Silencing of Sm Proteins in Trypanosoma brucei by RNA Interference Captured a Novel Cytoplasmic Intermediate in Spliced Leader RNA Biogenesis*

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In Trypanosoma brucei the small nuclear (sn) RNAs U1, U2, U4, and U5, as well as the spliced leader (SL) RNA, bind the seven Sm canonical proteins carrying the consensus Sm motif. To determine the function of these proteins in snRNA and SL RNA biogenesis, two of the Sm core proteins, SmE and SmD1, were silenced by RNAi. Surprisingly, whereas the level of all snRNAs, including U1, U2, U4, and U5 was reduced during silencing, the level of SL RNA was dramatically elevated, but the levels of U6 and spliced leader-associated RNA (SLA1) remained unchanged. The SL RNA that had accumulated in silenced cells lacked modification at the cap4 nucleotide but harbored modifications at the cap1 and cap2 nucleotides and carried the characteristic ψ. This SL RNA possessed a longer tail and had accumulated in the cytoplasm in 10 and 50 S particles that were found by in situ hybridization to be present in “speckles.” We propose a model for SL RNA biogenesis involving a cytoplasmic phase and suggest that the trypanosome-specific “cap4” nucleotides function as a signal for export and import of SL RNA out and into the nucleus. The SL RNA biogenesis pathway differs from that of U sn ribonucleoproteins (RNP)s in that it is the only RNA that binds Sm proteins that were stabilized under Sm depletion in a novel RNP, which we termed SL RNP-C.

Pre-mRNA splicing in trypanosomes is an essential step in gene expression, because all mRNAs undergo trans-splicing (1). trans-Splicing evolved to separate long polycistronically transcribed mRNAs that are dissected by the concerted action of the trans-splicing and polyadenylation (1). Recent studies revealed the existence of cis-splicing, but it is currently unknown how many genes are processed via this mechanism (2).

During the trans-splicing reaction, the spliced leader is donated to pre-mRNA from a small RNA, the SL1 RNA (1). Mechanistically, trans-splicing is related to cis-splicing and requires the concerted action of snRNPs carrying the U2, U4, U5, and U6 snRNAs (3). Trypanosomes encode for splicing factors such as U2AF35, helicases, heterogeneous nuclear RNP proteins, and serine/arginine rich proteins, but their role in trans- and cis-splicing has not yet been established (4).

One unique property of the snRNAs that participate in trans-splicing is their different cap structure. The SL RNA possesses a complex cap structure known as “cap4,” which consists of 2′-O-methylation of the sugar groups of the 4-nt AACU, with additional methylation on the bases of the first (m6A) and the fourth (m7U) nucleotides (5–7). A correct hypermodified cap4 was shown to be essential for trans-splicing (8, 9). The U2 and U4 possess a 2,2,7-trimethylated guanosine (TMG) cap, whereas the U6 has an inverted cap (10) and the U5 lacks TMG and possesses a 5′-phosphate terminus (11, 12).

The SL RNA is transcribed from a distinct well defined promoter by RNA polymerase II (13). Termination of SL RNA transcription occurs on a T-tract, and longer 3′ tails were observed in Leishmania tarentolae mutants altered at either the Sm site or terminal stem-loop III, suggesting that the SL RNA undergoes trimming at the 3′ end (14).

The SL RNA was shown to exist in a small RNP, namely the SL RNP (15, 16). Structure-function analysis of the SL RNA identified domains within the exon (9, 17) and the intron (9, 18) that are essential for trans-splicing. A co-transcriptional model for cap4 methylation was proposed that provides evidence that modification of the first 2 nt takes place on short nascent SL RNA transcripts. Modification of the fourth cap nucleotide took place only after the Sm binding site was transcribed (19). Recently, studies in L. tarentolae and Trypanosoma brucei provided evidence that the SL RNA is also present in the cytoplasm and does not reside only in the nucleus (20). Treatment of these parasites with the karyopherin-specific inhibitor leptomycin B eliminated the cytoplasmic SL RNA, and SL RNA export was shown to be mediated by the nuclear exporter, exportin 1 (XPO1). Interestingly, treatment with leptomycin B resulted in the accumulation of SL RNA with a 3′ tail, which lacked the modification on the cap4 position, suggesting that a cytoplasmic stage is necessary for SL RNA biogenesis (20).

In metazoa, U snRNPs biogenesis is a complex process. Briefly, the snRNAs are transcribed by polymerase II and acquire a monomethylated (m7G) cap (21). These snRNAs are first exported to the cytoplasm assisted by several factors, including the cap-binding complex (22), the export receptor Xpo1p (23), and an additional adaptor for RNA export, PHAX (24). Upon their export to the cytoplasm, U snRNAs interact with the seven Sm proteins to form the snRNP core complex. In vitro experiments indicate that the Sm core is assembled in a stepwise manner. The subcomplexes, D1-D2 and E-F-G, bind concomitantly to the Sm site, forming a subcore complex, and assembly is completed by its association with the B-D3 complex.

