neither
of the compensatory base pairing mutations restored trans-spli-
ing (Fig. 3B). 77/80 alone or in combination with 42/48
resulted in extended, heterogeneous 3’ end formation (data not
shown) consistent with the 70’79 phenotype (24), whereas
52/59 and 52/59 + 65/72 possessed correct 3’ ends (data not
shown). 42/48 + 77/80, 52/59 + 65/72, and 77/80 showed
undermethylated cap 4 structures (data not shown), as did 42/48,
52/59, and 70/79 (24).

Both the structure and sequence content of stem-loop II are
thus important features in the maturation of the SL RNA
precursor. The structure alone is not sufficient to direct either
cap 4 methylation or splicing. The stem-loop II extension struc-
ture may play an intermediate role in the splicing pathway, but
it is not sufficient to restore processing or splicing.

The Stem-Loop II Single Nucleotide Bulge and Sm-binding
Site Affect trans-Splicing and Cap 4 Methylation—Because
70/79 altered most of the Sm-binding site and resulted in no
trans-spliing and defects in both 5’ and 3’ end formation, we
examined the area in finer detail. A 2-bp transversion (TV)
series was created from position 70 to 81; in addition, 70/79
and 75/81 TV mutations were made (Fig. 4A). It should be
 noted that 70/71 and 72/73 comprise part of stem-loop II (see
Fig. 1) and that 74/75 TV may extend the Sm sequence
(AAUCUUUGG).

The total RNA of these transfecteds revealed a variety of
phenotypes for trans-spliing and methylation. By formalde-
yde-agarose gel analysis, only the 74/75-tSL RNA was an
efficient trans-spliing substrate, with low levels of splicing
evident in 72/73 (Fig. 4B). The presence of tSL RNA but lack of
the 1.45-kb artifact RNA in mutants 78/77, 70/79 TV, and
75/81 suggested additional increased stability phenotypes.
Primer extension revealed an intriguing gradient of SL RNA cap 4
methylation in the 70/71, 72/73, and 74/75 mutants (Fig. 4C),
which showed low (5%), medium (40%), and normal (75%)
methylation, respectively, and were trans-spliing proportional
to their methylation state. Thus, as a component of the
Sm-binding consensus, A75 does not appear to be an essential
site for cap 4 formation, transcription termination, and 3
end process-
ing (data not shown), as did 42/48, 52/59, and 70/79 (24).

We have made a series of mutations in the region down-
stream of the exon in the SL RNA gene to examine effects on
trans-spliing. We have assayed for the ability of mutated SL
RNA to trans-spli, and we have correlated this with correct
4 formation, transcription termination, and 3’ end processing
as determined here and elsewhere (11, 24). A summary of
nine phenotypes associated with the intron mutations is pre-
sented in Table I. In the majority of mutants, reduction or loss

Fig. 4. The stem-loop II bulge and Sm-binding site affect trans-
spliing and methylation. A, mutations in the 70/81 region. Nucleo-
tide transversions were used to maximize structural disruption. The
Sm-binding site (black background) and stem-loop II bulge nucleotide
(oval) are indicated. B, the trans-spliing ability of the mutants de-
scribed in A is shown in a blot of a 1.1% agarose-formaldehyde gel
hybridized with the 28/39 tag oligonucleotide. tSL is included as a
positive splicing control. Size markers (M) and 3’ end phenotypes are as
described in Fig. 2. C, variable methylation in the 70–81 mutants.
Primer extension analysis was performed. tSL is shown as a repre-
sentative of wild-type cap 4 formation. The +1 to +5 RT termination sites
are marked.

DISCUSSION

We have made a series of mutations in the region down-
stream of the exon in the SL RNA gene to examine effects on
trans-spliing. We have assayed for the ability of mutated SL
RNA to trans-spli, and we have correlated this with correct
4 formation, transcription termination, and 3’ end processing
as determined here and elsewhere (11, 24). A summary of
nine phenotypes associated with the intron mutations is pre-
sented in Table I. In the majority of mutants, reduction or loss

less than 5% methylation and had splicing that was only de-
tectable by the mutation-specific RT-PCR assay (data not
shown). In mutant 83/85 + 94/96, the tSL RNA cap 4 was
methylated to WT levels, thus the compensating mutations,
which restored stem-loop III, also restored a structural signal
for the cap 4 methylase. 88/91 showed approximately 50%
methylation (Fig. 5C) and displayed reduced splicing (Fig. 5B);
the intron tag previously used to follow SL RNA transcription
(9) was inserted into this loop and does not interfere with cap 4
methylation of tagged SL RNA (11). Thus, methylation is
linked to the formation of a minimum of 4/5 bp stem in stem-
loop III.
of trans-splicing correlates with defects in cap 4 methylation and 3' end maturation. Where cap 4 methylation and 3' end formation are WT, primary catalytic elements may have been mutated (e.g. 42/48).

The mutant phenotypes have allowed us to evaluate structures and elements that may be important for SL RNA maturation and to propose a possible pathway for discrete processing steps in L. tarentolae (Fig. 6). In this model, the T tract functions as a transcription termination element (24). The Sm-binding site and stem-loop III structure are required to allow precise 3' end maturation. Formation of the mature 3' end, along with elements within stem-loop II, are required for cap 4 synthesis, as is the 10-29 region of the exon (11). Nucleotides within the intron region of stem I are likely to be required for splicing catalysis.

