MOLECULAR AND CELLULAR BIOLOGY, Nov. 1992, p. 4844–4851
0270-7306/92/114844-08$02.00/0

Vol. 12, No. 11

Trypanosoma brucei Spliced-Leader RNA Methylations Are Required for *trans* Splicing In Vivo

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Received 28 May 1992/Accepted 31 July 1992

The *Trypanosoma brucei* spliced leader (SL) RNA donates its 5′ leader sequence to all nuclear pre-mRNAs via *trans* RNA splicing. The SL RNA is a small-nuclear U RNA-like molecule which is present in the cell as part of a small ribonucleoprotein particle. However, unlike the trimethylguanosine-capped small nuclear U RNAs, the SL RNA has a highly modified 5′ terminus containing an m^7G cap and methylations on the first four transcribed nucleotides. Here, we show that incubation of procyclic-form *T. brucei* in the presence of the *S*-adenosylmethionine analog, sinefungin, leads to a rapid inhibition of SL RNA methylation. A concomitant inhibition of *trans* splicing and an accumulation of high-molecular-weight tubulin transcripts were also observed. The effects of sinefungin on SL RNA methylation and on *trans* splicing were correlated by labeling of cells incubated in the presence of the antibiotic. The results indicate that 5′ modifications of the SL RNA are necessary for it to participate in *trans* splicing. SL RNA modification is not required for assembly of the core SL ribonucleoprotein, as these Cs_2SO_4-resistant particles can be formed with either methylated or undermethylated SL RNA.

trans RNA splicing in the protozoan *Trypanosoma brucei* is a process by which a short, 39-nucleotide leader sequence (the spliced leader [SL]) is joined to protein-encoding exons to generate mature mRNAs (for a review, see reference 1). The substrates for the reaction are independently transcribed and consist of an approximately 140-nucleotide SL RNA, containing the SL at its 5′ end, and the precursor mRNAs. The latter are, in many cases, large polycistronic units which can extend for as many as 55 kb. It has been suggested that a function of *trans* splicing may be to aid in the resolution of these precursors into capped, monocistronic mRNAs.

The mechanism of *trans* splicing is similar biochemically to that of pre-mRNA *cis* splicing and proceeds through a two-step cleavage-and-ligation reaction (22, 29). In the first step of the reaction, a branch site residue in the pre-mRNA participates in a nucleophilic attack on the 5′ splice site, leading to cleavage of the splice site and generation of a 2′-5′ branched intermediate. Because the two RNA substrates for *trans* splicing are not linked as they are for *cis* splicing, this branched intermediate has the shape of a Y structure rather than a lariat (22, 29). Resolution of the Y structure occurs with cleavage at the 3′ splice site and ligation of the two exons to form a mature mRNA. Although much about the biochemical components of the *trans*-splicing pathway remains to be understood, the process requires several trypanosomal analogs of the small nuclear U RNAs (U snRNAs) that participate in *cis* splicing (33). These are the U2, U4, and U6 snRNAs which were initially identified in trypanosomes by virtue of their trimethylguanosine (TMG) cap structures (20, 31, 32). However, the trypanosome U snRNAs differ significantly in sequence from the highly conserved snRNAs of higher eukaryotes. These RNAs lack consensus Sm-binding sites, which are present in single-stranded regions of the U1, U2, U4, and U5 snRNAs of all other species examined and which are required for their assembly into ribonucleoprotein (RNP) particles and their acquisition of TMG caps (for a review, see reference 17). The *T. brucei* U snRNAs also differ from the eukaryotic consensus sequences by the absence, in the U2 snRNA, of a conserved branch point recognition sequence and a stem-loop III region (14, 34). In addition, no U1 or U5 snRNA homologs are known to exist in trypanosomes despite extensive efforts toward their identification (36a).

