Chapter 2
SL RNA Biogenesis in Kinetoplastids: A Long and Winding Road

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Abstract The spliced leader (SL) RNA is a defining element in the gene expression of kinetoplastids. The first 39 nt of this small RNA are *trans*-spliced onto every nuclear message, providing a unique hypermethylated cap and sequence elements required for stability and translation. Transcribed from a large tandem array, the journey that each primary SL transcript takes en route to splicing is marked by molecular modifications. Methylation, pseudouridylation, and 3′-end nuclease processing contribute to the mature product. The consequences of this elaborate pathway are not understood fully, but may reveal distinctions that will make these oft-parasitic organisms yield their foothold in the vertebrate host.

2.1 Introduction

The spliced leader (SL) RNA defines the 5′ end of every nuclear mRNA in kinetoplastids, accounting for a huge chunk of transcriptional activity in the cell (Haanstra et al. 2008). In a multistep process, these small transcripts are extensively modified, including hypermethylation of the 5′-cap structure $m^7Gpppm^{6,6}AmpAmpCmpm^3Um$ referred to as cap 4, pseudouridylation at nucleotide 28, and removal of a 3′-extended tail (Bangs et al. 1992; Liang et al. 2002; Sturm et al. 1999).

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The SL RNA is required for kinetoplastid gene expression because of the strategy these organisms adopted for the generation of their mRNA. Transcription of protein-coding genes is polycistronic, with processing to essentially typical eukaryotic monocistronic mRNAs occurring through coupled *trans*-splicing and polyadenylation. *Trans*-splicing allows the use of RNA polymerase I in addition to RNA polymerase II for the synthesis of mRNA, as the mRNA cap structure is provided to both by the capped SL RNA transcript. Under normal circumstances, gene regulation at the level of transcription initiation is minimal, if at all, in these organisms (Clayton 2002). The predominant absence of transcriptional control sets the kinetoplastids apart from their host organisms and shunts the control of gene expression further downstream in the chain of events. As such, mRNA processing and stability, translation, and posttranslational signals functionally regulate the levels of any particular protein (Lahav et al. 2011).

In this review of SL RNA biogenesis, we will follow the path of the SL RNA from the gene to the mRNA. Although several steps in the process have been characterized in recent years, much remains to be understood about the underlying mechanisms.

2.2 Genomic Organization

The SL RNA genes in kinetoplastids are found in extensive head-to-tail tandem arrays (Fig. 2.1) that are sometimes interspersed with 5S rRNA genes (Santana et al. 2001). Gene copy number is in the range of 100–200 (Aksoy et al. 1992), making SL an excellent marker for kinetoplastid taxonomy (Murthy et al. 1992; Westenberger et al. 2004; Maslov et al. 2007). The arrays are located in one to two distinct nucleoplasmic locations proximal to the nucleolus (Dossin and Schenkman 2005; Hitchcock et al. 2007; Uzureau et al. 2008). Among different genera and/or species, the transcribed and nontranscribed regions of the array show substantial sequence variation, except for nucleotides 1–6 and 21–39 of the SL. Within a taxon, the active use of copy correction in the maintenance of gene and array integrity is indicated by the prevalence of specific alterations throughout given arrays, particularly in the intergenic regions, but also in the SL RNA gene itself (Thomas et al. 2005). The presence of a self-cleaving ribozyme in a transcript encompassing the SL RNA and 5S rRNA sequences of *Diplonema papillatum* hints at further RNA function and activity derived from these multicopy repeats (Webb et al. 2009).

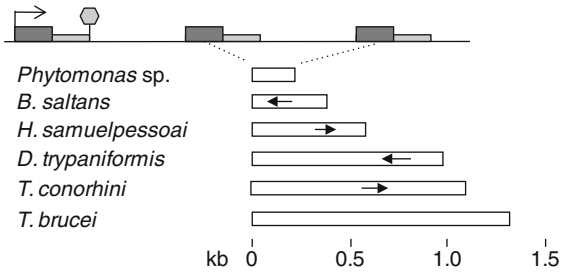

Fig. 2.1 Organization of the kinetoplastid SL RNA gene array. SL RNA genes are found as tandem head-to-tail arrays in all kinetoplastid genomes. Conserved SL sequences are shown as *dark gray boxes* and variable intron sequences are shown as *light gray boxes*. Transcription start sites and termination sites are indicated by a line *arrowhead* and a *hexagon*, respectively. The *single line* represents a variable length nontranscribed spacer that may contain a 5S ribosomal RNA gene (*solid arrowhead* within *rectangle*) transcribed by RNA polymerase III in either orientation relative to the SL RNA gene. SL RNA gene repeats representing the extremes of length and all combinations of SL RNA and 5S rRNA are shown for the trypanosomatids *Herpetomonas samuelpessoai*, *Trypanosoma brucei*, *T. conorhini*, the plant trypanosome *Phytomonas* (phloem-restricted group), and the bodonids *Bodo saltans* and *Dimastigella trypaniformis*

