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Trans splicing in trypanosomes requires methylation of the 5′ end of the spliced leader RNA

(RNA modification/RNA processing/cap)

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ABSTRACT **_Trypanosoma brucei_ spliced leader (SL) RNA contains an unusual cap 4 structure consisting of 7-methylguanosine linked to four modified nucleosides. During RNA maturation, trans splicing transfers the first 39 nucleotides of the SL RNA including the cap structure to the 5′ end of all mRNAs. Here we show that exposure of permeable trypanosome cells to *S*-adenosyl-L-homocysteine inhibits methylation of the nucleosides adjacent to 7-methylguanosine of newly synthesized SL RNA and prevents utilization of the SL RNA in trans splicing. However, trans splicing of the SL RNA preexisting in the cells is not inhibited by *S*-adenosyl-L-homocysteine as shown by the observation that newly synthesized α-tubulin RNA is trans spliced at the same level as in control cells. Therefore, it appears that the newly synthesized SL RNA is the only known component of the trans-splicing machinery that is impaired in its function by inhibition of methylation. Undermethylation does not alter either the stability of the SL RNA or the electrophoretic mobility and chromatographic behavior of the core SL ribonucleoprotein particle. Taken together, our data suggest that the cap 4 structure of the SL RNA plays an essential role in the trans-splicing process.**

All mRNA molecules in trypanosomes are chimaeras of two sequences. In *Trypanosoma brucei* the first 39 nucleotides at the 5′ end are common to all mRNAs and are referred to as the spliced leader (SL) or miniexon sequence (1). The joining of the SL sequence and the mRNA body takes place by trans splicing between two unlinked RNA molecules—namely, the SL RNA, which functions as donor of the SL sequence, and the pre-mRNA, which is the SL sequence acceptor and provides the mRNA body (1). Trans splicing has been shown to be mechanistically similar to cis splicing of intervening sequences of higher eukaryotes (2–4) and to require intact U2 and U4/U6 small nuclear ribonucleoprotein particles (RNPs) (5). No U1 or U5 small nuclear RNA analogues have been identified so far in trypanosomes.

In *T. brucei* SL RNA, the only modified nucleotides are clustered at the very 5′ end, forming the structure m^7GpppA*pAmpCmpU*pApA*pC, where asterisks indicate partially identified modifications on the preceding nucleotide and m represents a 2′-*O*-methyl group on the preceding nucleotide (6–8). The existence of 2′-*O* modifications in the first adenosine and in the uridine residue is implicated by the finding that T2 ribonuclease digestion of *T. brucei* SL RNA produces the T2-resistant structure m^7GpppA*pAmpCmpU*pAp consistent with the inability of this ribonuclease to hydrolyze pyrophosphate bonds or 5′ bonds adjacent to 2′-*O*-modified nucleosides. Finally, the first adenosine residue, although modified, does not appear to be the N^6,2′-*O*-dimethyladenosine located within the capped 5′ end of many viral and cellular mRNAs (8).

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The 5′ modifications of the SL sequence appear to have been conserved through evolution of Trypanosomatidae, as suggested by the finding that the SL RNAs of two distantly related Trypanosomatidae, namely *T. brucei* and *Leptomonas collosoma*, carry a similar cap structure (8). In addition, the corresponding first four nucleotides of the cap have been conserved among all the trypanosome SL RNA genes analyzed so far (1). Conceivably, modification of the trypanosomatid SL sequence might be required for the function of the SL RNA in trans splicing and/or for the function of the mRNA during translation. Using permeable trypanosome cells, we provide evidence that the complex methylated cap structure of the SL RNA is required for trans splicing.