It has been suggested that the SL RNA plays a bifunctional role in *trans* splicing by acting both as a substrate for the reaction and as a U1-like snRNA. This hypothesis is supported by the observation that the 5′ domain of a trypanosome SL RNA can promote pre-mRNA *cis* splicing in the absence of U1 snRNA in a mammalian in vitro system (6). Also consistent with this hypothesis is the observation that SL RNAs from several different nematode and trypanosome species can be folded into similar secondary structures in the absence of primary-sequence conservation (7). In nematodes, the resemblance between the SL RNAs and U snRNAs is most evident, as the SL RNAs are bound by Sm antigens and are TMG capped (30). The *T. brucei* SL RNA is also bound by proteins which are shared by the U snRNPs (8, 24); however, the SL RNA does not contain the characteristic U snRNA cap structure. Rather than containing a TMG cap, it has a highly unusual 5′ terminus with an m^7G cap and methylations on the first four transcribed nucleotides (12, 27). The base modifications on two of the transcribed nucleotides have recently been identified by mass spectroscopy, and the entire structure is now known to be $m^7G(5')ppp(5')m^6_2AmpAmpCmpm^3Ump$ (3).

In our previous studies, we observed that the 5′ methylations of the *T. brucei* SL RNA are conserved in a very distantly related trypanosomatid, *Leptomonas collosoma* (27). This observation suggested that the modifications play an important role in SL metabolism, perhaps during addition of the SL to pre-mRNAs by *trans* splicing. To test this hypothesis, we have incubated procyclic-form *T. brucei* in media containing sinefungin, an inhibitor of *S*-adenosylmethionine-dependent transmethylation reactions (5, 36). The

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results of our experiments indicate that sinefungin causes a rapid inhibition of *trans* splicing and an accumulation of high-molecular-weight pre-mRNAs. These effects of the antibiotic are due, at least in part, to an inhibition of 5′ SL RNA methylation.

MATERIALS AND METHODS

Cell culture and RNA isolation. Variant 1.1 of the IsTat serodeme has been previously described (28). Procyclic-form cells were grown in Cunningham's SM (semidefined) medium as modified by Bienen et al. (4) and by the addition of 10 mg of adenosine, 10 mg of guanosine, and 2.5 mg of bovine hemin per liter. For the in vivo labeling of trypanosomes, 8.0×10^8 cells, harvested at a density of 7.5×10^6/ml, were suspended in phosphate-free medium to a final density of 1.0×10^7/ml, and the culture was divided in two. The cells were incubated for 2 h at 26°C, and then sinefungin (Sigma) was added to one of the resulting cultures to a concentration of 1.0 μg/ml. Following an additional 1-h incubation, carrier-free $^{32}PO_4$ was added to both cultures at a concentration of 25 μCi/ml, and incubation continued for 6 h.

Trypanosomes were collected by centrifugation, and total RNA was purified by the guanidinium–hot-phenol method as described elsewhere (16). Poly(A)$^+$ RNAs were twice purified by oligo(dT)-cellulose chromatography (16); SL RNAs were obtained by hybridization selection (16).

Northern (RNA) analyses. For the identification of high-molecular-weight transcripts, 20-μg samples of total RNA were electrophoretically separated on a 1% agarose–6% formaldehyde gel and blotted to Nytran (Schleicher and Schuell). Filters were hybridized for 16 h at 60°C in a solution containing 60% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution (0.1% [wt/vol] each Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 0.1 mg of yeast tRNA per ml, and 0.1% sodium dodecyl sulfate (SDS), with ^{32}P-labeled RNA probes synthesized in vitro by SP6 polymerase runoff transcription. Filters were washed in 0.1× SSPE–0.1% SDS at 65°C, and the hybridization results were visualized by autoradiography.