2.3 Transcription and Cotranscriptional Processing

2.3.1 Promoter

Two upstream elements drive SL RNA transcription by RNA polymerase II (Yu et al. 1998; Campbell et al. 2003; Günzl 2003; Palenchar and Bellofatto 2006; Martínez-Calvillo et al. 2010). A third element referred to as an initiator was defined in *Leptomonas seymouri* (Das et al. 2008), however it does not function independently of other upstream elements in bona fide initiator fashion; the equivalent sequences in *L. tarentolae* affect transcript stability, but not transcription (Saito et al. 1994). The basal transcription factors, core small nuclear (sn) RNA gene transcription factors, and the mediator complex (Lee et al. 2010) associated with SL RNA gene transcription have been identified (Das et al. 2008; Günzl 2010b; see Chap. 1 by Günzl). The promoter and coding regions are free of nucleosomes (Hitchcock et al. 2007), as anticipated for highly transcribed genes. Under stress conditions, transcription of the SL RNA genes can be shut down, most likely through the degradation of transcription factors, via a process termed spliced leader silencing or SLS (Goldshmidt et al. 2010).

2.3.2 m^7G (Cap 0) Formation

SL RNA is modified early in its synthesis by a trio of activities starting with RNA triphosphatase TbCet1 (Ho and Shuman 2001), followed by RNA

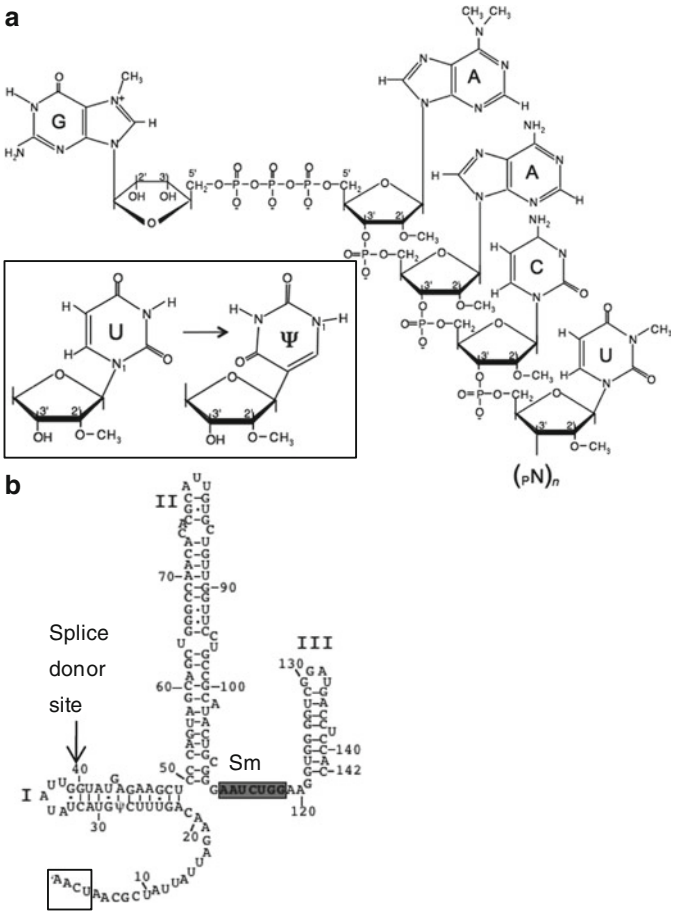

Fig. 2.2 RNA modifications and secondary structure of the SL RNA. (**a**) Chemical composition of the hypermethylated cap 4, which includes the following modifications: m^7G in the inverted cap 0 structure; 2′-*O*-ribose methylation of the first four transcribed nucleotides; ^{6,6}A-dimethylation of the first base; and ^{3}U methylation of the fourth base. The *inset* shows rotation of the uracil ring in Ψ_{28} formation from N1 to C5 relative to the C1 of ribose. (**b**) Secondary structure model of the *T. brucei* SL RNA. Stem loops I, II, and III are indicated, as are the splice-donor site and the single-stranded Sm-binding site. Cap 4 nucleotides are *boxed*