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1 The abbreviations used are: SL, spliced leader; snRNA, small nuclear RNA; snRNA, small nuclear RNA; SLA, spliced leader-associated RNA; TMG, 2,2,7-trimethyl guanosine; DIG, digoxigenin-11; ψ, pseudouridine; SMN, survival of motor neurons; CMc, N-cyclohexyl-N′-β-(4-methylmorpholinium/ethylcarboximidate p-tosylate; RNAi, RNA interference; RNP, ribonucleoprotein; RT, reverse transcriptase; nt, nucleotide(s); ds, double-stranded; PAP, poly(A) polymerase; Bicine, N,N-bis(2-hydroxyethyl)glycine; PIPES, 1,4-piperazinediethanesulfonic acid.

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2. Sm proteins, which share the conserved structural motifs of the U6 snRNP, are thought to take place entirely in the nucleolus. The U6 snRNA is the only spliceosomal RNA that has a different

material and methods

4. All the oligonucleotides were specific for T. brucei. Oligonucleotides for Synthesis of 7SL RNA and the SL RNA Probes—953618 (5'-CCCACGGAAAACACTAGGGC-3', antisense) was complementary to the SL RNA from positions 65 to 79. Oligonucleotides Used for RT-PCR of the Poly(A) Polymerase Gene—850143 (5'-CCCAACGAAAACACTAGGGC-3', antisense) was specific for the poly(A) polymerase gene (PAP), first exon, from positions 223 to 242.

3. After snRNP assembly in the cytoplasm, the snRNA is imported back to the nucleus by a special import factor, Snuporin-1, which also interacts with the general import factor importin b, both of which are required for U snRNP import (33, 34). The snRNAs re-enter into the nucleus, the snRNAs and snRNP biogenesis.

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1. In this study, we silenced two Sm proteins, namely SmE and SmD1, by RNAi, using either the expression of stem-loop RNA from the inducible procyclin (EP) promoter or from the inducible T7-opposing promoters (42). Silencing was lethal, and both trans- and cis-splicing were inhibited. The reduction of the Y structure intermediate suggests that trans-splicing was inhibited at the first step of splicing. As expected, the level of U1, U2, U4, and U5 snRNAs was reduced. Surprisingly, the level of SL RNA was elevated; the level of U6 and the spliced leader RNA (SLA1) did not change, suggesting that U6 and SLA1 RNAs bind different core proteins. The SL RNA accumulated in the cytoplasm; it carried a long 3' tail and lacked the modification at the cap 4 nt, but it carried the 3' end trimming (30). Both of these events are dependent on proper Sm core assembly. In budding yeast, it is not clear whether snRNP assembly involves a cytoplasmic phase. A yeast homologue of SMN is absent in T. brucei.

3. Trypanosomes carry Sm proteins that bind to the U snRNAs and SL RNA (38–40). As their counterparts in other eukaryotes, the trypanosome proteins have the potential to form the canonical heptameric ringlike structure. The Sm proteins carry a bipartite Sm motif, including interesting deviations from the Sm consensus. Interestingly, the SmD1 and D3 lack the C-terminal RG dipeptide repeats of the human homologue. The arginines in this domain are methylated in the mammalian proteins (28), constituting an important determinant of the SmD1 depletion of SmD1. Under SmD1 depletion, splicing was inhibited. The reduction of the Y structure was specific for

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mixture was diluted from 1:10 to 1:10,000, and 200 µl were portioned as aliquots onto the microtiter plate. Next, the microtiter plate was incubated in a humid chamber at 27 °C in a 5% CO₂ atmosphere. After 2–3 weeks, a clonal population was obtained in the microtiter plates and the cells were transferred to medium for propagation. Cells from cultures that showed typical growth arrest upon tetracycline induction were grown and frozen. Every 2 weeks a new culture was started from the original frozen stock.

Preparation of Nuclear and Cyttoplasmic Extracts—T. brucei procyclins (10%) were harvested and washed with phosphate-buffered saline. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 5 µM leupeptin). Next, the cells were broken by 20 strokes in a Dounce homogenizer in this buffer. Nonidet P-40 (4% v/v) nuclei were washed twice with 10,000 × g. The pellet and the cytoplasmic fractions were deproteinized and the RNA subjected to primer extension with the different probes.

Northern Analysis—Total RNA was prepared with TRIzol reagent (Sigma), and 20 µg/lane was fractionated on a 1.2% agarose, 2.2 M formaldehyde gel. The RNA was visualized with ethidium bromide. The SmE and SmD1 mRNA and tubulin mRNAs were detected with randomly labeled probes (Random Primer DNA Labeling Mix, Biological Industries Co.). For analyzing small RNAs, total RNA was fractionated on a 10% polyacrylamide gel containing 7 M urea. The RNA was transferred to a nylon membrane (Hybond; Amersham Biosciences) and probed with γ-³²P-end-labeled oligonucleotides or antisense SL RNA probe. The antisense SL RNA probe was transcribed in vitro by T7 polymerase using a PCR product encoding for the SL RNA gene and carrying the T7 promoter as a template.