Primer extension analyses. The oligonucleotide primer KW01, which is complementary to nucleotides 120 through 137 of the *T. brucei* SL RNA, and the oligonucleotide JD20, which is complementary to nucleotides 46 through 65 of the U2 snRNA, were radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. To 10 μg of total RNAs, or to 3% of the Cs_2SO_4 gradient fraction RNAs, was added approximately 10^6 cpm of each oligonucleotide, and the mixtures were dried. Samples were dissolved in 4.5 μl of 0.5 mM EDTA, and the mixtures were incubated at 90°C for 2 min and placed on ice for 5 min. A 0.5-μl sample of RNasin and 2 μl of 5× buffer (10 mM dithiothreitol, 250 mM KCl, 40 mM $MgCl_2$, 250 mM Tris hydrochloride [pH 8.0]) were added, and the mixture was incubated at 40°C for 30 min. A 2-μl sample of a 5 mM deoxynucleoside triphosphate mix and 1 μl (7.0 U) of avian myeloblastosis virus reverse transcriptase were added. Reaction mixtures were incubated at 40°C for 60 min, and the reactions were stopped by the addition of 100 μl of 2 M ammonium acetate and 110 μl of isopropyl alcohol. After precipitation, the samples were centrifuged, washed in 70% ethanol, dried, and suspended in formamide dye buffer (16). A sequencing ladder was prepared by using as a template pSPSL1, which contains the *T. brucei* SL RNA coding region and consists of the 156-bp *Nla*IV-*Fok*I fragment of pTb1.4-1 (23) cloned into the *Hin*dIII site of pSP64 by using *Hin*dIII linkers. Sequencing reactions were as described previously (25). Electrophoresis was done in 6% acrylamide–8 M urea sequencing gels. Gels were dried and autoradiographed.

Cs_2SO_4 gradient fractionation. For whole-cell extract preparation, a culture of approximately 2×10^9 procyclic-form trypanosomes at a density of 9×10^6 cells per ml was divided in two. Sinefungin was added to one of the resulting cultures to a final concentration of 2.0 μg/ml, and incubation continued for 30 min. Cells were then harvested by centrifugation and rinsed with lysis buffer (30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] KOH [pH 7.9 at 25°C], 5 mM $MgCl_2$, 50 mM KCl, and 5 μg each of pepstatin and leupeptin per ml), and an equal volume of silicon carbide was added to the resulting pellets. The cells were lysed by grinding with a Teflon pestle for approximately 20 gyrations and suspended in 3.0 ml of lysis buffer. The homogenates were centrifuged to remove the silicon carbide and the majority of insoluble debris. The supernatants were then collected and centrifuged for an additional 1 h at 45,000 rpm in a Ti-70.1 rotor (150,000 × *g*) to pellet ribosomes and other material not removed in the first centrifugation step. To the resulting supernatant were added solid Cs_2SO_4 and lysis buffer to a density of 1.4 g/ml and a final volume of 5.0 ml. After centrifugation for 72 h in an SW50.1 rotor at 35,000 rpm at 20°C, 0.5-ml fractions were collected from the top of each tube. The fractions were desalted by Sephadex G-25 gel filtration, and the RNAs were recovered by phenol-chloroform extraction and ethanol precipitation.

Cap analyses. Labeled RNAs [either poly(A)$^+$ or SL RNAs] were digested to completion with RNase T_2 in 50 mM Na^+ acetate (pH 5.2)–2 mM EDTA at 37°C for several hours. The digestion products were spotted on polyethyleneimine (PEI) cellulose plates and developed for 24 h in 2 M pyridinium formate (pH 3.4). Cap spots were located by autoradiography and eluted with 2 M triethylammonium bicarbonate. Triethylammonium bicarbonate was removed by lyophilization.

T_2-resistant oligonucleotides were digested with nucleotide pyrophosphatase (in 10 mM Tris [pH 7.6]–10 mM $MgCl_2$ for 1 h at 37°C) and nuclease P1 (in 50 mM Na^+ acetate, pH 5.2, for 1 h at 37°C). The digestion products were chromatographed on cellulose thin-layer plates by using as a first-dimension solvent isobutyric acid-concentrated NH_4OH-H_2O (66:1:33, vol/vol/vol) and as a second-dimension solvent 0.1 M sodium phosphate [pH 6.8]–ammonium sulfate–*n*-propanol (100:60:2, vol/wt/vol).