guanylyltransferase/(guanine N-7) methyltransferase, provided by the bifunctional protein TbCgm1 specialized for SL RNA processing (Ruan et al. 2007; Takagi et al. 2007). Chromatin immunoprecipitation studies support cotranscriptional recruitment resulting in the formation of the cap 0 structure on nascent SL. The precise cellular localization and functional significance of what follows is the subject of interest and debate, as studies of cap 4, the most extensive cap structure described in any system (Fig. 2.2a), and other unique aspects of SL RNA maturation, are critical for efficient function. Cotranscriptional cap 4 formation is championed by some

(Mair et al. 2000; Hury et al. 2009), while others argue for dissociation of the downstream methylations from RNA polymerase II activity (Zeiner et al. 2003a, 2004a; Zamudio et al. 2007).

2.3.3 Termination

A run of T nucleotides is the common downstream limit of the SL RNA gene, along with many other small RNA genes. This T tract leads to attenuated termination of transcription in *L. tarentolae* (Sturm et al. 1999) resulting in a primary transcript with a 3′-polyuridylate tail. The poly(U)-binding protein La has minimal or no effect on the maturation of the SL RNA (Foldynová-Trantírková et al. 2005; Arhin et al. 2005). The unpaired nucleotides at the 3′ end of the nascent SL RNA are trimmed off later in SL RNA biogenesis.

In different kinetoplastids, the mature SL RNA transcript varies in size between 96 and 142 nt (Hitchcock et al. 2004) and folds into a secondary structure consisting of three stem loops (Fig. 2.2b) that is common to SL RNAs from nematodes and many other *trans*-splicing eukaryotes (Bruzik et al. 1988); exceptions to this structural rule include the chordates *Ciona* and *Oikopleura* (Vandenberghe et al. 2001; Ganot et al. 2004) and the *Perkinsus*/dinoflagellate lineage (Zhang et al. 2007, 2011). The majority of the exon is single stranded (Harris et al. 1995), with the loop of the first stem loop adjacent to the splice donor site. The second and third stem loops, which can vary considerably in length and structure, flank a consensus single-stranded Sm-binding site that is required for SL RNA maturation and function.

Following cap 0 addition, the SL RNA embarks on a quest to obtain the additional modifications required for its maturation. How the molecule is directed through this pathway is largely undefined. The nonessential nature of the individual cap 4 modifications was surprising (Arhin et al. 2006a, b; Zamudio et al. 2006, 2007); however, the cumulative effects of these methylations are subtle but potentially vital in the natural setting (Zamudio et al. 2009a). An early step in maturation involves an SL RNA processing complex specialized for Sm-protein independent modifications in biogenesis.

2.4 Cap 1/Pseudouridylation

The activities of two SL RNA modification enzymes are linked in a molecular complex (Zamudio et al. 2009b) that resides in or near the nucleolus.

2.4.1 Pseudouridylation

Pseudouridine (Ψ) is a common RNA nucleotide modification resulting from the rotation of a uridine base relative to its ribose ring (Fig. 2.2a inset). These modifications are ubiquitous on tRNAs, snRNAs, and rRNAs, and optimize structural base-pairing interactions. The pseudouridylation at nucleotide 28 (Ψ_{28}) of SL RNA occurs prior to *trans*-splicing on the SL RNA substrate molecule, resulting in the presence of this modification on every mRNA. When and where this modification plays its biological role is unclear, but RNA–RNA interactions at both the substrate and mRNA levels leave many options.

SL RNA pseudouridylation is directed by the small nucleolar (sno) RNA SLA1 (Watkins et al. 1994; Liang et al. 2002; Hury et al. 2009). SLA1 snoRNA is transcribed with a group of three other small RNAs by RNA polymerase II (Roberts et al. 1996, 1998; Xu et al. 2001), and possesses an unusual, but undetermined 5′-cap structure (Watkins et al. 1994). Ablation of the pseudouridine synthase TbCBF5 eliminates Ψ_{28} formation on SL RNA and results in incomplete cap 4 formation (Barth et al. 2005); attempts at snoRNA silencing of the SLA1 RNA were not successful (Liang et al. 2003b). Mutagenesis of the exon at position 28 yielded no catastrophic phenotype (Sturm et al. 1998; Liang et al. 2002), however minor effects cannot be ruled out. Colocalization of SLA1 RNA with the transcription factor tSNAP42 is consistent with an early step in SL processing (Hury et al. 2009).