Primer Extension and Mapping the Pseudouridine on the SL RNA—Primer extension was as described previously (12). Total RNA or mRNA from nuclear and cytoplasmic fractions (10 µg) was used in a primer extension reaction with 100,000 cpm (γ-³²P)-ATP-labeled oligonucleotide. After annealing at 55–60 °C for 15 min and chilling on ice for 2 min, 1 unit of reverse transcriptase (Expand RT, Roche) and 1 unit of RNase inhibitor (Promega) were added and an extension reaction was performed at 42 °C for 90 min. The extension products were ethanol-precipitated and analyzed on 6% polyacrylamide, 7 M urea gel. For determination of the SL RNA, RNA from the nuclei was divided into two halves. Half was treated with 30 µl of CMC buffer (0.17 M CMC in 50 mM Bicine, pH 8.3, 4 mM EDTA, 7 M urea) at 37 °C overnight. After hybridization, the cells were washed three times with 50% formamide, 2 × SSC at 42 °C. Finally, slides were washed three times for 5 min at room temperature. For detection of the DIG-labeled probes, slides were incubated for 45 min at room temperature with 1/200 diluted fluorescein isothiocyanate-conjugated mouse anti-DIG (Roche). To stain the nucleus, the cells were stained with propidium iodide (10 µg/ml) for 10 min. The cells were visualized by using a Bio-Rad MRC 1024 upright confocal microscope with a krypton-argon ion laser, as previously described (46).

Western Analysis—Whole cell extracts of induced and uninduced cells were prepared from 5 × 10⁸ cells as previously described (16). The extracts were fractionated on a DEAE column, and the column eluate was analyzed by Western analysis. Proteins were fractionated on SDS-PAGE (12%) gel, transferred to NitroBind (Micron Separations, Inc.), and probed with the antibodies. The anti-CP antibodies (kindly provided by Albrecht Bindereif) were diluted 1:1000 (43). The bound antibodies were detected with goat anti-rabbit bound to horseradish peroxidase and visualized by ECL (Amersham Biosciences).

RNase Protection—Total RNA (10–30 µg) was mixed with 100,000 cpm of gel-purified RNA probe and concentrated by ethanol precipitation. The pellet was washed and dissolved in hybridization buffer (40 mM PIPES, pH 6.4, 80% formamide, 0.4 mM sodium acetate, 1 mM EDTA). After having been boiled for 1 min, the samples were incubated at 42 °C for 14–16 h. After the hybridization, the samples were diluted 1:10 with a solution consisting of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM sodium acetate, containing 2.5 units/ml RNase ONE (Promega). The digestion was performed at 30 °C for 1 h and terminated by 20 µl of 4 M ammonium acetate for 10 min. After phenol-chloroform extraction, the protected products were precipitated with ethanol in the presence of 20 µg of glycogen and analyzed on a 6% polyacrylamide, 7 M urea denaturing gel.

RT-PCR—The total RNA was extensively treated with DNase inactivation reagent (DNA-free; Ambion) to remove the DNA contamination. The transcription was performed with DmRED primers. The samples were heated for 5 min at 95 °C, followed by annealing for 15 min at 60 °C. After having been chilled on ice for 2 min, 1 unit of reverse transcriptase (Expand RT, Roche) and 1 unit of RNase inhibitor (Promega) were added, and the reaction was performed at 42 °C for 60 min. Next, the cDNA was used in PCR amplification. The nested PCR was performed with cDNA that was directly used for PCR.

In Situ Hybridization—PCR for preparing the digoxigenin-11 (DIG)-labeled DNA probe was performed under standard conditions using the expand high fidelity enzyme (Roche Molecular Biochemicals). The dNTP mixture contained 200 µM amounts of each of the nucleotides except dTTP (130 µM) and DIG-dUTP (70 µM). The conditions for fixation and hybridization were similar to those published previously (46). Microscopic slides were prepared by dropping 20 µl of fixed-cell suspension on glass slides. The slides were allowed to air-dry. Before hybridization, the slides were subjected to partial hydrolysis and proteolysis by using 0.1% pepsin in 0.01 M HCI for 5 min at 37 °C. The DIG-labeled PCR product was in 80% deionized formamide, 2 × SSC, 50 µM sodium phosphate buffer, and 500 µg/ml denatured salmon sperm DNA. Hybridization was performed at 42 °C overnight. After hybridization, the slides were rinsed three times for 20 min each in 50% formamide, 2 × SSC at 42 °C. Finally, slides were washed three times for 5 min at room temperature. For detection of the DIG-labeled probes, slides were incubated for 45 min at room temperature with 1/200 diluted fluorescein isothiocyanate-conjugated mouse anti-DIG (Roche). To stain the nucleus, the cells were stained with propidium iodide (10 µg/ml) for 10 min. The cells were visualized by using a Bio-Rad MRC 1024 upright confocal microscope with a krypton-argon ion laser, as previously described (46).

RESULTS

Silencing of SmE and SmD1 by the T7-opposing Promoters and by the Production of Stem-loop RNA—As a first step toward understanding the role of Sm proteins in trypanosomes, as well as to determine unequivocally which snRNAs bind to Sm proteins, we silenced, by using RNAi, two of the core proteins, SmE and SmD1, by utilizing two approaches. The SmE protein was silenced by producing dsRNA from two T7-opposing promoters. A 258-bp fragment was amplified from genomic DNA by using PCR and then cloned into the pZJM vector carrying the two T7-opposing promoters under the control of the tetracycline repressor (42). The SmD1 was also amplified from the genome, and a stem-loop construct was designed. The construct was composed of a 317-nt stem and a 500-nt loop derived from the pex gene (42). This construct was cloned into the pZJM vector, allowing its expression from the pex promoter. Its expression is controlled by the tetracycline repressor. In both constructs, the vector carried a phleomycin-resistant gene, which is under the control of the T7 promoter and RNA spacer region, which enables its integration into the non-transcribed rRNA spacer. After linearizing the constructs, the DNA was used to transfect T. brucei (29–13) cells that express the tetracycline repressor as well as the T7 polymerase (42). One day after transfection, the cells were diluted onto the microtiter plates to obtain a clonal population. After 3 weeks, the cloned cells were used to establish pure cultures that were examined for inhibition of their growth upon the addition of tetracycline. The clones that were clearly arrested upon the addition of the drug were chosen for further analysis. Fig. 1A shows the growth of the SmD1 cell line and clearly indicates that the cells depleted in SmD1 cannot survive. The arrest in growth was observed 2 days after tetracycline induction. Interestingly, despite the fact that cells cannot grow under conditions of SmD1 depletion and eventually died, no apparent morphological changes were observed such as the changes we observed during the silencing of the signal peptide-binding protein SRP54p (47).