RESULTS

Inhibition of *trans* splicing by the *S*-adenosylmethionine analog, sinefungin. To examine whether the 5′ methylations are necessary for the SL RNA to be recognized as a substrate for *trans* splicing, we incubated procyclic-form cells with sinefungin, a naturally occurring nucleoside antibiotic. Sinefungin has previously been shown to display antitrypanosomal activity against several species of *Leishmania* (2) and against *Trypanosoma cruzi* (9). The sensitivity of procyclic-form *T. brucei* to sinefungin was determined by incubating the cells in various concentrations of the drug. Complete growth inhibition was observed with sinefungin concentrations of 1.0 μg/ml or greater (Fig. 1). Although the trypanosomes did not reproduce in the presence of the antibiotic, the majority of the cells remained viable, as

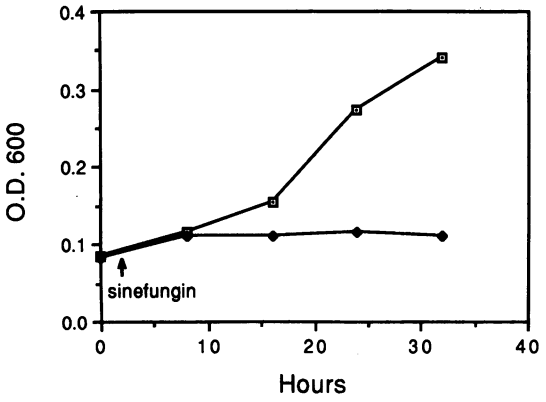

FIG. 1. Effect of the *S*-adenosylmethionine analog, sinefungin, on the growth of procyclic-form *T. brucei*. ⊡ and ◆, 0.0 and 1.0 μg of sinefungin per ml, respectively. O.D. 600, optical density at 600 nm.

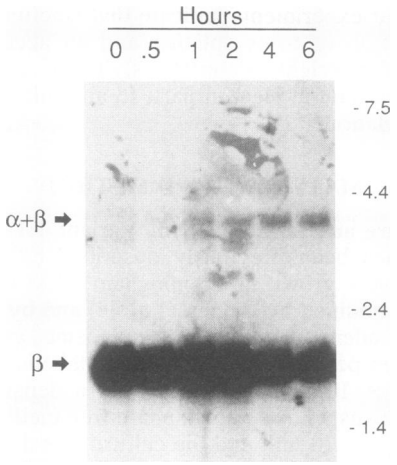

FIG. 2. Northern blot analyses of equivalent amounts (20 μg) of total cellular RNA extracted from cells cultured in 2.0 μg of sinefungin per ml. Cells were harvested at 0, 1/2, 1, 2, 4, and 6 h following addition of the antibiotic. The RNAs were electrophoresed through a 1% agarose–6% formaldehyde gel, blotted onto Nytran, and hybridized with a probe encompassing nucleotides −8 to +149 relative to the splice site of the β-tubulin gene. β, position of the mature, monomeric mRNA; α(+)β, position of the approximately 3.7-kb RNA containing both β- and α-tubulin exon sequences. Numbers on the right are sizes, in kilobases.

observed by light microscopy, for as long as 24 h following the addition of sinefungin to the media.

The effect of sinefungin on the *trans*-splicing pathway was first examined by assaying trypanosomes grown in the presence of the drug for the accumulation of potential reaction precursors. Many protein-encoding genes in *T. brucei* are initially transcribed as large polycistronic units which are subsequently processed by *trans* splicing and polyadenylation (1). To assay for these polycistronic transcripts, cells were harvested at various time points after the addition of the antibiotic to the culture, and their total RNAs were analyzed by Northern hybridization. The result of one such analysis, shown in Fig. 2, indicates that a decrease in the amount of mature 2.0-kb β-tubulin transcript was observed with increasing periods of incubation. Concomitant with this decrease was an accumulation of high-molecular-weight tubulin transcripts. These transcripts were observed as early as 30 min after addition of the drug, and they continued to accumulate for several hours.