2.4.2 Cap 1 2′-O-Ribose Methylation

The enzyme catalyzing the cap 1 2′-*O*-ribose methylation in *T. brucei*, TbMTr1 (Zamudio et al. 2007), is a member of the Rossman Fold family of methyltransferases (Feder et al. 2003) that possesses a cap-binding domain (Mittra et al. 2008) and is the first of a series of enzymes responsible for formation of the hypermethylated cap 4. TbMTr1 methyltransferase activity is optimized for the SL RNA primary sequence (Mittra et al. 2008), which is shared by the U1 snRNA substrate at six of the first seven nucleotides. A related cap 1 methyltransferase was confirmed subsequently in humans (Bélanger et al. 2010; Werner et al. 2011). Although *T. brucei* cells can survive in culture without TbMTr1 (Zamudio et al. 2007), the maturation profile of SL RNA shifts in its absence with the accumulation of cap 0 and 3′-extended intermediates, indicating that movement through the biogenesis pathway is hindered. The mRNA population in TbMTr1$^{-/-}$ cells is methylated at the remaining cap 4 positions, demonstrating that downstream capping activities are efficient once the substrate processing delay is overcome. Accumulation of underprocessed SL RNA is not observed with knockout of the downstream Sm-protein-dependent cap methyltransferases, supporting distinct steps and localizations for maturation. Neither TbMTr1$^{-/-}$ mRNA stability nor translatability is affected in TbMTr1$^{-/-}$ background (Zamudio et al. 2009a).

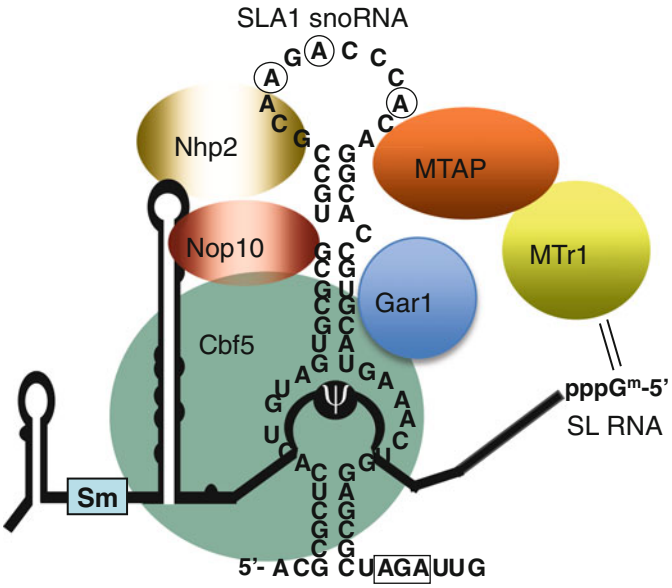

Fig. 2.3 Early modification of the SL RNA is performed by a bifunctional complex. Composition of the ribose-cap 1 methyltransferase/pseudouridylation-SLA1 snoRNA complex. The conserved pseudouridylation complex consists of four proteins (Cbf5, containing the enzymatic activity; Nop10; Gar1; and Nhp2) and a boxH/ACA small nucleolar RNA (snoRNA) scaffold (Hamma and Ferre-D'Amare 2006). The SLA1 snoRNA provides specificity for the Ψ_{28} modification by base pairing with the SL RNA (Liang et al. 2002). *Circled* nucleotides in the SLA1 RNA indicate the conserved box H; boxed nucleotides represent the AGA motif found in kinetoplastids rather than the canonical ACA (Myslyuk et al. 2008). The cap 1 methyltransferase, TbMTr1, is thought to associate with the pseudouridylation complex via the MTAP protein. Cap-binding activity of the TbMTr1 is indicated by the *double lines*. Thus far, the SL RNA has not copurified with the complex. Figure reproduced with permission from Zamudio et al. (2009b)

Furthermore, the remaining cap 4 2′-*O*-ribose methyltransferases cannot be deleted from TbMTr1$^{-/-}$ cells (Zamudio et al. 2009a), suggesting a lack of redundancy in function.