MATERIALS AND METHODS

RNA Synthesis and Ribonuclease Mapping. Cultured procyclic trypanosome cells were grown and permeabilized essentially as described (9). Briefly, cells at a density of 5–8 × 10^6 per ml (midlogarithmic phase) were washed twice with medium minus sera and twice with transcription buffer (20 mM Hepes·KOH, pH 7.9/20 mM KCl/3 mM $MgCl_2$/1 mM dithiothreitol/10 μg of leupeptin per ml/150 mM sucrose) and were permeabilized on ice for 1 min with palmitoyl L-α-lysophosphatidylcholine (lysolecithin; Sigma) at a final concentration of 500 μg/ml. After one wash with transcription buffer, the cells were resuspended in the same buffer at a concentration of 2.4 × 10^9 cells per ml, and RNA synthesis was initiated by the addition of an equal volume of a solution containing 20 mM Hepes·KOH (pH 7.9), 20 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg of leupeptin per ml, 50 mM creatine phosphate, 1.2 mg of creatine kinase (Boehringer Mannheim) per ml, 4 mM ATP, 2 mM GTP, 2 mM CTP, and 1 mCi (1 Ci = 37 GBq) of [α-^{32}P]UTP (3000 Ci/mmol; Amersham) per ml. After incubation at 30°C, cells were treated with DNase I and lysed by adding EDTA to 40 mM, proteinase K to 500 μg/ml, and SDS to 1%. Purified RNA samples from 6 × 10^6 cells (see Fig. 2) or 4 × 10^7 cells (see Fig. 3) were used for RNase protection analysis (9) and fractionated on 6% acrylamide gels containing 7 M urea.

TLC Analysis. The SL RNA was purified by hybrid selection with SL DNA (9) immobilized onto a nitrocellulose filter followed by fractionation of the hybridized RNA on 6% acrylamide/7 M urea gels and elution from gel slices. The solvent used for TLC (see Fig. 4*A*) was 2 M pyridinium formate (pH 3.4). Chromatography on Avicel plates (see Fig. 4*B*) was done as described (10) with the isobutyric acid/ammonia system as solvent. Unlabeled markers run in adjacent lanes were visualized by illumination with 254-nm UV

Abbreviations: Guo-5′-*P*, guanosine 5′-monophosphate; RNP, ribonucleoprotein particle; Ado-Hcy, *S*-adenosyl-L-homocysteine; Ado-Met, *S*-adenosyl-L-methionine; 7-MeGuo, 7-methylguanosine; 7-MeGuo-5′-*P*, 7-methylguanosine 5′-monophosphate; SL, spliced leader; Ado-Hcy-SL RNA, SL-RNA from Ado-Hcy-treated cells.

light. After chromatography, the spots corresponding to 7-methylguanosine 5′-monophosphate (7-MeGuo-5′-*P*), guanosine 5′-monophosphate (Guo-5′-*P*), and undigested material were scraped from the plate and assayed for radioactivity by liquid scintillation. Assuming that the digestion went to completion and taking into account the number of guanosine residues in the SL RNA, we calculated that the SL RNA from control cells yielded 0.82 mol of 7-MeGuo-5′-*P*, whereas the SL RNA from *S*-adenosyl-L-homocysteine (Ado-Hcy)-treated cells (Ado-Hcy-SL RNA) yielded 0.62 mol of 7-MeGuo-5′-*P* and 0.16 mol of Guo-5′-*P*.

Fractionation of Ribonucleoprotein Particles. Permeable cells were incubated in a complete transcription medium {final concentrations: 20 mM Hepes·KOH (pH 7.9), 75 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 1 mM GTP, 1 mM CTP, 10 mg of leupeptin per ml, 25 mM creatine phosphate, 0.6 mg of creatine kinase per ml, and 500 mCi of [α-^{32}P]UTP per ml}, and RNA synthesis was allowed to proceed for 10 min. At the end of the incubation, cells were pelleted and washed once in transcription buffer (20 mM Hepes·KOH, pH 7.9/20 mM KCl/3 mM $MgCl_2$/1 mM dithiothreitol/10 μg of leupeptin per ml/150 mM sucrose) containing 2 mM ATP. When Ado-Hcy was included in the transcription medium, 0.5 mM Ado-Hcy was present in the buffers throughout the isolation procedure. Cells were resuspended in transcription buffer containing 2 mM ATP in the same volume used for RNA synthesis and were lysed by the addition of Nonidet P-40 to 0.5%. Supernatants were prepared by centrifuging the lysates for 30 min at 12,000 × *g* at 4°C, applied onto DEAE-Sepharose CL-6B columns equilibrated with transcription buffer, and eluted stepwise with 100 mM increments of KCl between 0.02 and 0.5 M. The elution profile of the ^{32}P-labeled RNAs was analyzed by denaturing gel electrophoresis of aliquots of the various fractions after deproteinization. The 0.4 M KCl fractions, which contained >90% of the input SL RNA, were fractionated by electrophoresis through discontinuous native gels (11). The DNA oligonucleotides used for the identification of the SL RNP were added to the samples to a final concentration of 40 μg/ml.