The correlation between the arrest in growth and a reduction of the level of the corresponding mRNA was examined by Northern analysis, as presented in Fig. 1B. The results indicate a 100% reduction with SmD1 and a reduction of ~85% of the SmE that appeared 2–3 days following tetracycline induction. The reduction of the RNA was accompanied by an increase in dsRNA production, and its level was much higher in cells induced by the T7-opposing system compared with the cells silenced by producing dsRNA in the form of a stem-loop. However, silencing of SmD1 was more stable and the silencing was not lost even when incubation was prolonged, whereas SmE silencing was lost after 10 days. The instability of silencing by the T7-opposing system was recently reported and attributed to rearrangements and excision of the silencing information (48).
RNAi Silencing of Sm Proteins in Trypanosomes

To control the level of RNA loaded on these gels, we used the 5SrRNA as a control, because the level of mRNAs were also changed during the silencing (see next section).

To examine the depletion of the SmD1 protein under silencing, we fractionated extracts on a DEAE-Sephacel column, as previously described, to enrich the snRNPs (16). The particles obtained from the DEAE-eluate were subjected to Western analysis with anti-CP antibodies that were generated against the low molecular weight core proteins that were purified by affinity selection of the T. brucei U2 snRNP (43) and were later shown to be the Sm proteins (40). The results, presented in Fig. 1C, indicate a marked reduction in the level of core Sm proteins, not only at the level of protein that corresponded to the size of D1 (12.5 kDa). The anti-CP recognizes a strong band in the 12-kDa region, which corresponds to the reactivity with several Sm proteins. The reduction in the reactivity with the core proteins may suggest that, in the absence of a single Sm protein, the heptameric Sm ring cannot form and consequently these proteins are prone to degradation. The nonspecific reactivity of the −26-kDa protein was used as a control for equal loading of proteins on the gel.

Sm Silencing Inhibited trans- and cis-Splicing—SmD1 depletion in budding yeast resulted in inhibition of splicing already at the first step of splicing (41). To examine whether this is the case also in trypanosomes, we set up several assays to monitor inhibition in splicing. To monitor for trans-splicing inhibition, we examined the level of the Y structure intermediate; this level reflects the defects that affect the first or second step of splicing. trans-Splicing inhibition should result in a reduction of the level of mature mRNAs, along with an accumulation of pre-mRNA precursors. We chose the tubulins that are found in reiterated repeats coding for the α- and β-tubulin, to monitor the accumulation of pre-mRNA precursors. It is expected that when trans-splicing is inhibited, polycistrionic precursors will accumulate as was observed when trans-splicing was inhibited in the presence of the methylation-specific inhibitor sinefungin (49). The effect of SmD1 silencing on trans-splicing is presented in Fig. 2. The level of the Y structure was monitored by primer extension using an oligonucleotide complementary to the SL RNA intron region. The results demonstrate a large reduction in the level of the Y structure intermediate, suggesting that trans-splicing is inhibited in the initial step of splicing (Fig. 2A). To ensure that equal amounts of RNA were used in the experiments, we monitored the level of snRNA 92 by primer extension.

We next examined the reduction in mature mRNAs and the accumulation of precursors. RNA extracted from the SmD1 cell line 3 and 5 days after tetracycline induction was subjected to Northern analysis with the tubulin-specific probe. The results, presented in Fig. 2B, demonstrate a reduction in the level of the mature tubulin transcript 5 days after induction and an increase in tubulin precursors appearing as dimers, trimers, and tetramers by 2 days after induction. The level of rRNA was used to control the level of RNA loaded on the gel. The experiments in Fig. 2 clearly indicate that trans-splicing was inhibited as the level of Y structure decreased (Fig. 2A). In addition, the level of mature tubulin mRNA decreased, and the level of tubulin precursors increased (Fig. 2B).

To examine how Sm depletion affects cis-splicing, we deter-
mined the processing of the poly(A) polymerase (PAP) gene. An RT-PCR assay was used to monitor the level of the mature PAP-spliced product by using oligonucleotides that are situated in the two exons (depicted in Fig. 2C, a-1); the results indicate a reduction in the level of mature PAPs (Fig. 2C, b). In addition, the level of the PAP precursor that accumulated was judged by examining the level of the RT-PCR product that originated from amplification of a product that spans the intron and the second exon (Fig. 2C, a-2). The decrease in the level of mature PAP is higher than the level of the pre-PAPs, which suggests that the precursor may undergo rapid degradation under splicing inhibition. The level of RNA used in these experiments was equal, as can be observed by the level of U3 snoRNA in these RNA samples.