Purification of complexes containing TbMTr1 revealed an association with the pseudouridylation machinery (Fig. 2.3) that contains the SLA1 snoRNA as a specific component (Zamudio et al. 2009b). Localization of a TbMTr1-GFP fusion shows nuclear accumulation (Zamudio et al. 2007), but TbMTr1 tagged with the HA epitope results in a discrete spot within the nucleus that is likely to represent the nucleolus (Zamudio, Sturm, and Campbell, unpublished data), consistent with the typical pseudouridylation site within the cell, and possibly representing an SL RNA processing center. Characterization of the TbMTr1/pseudouridylation complex identified a component designated TbMTAP for *m*ethyl*t*ransferase *a*ssociated *p*rotein that is required for the physical link between the two enzymatic activities. This complex is specific to SL RNA processing as the SLA1 snoRNA, but not other snoRNAs, is associated with the purified complex, and elimination of TbMTAP

reduces both SL RNA Ψ_{28} and cap 1 formation, but does not affect U1 snRNA cap 1 levels (Zamudio et al. 2009b). Initially, the TbMTr1/pseudouridylation association was thought to be yet another kinetoplastid-specific development to accommodate SL RNA processing; however, a homologous WD40-domain protein has been linked to Cajal body localization of small Cajal body RNAs or scaRNAs in humans (Tycowski et al. 2009).

Acquisition of early modifications involves the transporter exportin 1, or Xpo1 (Zeiner et al. 2003a; Biton et al. 2006). TbXpo1 inactivation by the toxin leptomycin B results in nuclear accumulation of SL RNA that is trapped in a cap 0-modified and 3′-end extended state, and cell death. Given the localization of TbMTr1 and the SL RNA cap 0 phenotype in leptomycin B-treated cells, TbXpo1 interaction with the SL RNA appears to occur before the cap 1 methylation, and suggests a function of TbXpo1 in the transport of the nascent transcript into the nucleolus for the first set of posttranscriptional modifications, similar to its role in U3 snoRNA trafficking (Boulon et al. 2004) rather than the proposed nuclear export function that predicted a cytosolic localization of the cap 1 enzyme (Zeiner et al. 2004b). In mammals, Xpo1 binds to neither the 5′ cap nor the nuclear cap-binding protein CBP20 directly (Ohno et al. 2000), implying that other players (such as PHAX in humans) remain to be identified. Whether a putative cap-binding protein is temporarily displaced by TbMTr1 or replaced by another chaperone for subsequent maturation steps is unclear.

2.5 Sm Association

The assembly of the heptameric Sm protein complex on the SL RNA represents a distinct stage in the maturation of substrate SL without which the molecule is stalled at a cap 1, 3′-end extended form that does not participate in *trans*-splicing. The composition of the SL RNP has been inferred by tandem-affinity tagging (Luz Ambrosio et al. 2009; Palfi et al. 2009; Tkacz et al. 2010). The seven canonical Sm proteins are associated with the SL RNA (Table 2.1). In contrast, variant SmD3 proteins Ssm4 and 16.5 K/Ssm2-2 are associated with the U4 and U2 snRNA, respectively, the variant SmB protein 15 K/Ssm2-1 with the U2 snRNA (Wang et al. 2006; Tkacz et al. 2007; Günzl 2010a; Jaé et al. 2010). The question of where this Sm-complex assembly occurs is controversial, with support on both the nuclear and cytosolic sides of the argument.

The cytosolic trafficking model invokes the nuclear egress of substrate SL RNA at the cap 1/3′-extended form to acquire the Sm-protein complex that then signals reimport of the SL RNP into the nucleus for completion of SL maturation. Mutation of the Sm-binding site (Sturm and Campbell 1999; Sturm et al. 1999) or knockdown of an Sm subunit (Mandelboim et al. 2003; Zeiner et al. 2004a) yielded similar immature/nonfunctional cytosolic SL RNA species, supporting the transit of SL RNA through the cytosol to acquire the Sm-protein complex in a manner analogous to the pathway in humans (Neuenkirchen et al. 2008). Copurification of importin