RESULTS

To test whether methylation plays a role in trans splicing, we incubated permeable trypanosome cells with Ado-Hcy, a competitive inhibitor of *S*-adenosyl-L-methionine (Ado-Met)-mediated methylation reactions. Fig. 1 shows a profile of total ^{32}P-labeled RNA synthesized in permeable cells. Active trans-splicing in these cells is indicated by the production of the linear form of the SL intron (Fig. 1, lane 1, and ref. 9). We found that the addition of 0.5 mM Ado-Hcy abolished the appearance of the linear form of the SL intron (Fig. 1, lane 3), suggesting that Ado-Met-dependent reactions are involved in the trans-splicing mechanism. Ado-Hcy did not affect the overall synthesis of RNA or the synthesis of the SL RNA (Fig. 1 and data not shown). The effect of Ado-Hcy could be reduced by exposing the cells to equimolar amounts of Ado-Hcy and Ado-Met (lane 4), supporting the conclusion that Ado-Hcy inhibits trans splicing by competitive inhibition of an Ado-Met-dependent reaction.

To monitor the extent of modification of newly synthesized SL RNA and its trans-splicing activity in Ado-Hcy-treated cells, we first used RNase mapping of total ^{32}P-labeled RNA with RNase A and T1 (T1 was included to cleave the SL exon/mRNA boundary), using an antisense SL RNA probe that extends from nucleotide 7 to 128 (Fig. 2). This probe does not include the first six nucleotides complementary to the sequence AACUAA, where the modified nucleosides of the SL RNA are located. This sequence contains two pyrimidine residues that are potential cleavage sites for RNase A.

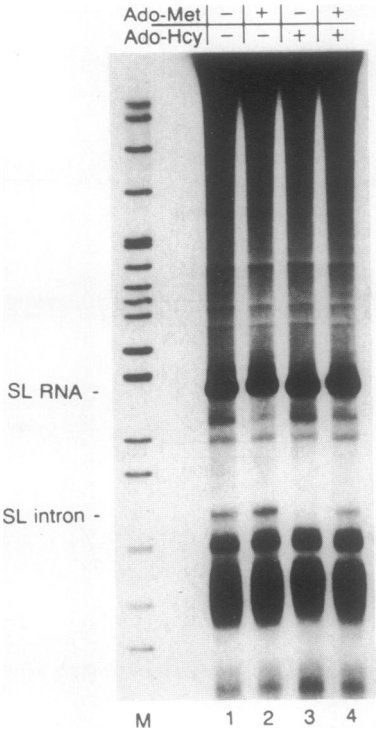

FIG. 1. Total RNA synthesis in permeable trypanosome cells. ^{32}P-labeled RNA was synthesized for 10 min in the presence or absence of 0.5 mM Ado-Met and 0.5 mM Ado-Hcy, as indicated above each lane, and fractionated by gel electrophoresis. The positions of the SL RNA and of the linear form of the SL intron are indicated.