The high-molecular-weight tubulin transcripts are the size (3.75 kb) expected of a dimer of the tubulin exon repeats. RNAs of this size hybridized with both the β-tubulin and the α-tubulin probes as well as with a probe specific for the tubulin β-α intergenic region (data not shown). Similar dimer-sized transcripts, as well as larger transcripts that are multimers of the tubulin exon repeats (i.e., β-α-β, β-α-β-α, etc.) have previously been identified in bloodstream-form *T. brucei* and have been shown to accumulate upon disruption of *trans* splicing by heat shock treatment (21). The transcripts were partially processed, as they are 3′ polyadenylated (21). These may be RNAs which are removed from the *trans*-splicing pathway prior to completion of their processing and which, once removed from the pathway, cannot be subsequently spliced. The accumulation of these multimeric tubulin transcripts upon incubation of trypanosomes in sinefungin (Fig. 2) is therefore consistent with the hypothesis that *trans* splicing is inhibited by the antibiotic.

The effect of sinefungin on *trans* splicing was examined directly by using a primer extension assay to detect branched Y structures, which are intermediates in the reaction. The oligonucleotide KW01, which is complementary to nucleotides 120 through 137 of the SL RNA, was 5′ end labeled and hybridized with total RNA prepared from trypanosomes grown for 0, 30, and 60 min in the presence of 2 μg of sinefungin per ml. The radiolabeled primer was extended by avian myeloblastosis virus reverse transcriptase in the presence of the four deoxynucleoside triphosphates. In the 0-min sample, a strong stop is observed opposite the G residue at the position of the splice junction (Fig. 3). This extension product has previously been shown to be the result of polymerization using branched 3′ intron fragments of the SL RNA as templates (22). Although, theoretically, the product could also be the result of polymerization using debranched introns as templates, that RNA species appears to be highly unstable, as it has not been detected by our assay. The quantity of the extension product is reduced by at least a factor of 4 when RNAs prepared from cells treated with sinefungin are used as templates. These results indicate that a reduction of branched Y structures occurs within 30 min of addition of the antibiotic to procyclic-form cultures, and they are consistent with the interpretation that *trans* splicing is inhibited. Furthermore, they suggest that this inhibition occurs early in the splicing pathway, prior to or at the initial step of cleavage and branch formation.

Sinefungin causes a decrease in SL RNA methylation. The primer extension analyses shown in Fig. 3 also reveal an effect of sinefungin on the 5′ modification of the SL RNA. Extension of the oligonucleotide KW01 using SL RNA from untreated cells as a template resulted in a major stop opposite the A residue which is the fifth transcribed nucleotide. As the SL RNA contains a cap 4 structure, this is the first nucleotide which is unmodified. The methylations of the first four nucleotides appear to interfere with base pairing and inhibit the progress of reverse transcriptase across this portion of the SL RNA (22). However, some extension of the KW01 primer to positions opposite the first and second A residues is also observed in this sample and represents approximately 32% of the SL RNA extension products. Extension of the primer to these positions is likely to be due to the presence of unmodified or partially modified SL RNAs in the RNA sample. We have obtained evidence that partially methylated molecules are present in the total SL RNA