**The Level of U snRNA and SLA1 RNA in Sm-silenced Cells**—
SmD1 depletion in yeast resulted in a reduction in the level of snRNAs (41). Protein components of small RNPs were shown to be required for the stability of the particle, as was demonstrated for the spliceosomal snRNAs (50), the components of signal recognition particle (51), as well as RNase P (52). We therefore determined the level of different snRNAs during SmE and SmD1 depletion. The level of the different RNAs was monitored by primer extension using an antisense oligonucleotide complementary to the 3' end of the RNAs. The results are presented in Fig. 3. Each lane represents a primer extension that was conducted simultaneously on two RNAs: the tested snRNAs and U3 snoRNA as a control. The results clearly indicate that the levels of U1, U2, U4, and U5 decreased, but not the levels of U6 and SLA1. These results indicate that, as in other eukaryotes and also in trypanosomes, the U6 does not bind Sm proteins but may in fact bind to Sm-like proteins (see "Discussion"). The SLA1 was previously shown to be immuno-oligonucleotide primer, using as a template a plasmid containing the T. brucei SL RNA gene (44). B, the level of tubulin mRNA during the SmD1 silencing. RNAs were obtained from cells that were either unin-duced (-Tet) or induced for 2 or 5 days (+Tet). Total RNA (20 μg) was subjected to primer extension with radiolabeled oligonucleotides complementary to each of the following RNAs: U1, U2, U4, U5, U6, and SLA1. The oligonucleotides and their sequences are summarized under "Materials and Methods." cDNA was separated on 6% sequencing gel. To control the level of RNA and each samples, primer extension was performed with two primers simultaneously complementary to U3 and the tested U snRNA.

**FIG. 2. Inhibition of trans- and cis-splicing upon Sm silencing.**
A, the level of the Y structure intermediate during Sm silencing. Total RNA was prepared from cells carrying the SmD1 RNAi construct, uninduced (-Tet) and 3 days after induction (+Tet). Reverse transcription was performed with a radiolabeled oligonucleotide complementary to the 3' end of the SL RNA. cDNA was separated on 6% sequencing gel. The position of the cDNA that corresponds to the Y structure is marked by an arrow. The DNA sequencing ladder was generated with the same oligonucleotide primer, using as a template a plasmid containing the T. brucei SL RNA gene (44). B, the level of tubulin mRNA during the SmD1 silencing. RNAs were obtained from cells that were either uninduced (-Tet) or induced for 2 or 5 days (+Tet). Total RNA (20 μg) was subjected to Northern analysis with a randomly labeled tubulin probe, pTbr15F1, a genomic clone carrying the T. brucei and T. brucei repeat unit (60). The mRNA and the multimeric pre-mRNA transcripts are indicated by arrows. The control examining the level of 5 S rRNA was as in Fig. 1B. C, the level of the cis-spliced poly(A) polymerase precursor and mRNA during SmD1 silencing. a, schematic representation of the PAP gene demonstrating the positions of the primers used for amplifying the mature and the cis-spliced precursor. The number of oligonucleotides used for the RT-PCR and the expected sizes of the products are indicated. b, the level of mature and pre-PAP RNA during SmD1 silencing. RT-PCR was performed on total RNA (DNA-free) prepared from uninduced (-Tet) or induced cells 3 days after induction (+Tet). c, to control the amount of RNA used in the experiments, the level of U3 was determined by primer extension.
precipitated by the anti-core proteins described above (53). However, this RNA, which was shown by us to belong to the H/ACA RNA family, and to guide pseudouridylation on SL RNA (44), most probably does not directly bind Sm proteins. Indeed, the level of SLA1 was reduced during the silencing of the pseudouridine synthase that binds the H/ACA RNAs.2

The Effect of Sm Silencing on the Level, 3' Tail, and cap4 Modification of the SL RNA—SL RNA was shown to bind Sm proteins in both Leptomonas collosoma and T. brucei (38, 39). We therefore examined the level of SL RNA in the Sm-silenced cell lines. The Northern analysis results, presented in Fig. 4A, indicate that, in contrast to the U snRNAs, the level of SL RNA increased. By using densitometric analysis, we found that the level of SL RNA increased at least 10-fold during silencing. Interestingly, the SL RNA was heterogeneous and longer than the transcript found in uninduced cells. To map the source of this heterogeneity, we mapped the SL RNA 5' and 3' ends by primer extension and RNase protection, respectively. The primer extension results are presented in Fig. 4B and indicate that major changes occurred in the cap4 modification. Most of the SL RNA in the induced cells lacked the modification at the cap4 position. The level of unmethylated (cap0) was higher in the induced cells, but clear stops corresponding to cap1 and cap2 nt were also observed, suggesting that the first and second modification but not the fourth cap modification, takes place during silencing. We next analyzed the exact 3' end of the SL RNA during Sm silencing. Previously, changes at the 3' end were noticed in SL RNA mutants with an altered stem-loop III or Sm site of the Leishmania SL RNA (14). In addition, treatment of T. brucei and Leishmania with leptomycin D resulted in the accumulation of SL RNA harboring a 3' long U tail (20).