However, if these two nucleosides contain a 2′-*O*-modification, RNase A does not cleave, and we expect a SL RNA protected fragment of 129 nucleotides. In contrast, a 124-nucleotide fragment is indicative that the SL RNA does not contain the above modifications. In agreement with our previous observations (9), RNase mapping of newly synthesized RNA from control cells revealed both the 129- and 124-nucleotide SL RNA species (Fig. 2, lane 1). Under the conditions used, this indicates that in a proportion of the SL RNA, the two pyrimidine residues are not modified. Analysis by TLC of T2 ribonuclease digestion products of gel-purified 129- and 124-nucleotide SL RNA fragments confirmed that the 129- but not the 124-nucleotide RNA contained 2′-*O*-modified nucleosides as revealed by the presence of a T2-resistant structure (data not shown). An identical pattern of RNase protected fragments was observed when 0.5 mM Ado-Met was included in the incubation mixture (Fig. 2, lane 2). In contrast, only the 124-nucleotide RNA fragment was detected in Ado-Hcy-treated cells (lane 3). The RNase mapping in Fig. 2 (Fig. 2, lanes 1, 2, and 4) also generated RNA fragments of 89 and 40 nucleotides, which correspond to the SL intron and SL exon, respectively, and are diagnostic of trans splicing of newly synthesized SL RNA and pre-mRNA (9). The absence of these RNA fragments in Ado-Hcy-treated cells (Fig. 2, lane 3) demonstrated that undermethylated SL RNA is not active in trans splicing. The simultaneous addition of equimolar amounts of Ado-Hcy and Ado-Met partially restored 5′ end modification of the newly synthesized SL RNA as well as trans-splicing activity (Fig. 2, lane 4).

To eliminate the possibility that Ado-Hcy is a general inhibitor of trans splicing, we asked whether the SL RNA present in trypanosome cells prior to the permeabilization procedure and to the addition of Ado-Hcy can serve as the SL donor to newly synthesized pre-mRNA. RNA samples similar to those of Fig. 1 were subjected to RNase mapping by

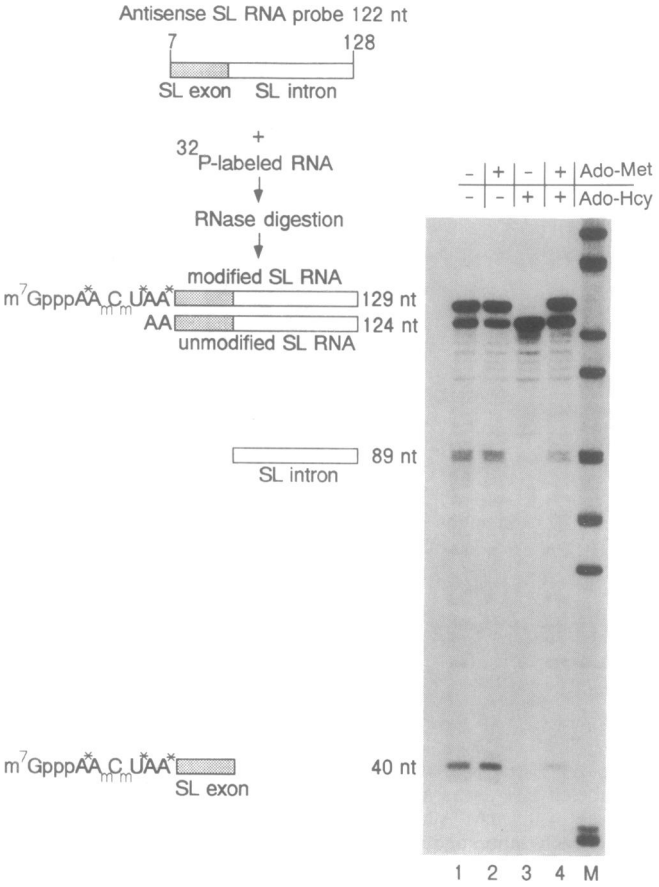

FIG. 2. RNase protection analysis with the SL antisense RNA probe. RNA samples similar to the ones shown in Fig. 1 were subjected to RNase protection analysis with RNase A and T1 as described (9). T1 was included to cleave the SL exon/mRNA boundary. A schematic representation of the probe and the expected protected fragments is shown on the left. The sizes of the protected fragments differ slightly from the ones published in ref. 9, since at that time we did not take into account the extensions generated by RNase A cleavage and the cap nucleotide. Lane M, ^{32}P-labeled *Msp* I-digested pBR322. nt, Nucleotide.