Table 2.1 Proteins involved in SL maturation

Sm-independent		Sm assembly		Sm-dependent	
Cap 0		SMN	Tb11.01.6640	**Cap 2**	
TbCet1	Tb927.3.2190	Gemin2	Tb927.10.5640	TbMTr2/TbCom1/ TbMT48	Tb11.02.2500
TbCgm1	Tb927.7.2080	SmB	Tb927.2.4540	TbMTr3/TbMT57	Tb09.211.3130
Cap 1		SmD3	Tb927.4.890	**Cap 4**	
TbMTr1	Tb927.10.7940	SmD1	Tb927.7.3120	[TbMTr3?][a]	
[TbMT^{66}A]		SmD2	Tb927.2.5850	[TbMT^{3}U]	
Ψ_{28}		SmF	Tb09.211.1695	**3′ end formation**	
TbCBF5	Tb10.100.0060	SmE	Tb927.6.2700	[poly(U) removal]	
TbGar1	Tb927.4.470	SmG	Tb11.01.5915	TbSNIP	Tb927.8.3710
TbNhp2	Tb927.4.750	PRP19	Tb927.2.5240		
TbNop10	Tb10.70.2465				
TbMTAP	Tb11.01.8210				
Other					
TbXpo1	Tb11.01.5940				

Square brackets [ ] represent proteins/activities that have yet to be identified
TbMTr3 may have dual activity including cap 4 ribose methylation

α with PTP-tagged SmB (Palfi et al. 2009) and SmD3 (Tkacz et al. 2010; Martinez, Zamudio, Sturm and Campbell, unpublished) may be a footprint of a trafficking pathway.

The nuclear Sm-assembly model was hypothesized as part of a cotranscriptional processing scenario (Mair et al. 2000) and is supported by colocalizations within a proposed SL RNP factory (Tkacz et al. 2007; Hury et al. 2009). The Sm-assembly factor SMN localizes to the nucleus also (Palfi et al. 2009; Jaé et al. 2011), consistent with nuclear Sm-complex formation. SMN specificity for assembly of the SL RNP, but not the U2 snRNP, has been demonstrated (Palfi et al. 2009) and is likely due to interaction of SMN with the canonical SmB/SmD3, but not the corresponding Sm variants found in the U2 snRNP. Additionally, the trypanosome SmB possesses a nuclear localization signal that is functional in human cells (Girard et al. 2004). Under this scenario, assembly of the Sm complex within the nucleus, likely after cap 1 2′-*O*-ribose methylation, would be required to transport the immature substrate to the next stage of processing. In this model, the cytosolic accumulation of SL RNA would be part of a turnover pathway for defective molecules termed spliced leader discard or SLD (Lustig et al. 2007; Goldshmidt et al. 2010). Conditional knockdown of the assembly factor SMN and the general splicing factor PRP19 also results in incomplete cap 4 formation on the SL RNA, but not cytosolic accumulation (Tkacz et al. 2010).

2.6 Cap2/Cap3/Cap4/3′ Processing

2.6.1 Sm-Dependent Cap 2′-O-Ribose Methylation

Two related enzymes supply the cap 2′-*O*-ribose methylations at positions 2 and 3, called TbMTr2 (aka TbCom1 and TbMT48) and TbMTr3 (aka TbMT57), respectively (Arhin et al. 2006a, b; Hall and Ho 2006; Zamudio et al. 2006). One possibility is that TbMTr3 may catalyze the position 4 methylation as well (Arhin et al. 2006a; Zamudio et al. 2006) as cap 3 is not seen in the absence of cap 4 in vivo; however, this remains to be demonstrated experimentally. In contrast to TbMTr1, phylogenetic analysis suggests a common origin with the Vaccinia virus cap 1 2′-*O*-ribose methyltransferase with subsequent specialization for modification of downstream nucleotides (Feder et al. 2003).

Generally diffuse, nucleoplasmic localizations for both enzymes are indicated, segregating their activities from that of the nucleolar TbMTr1/pseudouridylation steps. The arguments for cotranscriptional cap 4 formation include the finding that the position 2, 3, and 4 2′-*O*-ribose methylations did not appear on tethered, nascent transcripts until the SL RNA length reached position 117 and beyond in *T. brucei* (Mair et al. 2000). That position corresponds to the 3′-end of the Sm-binding site, so if these truncated transcripts were released from the transcription apparatus, the ability for the Sm-protein complex to form may have been the discriminating factor for the subsequent methylation events. The spatial separation of the cap 1 TbMTr1 in the nucleolus and TbMTr2 and TbMTr3 in the nucleoplasm as well as different processing defects upon cap methyltransferase depletion supports the original observation that cap 4 formation occurs posttranscriptionally (Ullu and Tschudi 1995).