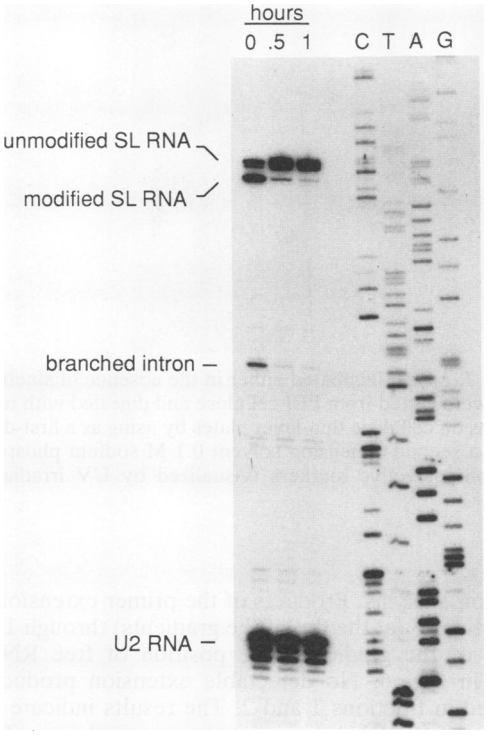

FIG. 3. Primer extension analysis of RNA extracted from procyclic-form *T. brucei* that was incubated in media containing 2 μg of sinefungin per ml for 0, 1/2, and 1 h. The assays were performed by using an oligonucleotide primer complementary to the 3′ end of the SL RNA. A second oligonucleotide primer complementary to nucleotides 46 to 65 of the U2 snRNA was included as an internal control. Primer extension products of a dideoxynucleotide sequencing reaction performed on a clone of the SL RNA are included for reference (C, T, A, and G). Positions of products that result from extension of the SL RNA primer hybridized either to SL RNA that is unmodified by methylation or is partially methylated, to fully modified SL RNA, or to branched splicing intermediates (branched intron) and positions of those resulting from extension of the U2 snRNA primer are indicated on the left.

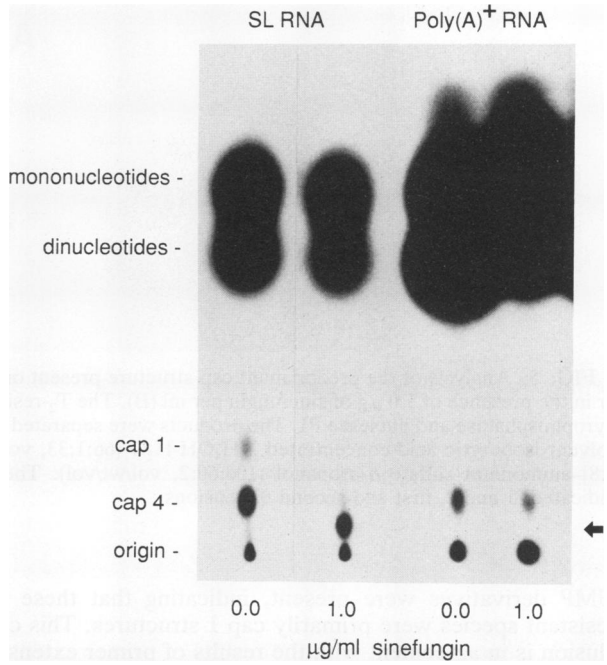

FIG. 4. Detection of T_2-resistant cap structures. Procyclic-form *T. brucei* grown in media containing either 0 or 1.0 μg of sinefungin per ml were labeled by the addition to the media of $^{32}PO_4$. Radiolabeled Poly(A)$^+$ RNAs and SL RNAs were purified and digested with RNase T_2, and the products were chromatographed on PEI cellulose thin-layer plates. Positions of cap 4 species and partially modified cap 1 species are indicated on the left. The position of the predominant T_2-resistant species present in SL RNA from sinefungin-treated trypanosomes is indicated by the arrow on the right.

population both by alkaline hydrolysis of end-labeled RNAs (27) and by direct analysis of SL RNAs labeled uniformly in vivo (see below).

Incubation of trypanosomes in media containing 2 μg of sinefungin per ml for 30 or 60 min dramatically reduces the block to reverse transcription at the 5′ end of the SL RNA (Fig. 3). Approximately 95% of the products extend to positions opposite the first or second transcribed nucleotide when SL RNAs from cells treated for 30 min with sinefungin are used as templates. These results are consistent with the interpretation that 5′ methylation of the SL RNA is rapidly blocked by the drug sinefungin.