To analyze the tail, we devised an RNase protection assay. An antisense probe was synthesized to the sequence 5' UUUUUUAAUUUUAUU-3' present at the 3' end of the SL RNA gene. RNA from induced and uninduced cells was subjected to RNase protection with this probe, and the results, presented in Fig. 4C, indicate that the SL RNA that had accumulated in the silenced cells had a tail that is longer by 5 nt compared with the RNA present in uninduced cells. The data suggest that, during Sm silencing, the trimming at the 3' end of the RNA is inhibited.

To examine where in the cell the SL RNA accumulated during Sm depletion, especially because recently it was demonstrated that the SL RNA has also a cytoplasmic phase (20), we analyzed the cellular distribution of the SL RNA during SmD1 silencing. To this end, nuclear and cytoplasmic fractions were prepared from induced and uninduced cells, and the quality of the cellular fractionation was examined by monitoring...
RNAs that localize either to the cytoplasm (7SL RNA) or exclusively in the nucleus (U6). The primer extension with these RNAs indicates that, although there is leakage during fractionation, most of the RNAs were found in the expected compartments. The SL RNA was analyzed by primer extension, and the results suggest that SL RNA that had accumulated in the cytoplasm lacked the modification on cap4 nt but harbored the modifications on cap1 and cap2, suggesting that these modifications take place co-transcriptionally (19) before the SL RNA exit the nucleus. Not much is known about the cap3 modification, and the lack of stops caused by cap3 modification most probably is the reason for a characteristic space observed in the primer extension of the SL RNA presented in Fig. 4B, as well as in all the studies that analyzed the cap4 modification by primer extension (9, 17, 18, 20).

The SL RNA That Accumulated in Induced Cells Carries the Pseudouridine at −12—We have recently demonstrated that SL RNA undergoes pseudouridylation at position −12, which is guided by SLA1 (44). However, it is currently unknown where and when this modification takes place. SLA1 was found in both the nucleolus and the nucleoplasm, but no evidence exists of the presence of SL RNA in the nucleolus (20, 44). To examine whether pseudouridylation takes place in the nucleus before the migration to the cytoplasm, we examined the status of pseudouridylation on SL RNA extracted from induced and uninduced cells by mapping the ψ after treatment with CMC. The results, presented in Fig. 5, suggest no difference in the level of ψ on SL RNA from induced and uninduced cells, whereas very clear differences exist in the cap4 modification, as discussed above. These results suggest that pseudouridylation takes place before the migration to the cytoplasm and is an early event in SL RNA maturation, perhaps like the modification on cap1 and cap2 that takes place co-transcriptionally (19).

The SL RNA Accumulates in the Sm-silenced Cells in Two Particles—The increase in the SL RNA level during Sm silencing suggests that it binds other subsets of proteins that are different from the Sm core. As a first step toward the identification of these particles, whole cell extracts were fractionated on sucrose gradients and the samples were analyzed for the distribution of the SL RNA. The distribution of U3 snoRNA and 7SL RNA served as controls. RNA was extracted from the

sucesrose gradient fractions, stained with ethidium bromide and analyzed by Northern analysis. Total RNA from SmD1-induced cells after 3 days of induction (+Tet) or uninduced (−Tet) cells (15 μg) was treated with CMC, as described under “Materials and Methods.” The RNA treated with CMC (+CMC) or control RNA (−CMC) was subjected to primer extension with oligonucleotide complementary to SL RNA. The products were separated on 6% polyacrylamide denaturing gel next to RNA sequencing of the corresponding SL RNA gene. The stop 1 nt before the pseudouridine site is marked by an arrow. The position of the Y structure is indicated.

FIG. 5. Mapping the pseudouridine on SL RNA before and after SmD1 silencing. Total RNA from SmD1-induced cells after 3 days of induction (+Tet) or uninduced (−Tet) cells (15 μg) was treated with CMC, as described under “Materials and Methods.” The RNA treated with CMC (+CMC) or control RNA (−CMC) was subjected to primer extension with oligonucleotide complementary to SL RNA. The products were separated on 6% polyacrylamide denaturing gel next to RNA sequencing of the corresponding SL RNA gene. The stop 1 nt before the pseudouridine site is marked by an arrow. The position of the Y structure is indicated.
RNA probe. The nuclei (induced and uninduced cells hybridized with DIG-labeled PCR 7SL
Panels A and B, uninduced and induced cells hybridized with DIG-labeled PCR SL RNA probe; panel C, induced and uninduced cells hybridized with DIG-labeled PCR 7SL RNA probe. The nuclei (N) are indicated by arrows. The scale bar is 10 µm.

Fig. 7. Localization of SL RNA during SmD1 silencing by in situ hybridization. Cells uninduced (–Tet) and after 3 days of tetracycline induction (+Tet) were fixed and hybridized with DIG-labeled PCR probes to SL RNA and 7SL RNA. Panels A and B, uninduced and induced cells hybridized with DIG-labeled PCR SL RNA probe; panel C, induced and uninduced cells hybridized with DIG-labeled PCR 7SL RNA probe. The nuclei (N) are indicated by arrows. The scale bar is 10 µm.