using as a probe an anti-sense RNA complementary to the first 164 nucleotides of the mature α-tubulin mRNA, which contains the SL sequence at the 5′ end (5, 9). By this assay trans splicing of newly synthesized α-tubulin RNA was not inhibited when the cells were incubated with Ado-Hcy (Fig. 3, lane 3). However, in several experiments, a slight increase in the amount of unspliced α-tubulin RNA was observed in the presence of Ado-Hcy (lane 3), maybe suggesting that the SL RNA becomes limiting at later times in trans splicing. The result in Fig. 3 shows that during the time of our assay (10 min), permeable trypanosomes were fully competent for trans splicing. Therefore, it must be that the preexisting SL RNA functions as the SL sequence donor for trans splicing of newly synthesized pre-mRNA.

A more detailed structure of the 5′ end of the Ado-Hcy-SL RNA was obtained by direct RNA analysis. Intact SL RNA was purified from control and Ado-Hcy-treated cells and digested with ribonuclease T2. TLC of the digestion products of control SL RNA generated a slowly migrating T2-resistant species (Fig. 4*A*), consistent with the presence of a cap structure (6–8). In contrast, no T2-resistant structure was observed with Ado-Hcy-SL RNA, indicating that SL RNA synthesized in Ado-Hcy-treated cells is incompletely modified. To determine the methylation state at the very 5′ end of the Ado-Hcy-SL RNA, permeable cells were incubated with [α-^{32}P]GTP to label the capping nucleotide. Intact SL RNA

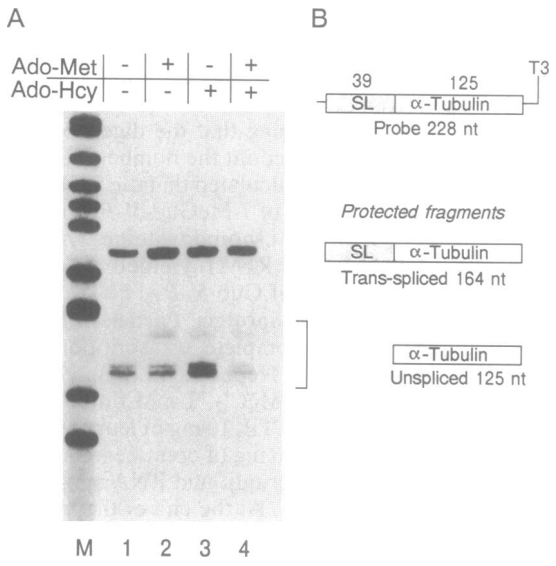

FIG. 3. Trans splicing of α-tubulin RNA is not inhibited by Ado-Hcy. (*A*) RNase protection analysis with the antisense α-tubulin probe of ^{32}P-labeled total RNA synthesized under the conditions indicated at the top of the lanes. Lane M contains the ^{32}P-labeled *Msp* I digest of pBR322. In lanes 2, 3, and 4 the unspliced α-tubulin RNA is detected as two bands as we reported previously (5). (*B*) Structure of the α-tubulin probe and the expected protected products. The probe is complementary to 164 nucleotides of the mature 5′ end of α-tubulin mRNA (5, 9). Active trans splicing is revealed by a protected fragment of 164 nucleotides. Unspliced α-tubulin RNA generates RNA fragments of ≈125 nucleotides.