Transcription of the SL RNA gene in vivo terminates in a T tract of greater than six T residues (Fig. 6, step 1). Mutation of the Sm-binding site (e.g. 76/77) and stem-loop III (e.g. 90/99) yielded mutants with defects in the nucleolytic formation of the mature 3' end of the SL RNA, demonstrating a cooperative

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**Table I**

Summary of mutant phenotypes

The symbols used are: +++, WT activity; +, reduced activity; -, <5% activity.

| trans-Splicing | 5' end, cap 4 | 3' end | Mutants, +tSL |
|---------------|---------------|--------|---------------|
| Agarose RT-PCR |               |        |               |
| + + +         | +++           | +++    | tSL (=WT), 43/44, 110/119, 120/129, 74/75, 83, 83/85 + 94/96, 106/107<sup>c</sup> |
| + + +         | +++           | +      | 100/109, 102/107<sup>c</sup>, 104/107<sup>c</sup> |
| +             | +++           | +      | 88/91         |
| +             | +             | +      | 72/73         |
| -             | +             | +      | 42/48         |
| -             | +             | +      | 52/59, 62/69, 52/59 + 65/72, 70/71 |
| -             | +             | +      | 80/89, 90/99, 83/85, 94/96 |
| -             | +             | +      | 80/81         |
| -             | +             | +      | 70/79, 75/81, 76/77, 78/79, 80/81 |

<sup>a</sup> Nucleolytic processing to base of stem-loop III.

<sup>b</sup> Staggered T track termination products.

<sup>c</sup> See Ref. 24.
FIG. 6. A summary of SL RNA elements important for 3' and 5' end formation and trans-splicing. The *L. tarentolae* SL RNA structure shown in Fig. 1 has been modified according to the data presented in this paper and elsewhere (24). The 3' end of the SL RNA precursor is shown by the “staggered termination” in the T tract, and the mature 3' end is indicated by an arrow at the base of stem-loop III. Nucleotides that have been examined by mutagenesis and do not disrupt normal SL RNA maturation or splicing have been changed to “N”; proposed base pairing interactions that are not necessary are deleted. The structures and sequences proposed to interact with maturation components, especially for cap 4 methylation, are included in the shaded oval and are discussed in the text.

function between these two elements (step 2). Structure rather than primary sequence of the stem-loop III stem was required for trans-splicing. Consistent with this, an 8-nucleotide insertion in the loop of stem-loop III in *L. collosoma* did not affect trans-splicing (25); however, in *L. seymouri* deletion of stem-loop III resulted in an actively trans-spliced and normally methylated SL RNA (12). Mutants that do not terminate accurately due to the disruption of their downstream T tract show an intermediate cap 4 phenotype (*e.g.* 100/109) that we interpret as indicative of a temporal order of 3'-processing (step 2) followed by cap 4 methylation (step 3). Methylation alone is not sufficient to confer splicing potential, since the 42/48 mutant is sufficient or no trans-splicing (22).

The essential nature of the Sm-binding site for *in vivo* trans-splicing in *L. tarentolae* agrees with data from *Ascaris*, where *in vitro* studies showed that the Sm-binding site was required for SL RNA trans-splicing (23). We are aware of the limitations in comparing *in vitro* trans-splicing assays with *in vivo* splicing phenotypes (12). We generally interpret lack of splicing phenotypes as due to splicing catalysis or, when they are detected, to maturation-related defects, but at this level of analysis our studies cannot exclude other explanations, for example impaired nucleus-cytoplasm-nucleus shuttling of the SL RNA. In contrast, a *Leptomonas* Sm-binding site mutant (“sub-Sm” (12)) that closely approximated a non-splicing, 3'-extended *L. tarentolae* counterpart (78/79) was viable for ribonucleoprotein assembly and splicing. Splicing in the 74/75 mutant, which has a transversion of the A of the Sm site, may reflect flexibility within the conserved Sm-binding site, as found in the U5 snRNA of *Saccharomyces* (33). An additional experimental difference to be considered between the two studies in trypanosomatids is that the exon tag in *L. tarentolae* consisted of 11 mutated nucleotides, whereas that in *Leptomonas* consisted of one mutated nucleotide. The contradictory results for Sm-binding site and stem-loop III in the kinetoplastids may be informative in interpreting our results as follows: given that stem-loop III does not contain primary sequence necessary for trans-splicing in *Leptomonas*, our non-splicing phenotypes may be secondary effects (*e.g.* additional 3'-extended sequences may inhibit the folding of stem-loop II).

As indicated by mutant 70/71, elements in stem-loop II are required for the intron component of cap 4 formation. In *Leptomonas*, deletion or substitution of stem-loop II above the bulge position did not affect cap 4 methylation (12), suggesting that some of the methylation phenotypes that we observed may be secondary effects due to interference with secondary or tertiary structure formation within the SL RNA itself or between the SL RNA and other splicing components. Similar to *L. tarentolae*, nucleotides in stem-loop II of the *Ascaris* SL RNA (positions 39–42 and 61–65) are essential for trans-splicing and include a single nt (U, position 62) bulge (26). Consistent with the *L. tarentolae* results and contrasting the *Leptomonas* results, deletion of nucleotides 59–68 in stem-loop II of the *Leishmania amazonensis* SL RNA (Δ1) resulted in either inefficient or no trans-splicing (8).

In this study we have identified how various structures within the intron of the SL RNA are interdependent in 3' end formation and cap 4 methylation, and we provide a possible pathway to describe the processing steps. We distinguish among trans-splicing negative mutants that are defective for discrete steps in SL RNA maturation and a mutant that may be affected in catalytic steps. These and subsequent mutants will facilitate studies on the intracellular trafficking of SL RNA, the identification of new trans-spliceosomal proteins and protein-RNA interactions, and allow testing of new models of interactions with other splicing RNA/ribonucleoproteins.

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