We termed the particle carrying the SL RNA that accumulated during Sm silencing SL RNP-C, because this particle accumulated in the cytoplasm. It is currently unknown whether the 10 S particles dislodged from the 50 S particle during extraction and fractionation on a sucrose gradient or whether the 10 S particles dislodged from the 50 S particle during Sm silencing. The accumulation of SL RNP-C in speckles may mark a special site where SL RNP assembly takes place. The status of cap4 of the SL RNA that accumulated in the SmD1-silenced cells suggests that modification on cap1 and cap2 takes place in the nucleus, most probably co-transcriptionally, as was previously suggested (19), whereas the cap4 nt modification takes place in the cytoplasm after Sm assembly. The differential capping of the SL RNA may play a role as export and re-entry signals, much like the function of m7G and TMG for U snRNP biogenesis.

Why SL RNA Biogenesis Requires a Cytoplasmic Phase—The data presented here support the notion that SL RNA has a cytoplasmic phase. This finding was at first surprising, taking into account the short half-life of the RNA (only a few minutes) (8). Earlier studies suggested that the T. brucei U4 and U5, like U snRNPs in metazoa, shuttle through the cytoplasm (54, 55). The fact that SL RNA has a cytoplasmic phase is supported by a recent study in Leishmania and T. brucei, demonstrating by in situ hybridization that SL RNA is found in the cytoplasm and is exported from the nucleus via Xpo1p (20). Why snRNP assembly requires a cytoplasmic phase is an intriguing question. In mammalian cells it was recently demonstrated that it is necessary for the Sm proteins to bind to the SMN complex immediately upon their translation, otherwise these proteins can bind nonspecifically other cytoplasmic RNAs such as tRNAs (27), suggesting the need to couple translation to snRNP assembly. However, in yeast S. cerevisiae snRNP assembly may take place in the nucleus and is assisted by the La protein, because the SMN protein does not exist in this yeast species (56). snRNP shuttling was most probably acquired early in evolution, because it is present in trypanosomes, which diverged very early in the eukaryotic lineage (57). However, the mechanism of snRNP assembly in trypanosomes, although possessing a cytoplasmic phase, may differ from the same process in metazoa. Notably, SMN was not identified in any of the trypanosome genome projects including Leishmania, Trypanosoma cruzi, and T. brucei. Moreover, the binding of Sm proteins to SMN requires that SmD1 and SmD3 carry dimethylarginine modification (28). Interestingly, the trypanosome proteins lack the RG repeats on these homologous Sm proteins; therefore, these proteins do not have the potential to undergo this specific methylation (40). In contrast to other eukaryotes, in trypanosomes almost every snRNA possesses a different cap structure. The most striking difference is the lack of a TMG cap on the U5 snRNA (11, 12) and the unique cap structure of the SL RNA (5–7). Despite the differences in cap structure, all of these snRNAs, including U1, U2, and U4, bind Sm proteins (38). Cytoplasmic assembly may direct the RNAs to different sub-compartments in the cytoplasm where assembly of these particles takes place. It will be of interest to determine whether the speckles identified in this study contain other snRNAs.

Which snRNA in Trypanosomes Bind Sm Proteins—Silencing of Sm proteins enabled us to unequivocally determine
which RNAs bind these proteins. The data in Fig. 3 indicate that U1, U2, U4, and U5 associate with Sm proteins but not with U6 or SLA1. Previous studies suggested that U6 binds to Sm proteins in trypanosomes, because the same core proteins were selected when antisense biotinylated oligonucleotides were used to select U2, U4, and U6 (38). However, the selected proteins with U6 may represent Sm-like proteins. Indeed, we have recently identified Sm-like proteins in T. brucei, and silencing of these factors resulted in a decrease in U6 snRNA but not in the U snRNAs demonstrated here to bind to Sm proteins, suggesting that U6, like in other eukaryotes, associates with Sm-like proteins.3

The previous report of the ability to immunoprecipitate SLA1 with the anti-CP antibodies is not consistent with the results obtained in this study (53). Moreover, we have demonstrated that SLA1 belongs to the H/ACA RNA family and guides modification on the SL RNA (44). Indeed, we have recently silenced the pseudouridine synthase in T. brucei and demonstrated that under this depletion the level of SLA1 was reduced, suggesting that SLA1 binds the core proteins characteristic to guide RNAs which direct pseudouridylation.2

When and Where RNA Modification on the SL RNA Takes Place—The finding that the SL RNA accumulated during Sm silencing already carries the pseudouridine is intriguing and may shed light on the function of SLA1 and pseudouridine for the SL RNA. The pseudouridine itself may have a very crucial function during the splicing reaction, because the presence of pseudouridine in the RNA may stabilize important interactions during activation of the trans-splicing reaction (44), analogous to the role of the pseudouridine present in the U2 snRNA domain that base pairs with the branch point sequence of the pre-mRNA (58). However, the binding of SLA1 may also function as a chaperone in keeping the SL RNA in a certain structure needed for the interaction with the methyltransferases involved in cap4 formation or for transporting the molecule to the cytoplasm. The interaction of SL RNA with SLA1 may therefore serve dual functions, one in “forcing” the SL RNA to fold in a certain important secondary structure, and another in directing the pseudouridine to this crucial position. Indeed, the A−12 position that is pseudouridylated is situated in a region across the 5′ splice site that is involved in intramolecular base pairing (44). This pseudouridine can fine-tune the inter- versus intramolecular base pairing during the splicing reaction. The finding reported here that the pseudouridine is added very early in SL RNA biogenesis suggests that the interaction with SLA1 or the itself is essential for keeping the SL RNA in the distinct folding necessary for transport and assembly with the Sm core proteins.