was purified and treated with tobacco acid pyrophosphatase, and the products of digestion were separated by TLC (Fig. 4*B*). As expected, 7-MeGuo-5′-*P* was released from control SL RNA, whereas Ado-Hcy-SL RNA produced both 7-MeGuo-5′-*P* and Guo-5′-*P* with a cumulative yield of 93% relative to the molar representation of 7-MeGuo-5′-*P* in control SL RNA (see *Materials and Methods* for details). The ratio of 7-MeGuo-5′-*P* to Guo-5′-*P* in Ado-Hcy-SL RNA varied from experiment to experiment. In the TLC plate shown, Ado-Hcy-SL RNA released ≈80% 7-MeGuo-5′-*P* and 20% Guo-5′-*P*, but SL RNA from Ado-Hcy-treated cells was also found to contain equimolar amounts of methylated and unmethylated capping nucleotide. Therefore, the majority of the Ado-Hcy-SL RNA was capped with a guanosine residue, and a great proportion of the molecules carried a methyl group on the capping nucleotide, suggesting that the trypanosome guanine 7-methyltransferase is less sensitive to Ado-Hcy than the 2′-*O*-methyltransferase. A similar result was obtained in the vaccinia virus capping system (12).

To exclude that undermethylated SL RNA was preferentially degraded by endogenous ribonucleases, we compared the decay rate of control and Ado-Hcy-SL RNA in a pulse–chase experiment (Fig. 5). Permeable trypanosomes were pulsed for 3 min with [α-^{32}P]UTP and then chased for 1, 2, 4, and 7 min in the presence of 50 μg of α-amanitin per ml to block transcription of the SL genes, and by adjusting the incubation cocktail to 90 mM KCl to inhibit trans splicing (9). The decay rate of SL RNA synthesized in Ado-Hcy-treated cells was indistinguishable from that of the SL RNA from control cells, demonstrating that RNA instability is unlikely to account for the lack of trans-splicing activity of undermethylated SL RNA.

Finally, we asked whether undermethylation of the SL RNA affects its packaging into a ribonucleoprotein particle. To this end control and Ado-Hcy-treated cells were labeled with [α-^{32}P]UTP, and a fraction enriched for RNPs was prepared by DEAE-Sepharose chromatography as de-

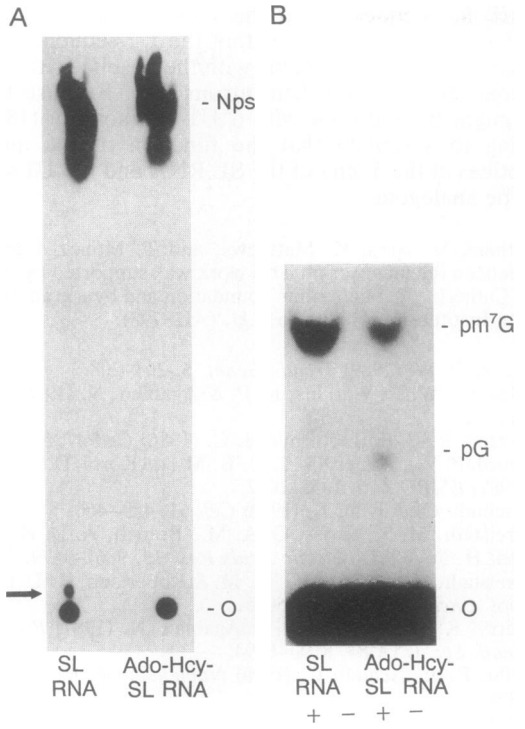

FIG. 4. Characterization of the SL cap structure by TLC. (*A*) Analysis of SL RNA by T2 ribonuclease digestion. [α-^{32}P]UTP-labeled SL RNA was synthesized in permeable trypanosome cells in the presence (lane Ado-Hcy-SL RNA) or absence (lane SL RNA) of 0.5 mM Ado-Hcy. Purified SL RNA was digested with T2 ribonuclease, and the products of digestion were analyzed by chromatography on PEI-cellulose plates. The arrow indicates the T2-resistant structure. Nps indicates the position of nucleoside 3′ monophosphates, and material at the origin represents incompletely digested RNA. (*B*) Chromatography of tobacco acid pyrophosphatase digestion products on Avicel plates. The SL RNA was labeled with [α-^{32}P]GTP. Lanes: +, digested SL RNA; −, undigested SL RNA. Unlabeled Guo-5′-*P* (pG) and 7-MeGuo-5′-*P* (pm^{7}G) were analyzed in adjacent lanes, and their positions are indicated. The O indicates the origin.