Procyclic *T. brucei* survive the loss of either or both TbMTr2 and TbMTr3 in culture, and mRNA cap analysis determined that undermethylated substrate SL RNA are comparable substrates for *trans*-splicing suggesting downstream function. Consistently, translation rates decrease in proportion to the loss of cap methylation, an effect further exacerbated when the cells are under stress (Zamudio et al. 2009a). The basis for this reduction is unclear, but as stability of the SL RNA is unaffected, the answer could lie in reduced levels of cap recognition by the multitude of trypanosome cap-binding proteins (Li and Tschudi 2005; Dhalia et al. 2005; Yoffe et al. 2006) producing downstream effects on nuclear to cytoplasmic transport and/or translation initiation. The consequences of reduced translation rates are tolerated in the isolation of culture conditions; however, a cell population at such a disadvantage would be eliminated in short order by their brethren with fully capped SL.

SL RNA in *L. tarentolae*, *T. brucei*, and *L. collosoma* mutated in the central portion of the exon present intriguing combinations of cap 4 phenotypes (Lücke et al. 1996; Sturm et al. 1998; Xu et al. 2000) in light of the *T. brucei* capping-enzyme deletion mutants. Transcripts with sequence alterations in the exon region of nucleotides 10–29 are not recognized by TbMTr2 or TbMTr3. These transcripts

are *trans*-spliced (Sturm et al. 1998), but are not loaded into polysomes (Zeiner et al. 2003b). Since the cap 1 mRNA is translatable in *T. brucei*, the reason for the absence of polysome formation on the mutated *L. tarentolae* SL may not be a function of cap recognition, but of base complementarity with the translation machinery. Still, undermethylation suggests a delay in processing, possibly due to defective recognition by the cap 2, 3, and 4 2′-*O*-ribose methyltransferases. Another interesting possibility is the presence of a physical block on the cap 1 structure in the *L. tarentolae* cells that cannot be removed to allow further cap processing or translation in the exon-mutated lines. The presence of this block could be a useful, and likely essential, element in the cytosolic trafficking scenario, in which substrate SL could efficiently compete for mature mRNA, to the detriment of the translation rate. Removal of the 5′ block upon re-entry into the nucleus would expose the 5′ end to further cap 4 maturation and allow subsequent access by translation initiation factors. Hence, exon mutagenesis disrupted recognition by the same machinery that interacts with the central exon region, and the 5′ block is not effectively removed. In the nuclear Sm assembly scenario, shielding of the substrate SL RNA cap structure would equally be a necessary precaution to prevent premature export of the unspliced molecule.

2.6.2 3′ Processing

Maturation of the SL RNA 3′ end involves the removal of the last 4–6 transcribed nucleotides and is performed by a minimum of two nucleases. The identity of the first of these enzymes is unknown. The final product is created by a general nuclease, named SNIP for *sn*RNA *i*ncomplete *p*rocessing (Zeiner et al. 2004b), that trims off the final nucleotide or two from a variety of snRNAs including the SL RNA, where the mature end is defined by the base of stem loop III. Knockdown of TbSNIP is lethal; however, the direct cause of this effect is unclear as multiple substrates are affected. As 3′ processing does not initiate until after Sm-complex formation, either some physical barrier exists to prevent association of the immature substrate SL and the 3′ nucleases, or the physical binding by the Sm proteins is required for the stable formation of stem loop III and definition of the mature 3′ end. If a spatial separation takes place within the nuclear environment or via partitioning by the nuclear envelope remains to be determined.

2.6.3 Base Methylation

In addition to the 4 cap 2′-*O*-ribose methylations, the SL receives three additional methylations, two on the first base ($m^{6,6}A$), and one on the fourth that is unique to kinetoplastids (m^3U). The identity of the methyltransferases performing these modifications is unknown, but their activity appears to follow a 5′-to-3′ polarity

(Mair et al. 2000) and is reduced in the absence of the 2′-*O*-ribose methylations (Zamudio et al. 2009a). Two candidate $m^{6,6}A$ dimethyltransferases are present in kinetoplastid genomes that are thought to act upon the ribosomal RNAs of the cytosol and mitochondrion. If the nuclear enzyme also catalyzes base methylation on the SL RNA remains to be determined. The likely nucleolar localization of this enzyme for both SL RNA processing in tandem with cap 1 2′-*O*-ribose methylation and ribosomal modification is consistent with a dual substrate function. The distinctive features that the substrates present to the enzyme, with this conserved ribosomal modification found near the 3′ end of the small subunit versus the extreme 5′-end position of the SL RNA site, cast some doubt on a combined action. No specific candidates have come to light for the m^3U methylation, but other methyltransferases with undetermined specificities remain to be explored experimentally.