This study highlights the long standing question regarding the role of cap4 in trypanosomes, where in the cell it takes place, and what specific methyltransferase carries out this reaction. Previous studies using the permeable T. brucei cells suggest that cap4 formation is co-transcriptional (19). cap4 analysis of pre-matured transcripts indicated that cap1 and cap2 modifications were successfully added onto short SL RNA transcripts (56 and 67 nt), but a fully modified cap was observed only after the Sm site was synthesized. These results suggest that, unlike capping of U snRNAs in mammalian cells, SL RNA modification takes place co-transcriptionally (19). Recent studies using the leptomycin B treatment identified SL RNA molecules that accumulated in the cytoplasm and lacked the cap4 nt modification (20). Based on these results, the authors concluded that capping is post-transcriptional and takes place immediately after the release of the SL RNA from the transcription complex (20). Mutational analysis on the SL RNA also indicated that mutants in the Sm site affected the modification of cap4 nt (9, 18). In addition, capping in vitro required that SL RNA be assembled with its core Sm proteins (8). The cellular fractionation results (Fig. 4D) suggest that modification on cap1 and cap2 most probably takes place co-transcriptionally, as was previously suggested (19), because these modifications were found on the SL RNA that accumulated in the cytoplasm. However, the modification on cap4 nt takes place in the cytoplasm only after Sm assembly. In fact, cap modification includes modification of the sugar to form the 2′-O-methyl nucleotides and base modification on cap1 and cap4; these two events may be carried out by different sets of methyltransferases. Based on the modification status of cap4 during Sm silencing, we propose that modifications on cap1 and cap2 mark the SL RNA for export to the cytoplasm, whereas the modification on cap4 nt that most probably takes place in the cytoplasm serves as an import signal for the SL RNA back to the nucleus, much like the TMG modification that takes place in the cytoplasm and serves concomitantly with the Sm proteins as a signal for import of snRNPs to the nucleus (29). Note that the issue of where these cap modification events take place awaits the characterization and cellular localization of the methyltransferases that conduct these specific reactions.

SL RNA 3′ End Formation as a Quality Control for SL RNP—An important modification that the SL RNA undergoes during its biogenesis is the 3′ trimming. The T. brucei SL RNA carries a long polypyrimidine tract at the 3′ end of the gene that may function in transcription termination (14). Under normal conditions, SL RNA appears as a homogeneous population of molecules; however, Leishmania mutants in either the stem-loop III or the Sm site possess long 3′ tails (14). In this study, we observed that the SL RNA that had accumulated in the Sm-silenced cells carried a short tail of 5 nt. We propose that initially the SL RNA tail is longer because there is a long T tract at the end of the SL RNA gene. Trimming may take place in two steps, first to convert the long tail to a short one with only 5 nt, which is later trimmed when the SL RNA returns to the nucleus. The last trimming event may serve as a last quality control checkpoint that marks the end of SL RNA maturation and is a sign for the SL RNP to join the spliceosome. The SL RNA that accumulated in the cytoplasm during Sm silencing only undergoes the first step of trimming leaving a 5-nt tail. This SL RNA does not return back to the nucleus and can therefore not undergo the last trimming event.

Why and How SL RNP Biogenesis Differs from That of U snRNPs—Several factors were shown to be involved in the export complex of U snRNA from the nucleus to the cytoplasm including the cap binding complex, Xpo1p, and PHAX (see Introduction). Indeed SL RNA was shown to be transported by Xpo1p (20). The cap-binding complex CB20 and CB80, as well as a PHAX, were identified in the trypanosomatid genome projects. We are currently examining whether these proteins are part of the SL RNP-C complex. However, binding of these factors could not explain the preferential stabilization of the SL RNA during Sm silencing. Factors that may preferentially differentiate the SL RNA from the U snRNAs are factors that bind the SL RNA co-transcriptionally as the SL RNA is transcribed by RNA poly II (13), whereas the U snRNAs are transcribed by RNA poly III (59). Another candidate that may help in stabilizing the SL RNP-C is the La protein. The La protein is known to bind to RNAs harboring long U tails (56). Although all snRNAs should possess U tails, because these RNAs are transcribed by polymerase III, the SL RNA has a longer U tail and can, under Sm silencing, compete out the cellular La. Interest-

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3 Q. Liu, X.-H. Liang, M. Belachcen, S. Ulie, R. Unger, and S. Michaeli, unpublished data.
ingly, La in yeast is believed to possess a function similar to SNM in metazoa, because it was demonstrated that the yeast La protein Lhp1p binds precursors U1, U2, U4, and U5 snRNAs and therefore may play a more general role in small RNA biogenesis (32). Therefore, it will be of interest to examine whether La is one of the proteins that stabilizes the SL RNA during Sm silencing. The factors that bind the SL RNP-C await further characterization.

The data presented in this study, together with information obtained from other studies (20), enabled to propose a unique pathway for SL RNA biogenesis that surprisingly has a clear cytoplasmic phase. The methylation on cap4 nt takes place in the cytoplasmic phase. The methylation on cap4 nt takes place during Sm silencing (32). Therefore, it will be of interest to examine whether La is one of the proteins that stabilizes the SL RNA during Sm silencing. The factors that bind the SL RNP-C await further characterization.

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