scribed. The purification was monitored by analyzing the ^{32}P-labeled RNAs on denaturing polyacrylamide gels (data not shown). No detectable difference was observed in the chromatographic behavior of the two RNP preparations: both were eluted between 0.4 and 0.5 M KCl and further fractionation by heparin-agarose chromatography also failed to reveal any difference (data not shown). To analyze in more detail the SL RNP structure, DEAE-Sepharose-purified RNPs were electrophoresed through a native polyacrylamide gel (11) shown in Fig. 6. RNPs prepared from ^{32}P-labeled permeable cells by this procedure consisted primarily of the SL RNP and of RNPs containing tRNA size molecules. Other RNPs (i.e., U2, U4/U6 RNPs) constitute a minor fraction of the labeled material and are not visible in Fig. 6. To identify the SL RNP, a DNA oligonucleotide (Y-21) complementary to nucleotides 40–60 of the SL RNA was incubated with the samples prior to electrophoresis (lanes 3 and 8). We have shown (5) that Y-21 can anneal to the SL RNP. Addition of Y-21 resulted in a clear mobility shift of the SL RNP, whereas no shift in mobility was observed when an oligonucleotide complementary to U2 snRNA (lanes 4 and 9) or an oligonucleotide with the same sequence as the SL RNA (lanes 5 and 10) was included in the samples. By this analysis the SL RNPs from control and Ado-Hcy-treated cells had indistinguishable electrophoretic mobilities implying that Ado-Hcy treatment did not affect packaging of the SL RNA into the core RNP complex.

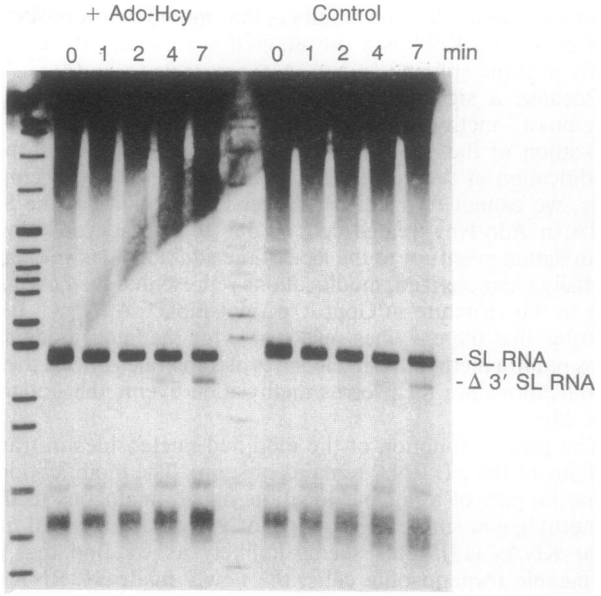

FIG. 5. Stability of the SL RNA. Permeable cells were pulsed in the absence (control) or presence of 0.5 mM Ado-Hcy (lanes +Ado-Hcy) for 3 min (lane 0) with [α-^{32}P]UTP and then chased for 1, 2, 4, and 7 min after transcription of the SL RNA, and trans splicing had been stopped by adding α-amanitin to 50 μg/ml and raising the KCl concentration to 90 mM (9). Aliquots of the RNA samples were analyzed by gel electrophoresis as described in Fig. 1. The positions of the SL RNA and of a 3′-end degradation product of the SL RNA (Δ3′ SL RNA, which is not utilized for trans-splicing) are indicated.