2.6.4 Cap 4 Binding by CBP20

In mammalian cells the CBP20 component of the nuclear cap-binding complex CBC is involved in transport of snRNAs to the cytosol. In trypanosomes, the CBP20 homolog preferentially binds cap 4 over cap 0, suggesting its interaction with the SL RNA is late in the biogenesis pathway (Li and Tschudi 2005). RNAi inhibition of TbCBP20 results in the accumulation of the SL RNA, suggesting it functions in bringing the mature SL RNP to the site of *trans*-splicing (Li and Tschudi 2005). By analogy with the mammalian homolog, TbCBC may accompany *trans*-spliced mRNA to the cytosol (Li and Tschudi 2005). The interaction of TbCBP20 with substrate SL, mRNA, or both SL forms remains to be demonstrated in vivo.

2.7 *Trans*-Splicing

From the coding region perspective, the requirements for *trans*-splicing are defined loosely as the presence of a GU splice-donor site in the SL RNA and two elements in the pre-mRNA: an AG splice-acceptor site and an upstream polypyrimidine tract (Matthews et al. 1994). Transcriptome analysis documents differential usage of alternative splice acceptor sites for a large number of genes (Siegel et al. 2010; Nilsson et al. 2010; Kolev et al. 2010), while experimental and computational approaches should elucidate the mechanistic rules for the selection of specific sites (Benz et al. 2005; Gopal et al. 2005; Siegel et al. 2005). These splicing events have the potential to impact transcript translation through control of stability, localization, and translatability based on the sequence present or absent from the 5′ untranslated region of the mRNA. The much-anticipated development of an

in vitro *trans*-splicing system (Shaked et al. 2010) will enable functional testing of the *trans*-splicing mechanism.

Mechanistically, *trans*-splicing is similar to *cis*-splicing (Liang et al. 2003a; see Chap. 3 by Jaé et al.) except that the SL RNA itself appears to replace the U1 snRNA as a component of the spliceosome. Any truly catalytic residues of SL RNA have not been determined, but some of the intron residues in the vicinity of stem loop II have been implicated. Exon-tagged SL that carry mutations in the 40–50 nt region yield molecules that are processed accurately, but not *trans*-spliced in *L. tarentolae* (Sturm and Campbell 1999). Various proteins copurify with tagged Sm proteins representing spliceosomal snRNPs and general splicing factors (Luz Ambrosio et al. 2009; Palfi et al. 2009; Tkacz et al. 2010). Unlike for the nematode SL RNP (Denker et al. 2002; MacMorris et al. 2007), no proteins unique to the kinetoplastid SL RNP have been identified so far.

2.8 Translation

The eukaryotic translation process begins with an interaction between the mRNA cap and cap binding protein eIF4E, which displaces the CBC in higher eukaryotes. Kinetoplastids follow this convention, but possess a wider array of eIF4E family members with a variety of cap binding affinities for cap 0 through cap 4 (Clayton and Shapira 2007; see Chapt. 9 by Shapira). Likewise, other proteins associated with ribosomal loading have a multiplicity of homologs, potentially resulting in a level of nuanced translational control not found in other eukaryotes. The other participants in the eIF4F complex, eIF4G (Dhalia et al. 2005; Yoffe et al. 2009) and eIF4A (Dhalia et al. 2006), have six and two homologs, respectively. This shift in gene expression control may be a functional consequence of the absence of individual gene promoters in these organisms. Differential assembly of these factors must use mRNA information beyond the SL, such as that contained in the 5′- and 3′-untranslated regions.

2.9 Conclusion

Production of the SL RNA is a major undertaking for kinetoplastids and has evolved into an efficient, specialized process. From the hypermethylated 5′ end through Ψ_{28} formation, protein assembly, and the trimming of the 3′ end, each molecule is shuttled through a chain of events that each play an important role in SL substrate or mRNA function. The sheer quantity of RNA flowing through this pathway makes the existence of devoted SL processing centers likely. As a strategy in gene expression for eukaryotes, the *trans*-splicing of polycistronically transcribed transcripts has either been retained or has emerged multiple times throughout the tree of life with a remarkably low level of variation on the SL RNA theme

(Nilsen 2001; Hastings 2005). Thus, kinetoplastids have not simply maintained an archaic mode of genetic control, but have maximized the use of a viable pathway and the downstream processes that characterize this group of widespread and highly successful organisms.

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