DISCUSSION

Our experiments demonstrate that the SL RNA, which is synthesized *de novo* in permeable trypanosome cells in the presence of Ado-Hcy and which carries an incompletely modified cap structure, is inactive in trans splicing. In contrast, trans splicing of newly synthesized α-tubulin pre-mRNA and of the SL RNA preexisting in the cells is not affected by Ado-Hcy treatment. This latter observation has important implications because it demonstrates that Ado-Hcy-treated permeable trypanosomes are equipped with a full complement of functional trans-splicing factors and that Ado-Hcy only affects the activity of newly synthesized SL RNA. Although other interpretations are possible, the sim-

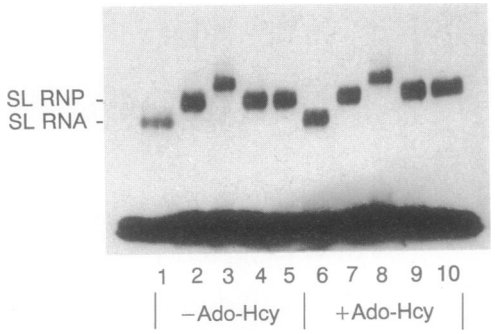

FIG. 6. Electrophoretic analysis of SL RNPs. Partially purified ^{32}P-labeled RNP preparations from control (lanes 1–5) and Ado-Hcy-treated cells (lanes 6–10) were separated by electrophoresis through a discontinous native gel (11). In this gel system, RNPs have a slower mobility than deproteinized RNA (compare lanes 1 and 2 or 6 and 7). Lanes: 1 and 6, deproteinized RNPs; 2 and 7, RNPs; 3 and 8, RNPs incubated with the Y-21 oligonucleotide complementary to nucleotides 40–60 of the SL RNA (5); 4 and 9, RNPs incubated with the oligonucleotide TRU2 complementary to U2 snRNA (5); 5 and 10, RNPs incubated with the SL-25 oligonucleotide that has the same sequence as the SL RNA from nucleotide 1 to 25.

plest explanation of our results is that modification of the 5′ end of the SL RNA is a prerequisite for activity of the SL RNA in trans splicing.

Because a significant fraction of the Ado-Hcy-SL RNA contains a 7-methylguanosine (7-MeGuo) "cap," it is likely that utilization of the SL RNA in trans splicing requires proper modification of the nucleotides adjacent to the "cap." However, we cannot distinguish whether the inactivity of the SL RNA in Ado-Hcy-treated cells is due to the lack of known methylation events or to the lack of the additional, as yet, only partially characterized, modifications represented by the asterisks in the structure m^7GpppA*pAmpCmpU*pApA*pC. It is possible that the enzymes responsible for the latter modifications require methylated nucleotides as substrates or that these modifications are themselves methylation events that require Ado-Met.

The precise function of the modified nucleosides in trans splicing of the SL RNA remains obscure. The modifications could be part of a nuclear location signal, analogous to the trimethylguanosine cap structure of vertebrate U small nuclear RNAs (13). This seems unlikely as we find that in permeable trypanosome cells, the newly made SL RNA is utilized for trans splicing within 3–5 min (unpublished observation), making it doubtful that maturation of trypanosome SL RNA involves migration from the nucleus to the cytoplasm and back into the nucleus. Alternatively, the cap 4 could be part of a recognition signal for some component of the splicing apparatus, since it has been shown that the presence of a 7-MeGuo "cap" enhances cis splicing of pre-mRNAs *in vitro* and in *Xenopus* oocytes (14, 15). In the trypanosome SL RNA, this putative recognition signal could be more extensive and encompass both the cap nucleotide and the adjacent modified nucleotides. For instance, the function of this recognition signal could be to target the SL RNA to the trans-spliceosome by interaction with an RNA or protein component.

Finally, it is interesting to note that the modified nucleotides present in mammalian U small nuclear RNAs (reviewed in ref. 16) are clustered toward the 5′ half of the molecules. In the case of the U1 small nuclear RNA, four of five of the modified nucleotides are at the very 5′ end of the RNA, immediately adjacent to or within the U1 sequence, which interacts by hydrogen bonding with the 5′ splice site consensus sequence of mammalian introns (17). Because the SL RNA might be endowed with a U1-like activity (18), it is tempting to speculate that the function of the modified nucleotides at the 5′ end of the SL RNA and the U1 snRNA might be analogous.

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