5′ SL RNA modifications are required for *trans* splicing. A correlation between the effect of sinefungin on *trans* splicing and its effect on SL RNA methylation was found by examining the cap structure of poly(A)$^+$ RNAs that had been spliced in the presence of the drug. These RNAs were specifically labeled by the addition of $^{32}PO_4$ to a culture of procyclic-form trypanosomes that had been incubated for 1 h in media containing 1.0 μg of sinefungin per ml. $^{32}PO_4$ was similarly added to a control culture of trypanosomes which contained no sinefungin, and after a 6-h period of incubation, the RNAs from the two cultures were recovered. A comparison of the amount of ^{32}P incorporation into total RNA from the sinefungin-treated cells and from the untreated cells indicated that stable RNA synthesis is reduced at this concentration of sinefungin by approximately 47%. Incorporation of the ^{32}P label into poly(A)$^+$ RNA was decreased by an additional factor of 3 in the sinefungin-treated cells, indicating that mRNA synthesis was specifically affected by the antibiotic.

To examine their 5′ termini, the labeled poly(A)$^+$ RNAs and SL RNAs were digested with RNase T_2, a nonspecific endonuclease which reduces the molecules primarily to mononucleotides and dinucleotides but which cannot hydrolyze the PP_i bonds or the 5′ bonds adjacent to 2′-O modifications that are found in the SL cap structure. The T_2-resistant caps were then separated from the other reaction products on the basis of their charge by PEI cellulose thin-layer chromatography (TLC). The results, shown in Fig. 4, indicate that the T_2-resistant species produced upon digestion of SL RNAs from the untreated cells migrated as two spots under the conditions used. The two spots were eluted from the PEI cellulose, and their nucleotide compositions were determined by two-dimensional cellulose TLC after their complete digestion with pyrophosphatase and nuclease P1. The T_2-resistant species present in the predominant spot was shown by this analysis to be the fully modified cap 4 structure (Fig. 5A). Present in the minor spot were several capped species derived from partially modified SL RNAs. These species appeared to contain either m^7G or G cap residues as well as the A residues which were the first two transcribed nucleotides (data not shown). No CMP or

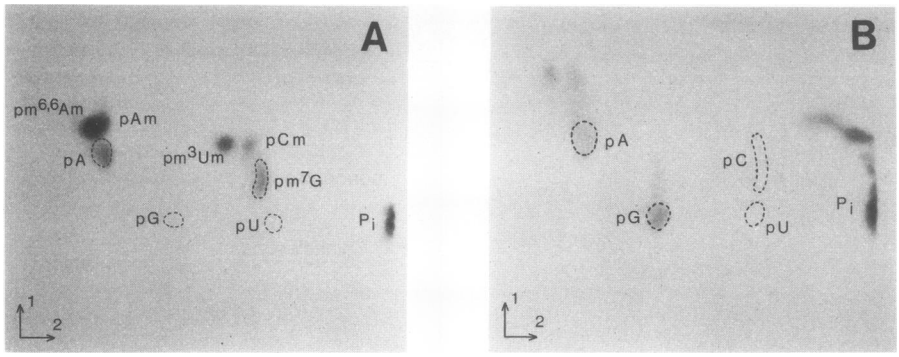

FIG. 5. Analysis of the predominant cap structure present on SL RNAs from *T. brucei* incubated either in the absence of sinefungin (A) or in the presence of 1.0 μg of sinefungin per ml (B). The T_2-resistant structures were eluted from PEI cellulose and digested with nucleotide pyrophosphatase and nuclease P1. The products were separated in two dimensions on cellulose thin-layer plates by using as a first-dimension solvent isobutyric acid-concentrated NH_4OH-H_2O (66:1:33, vol/vol/vol) and as a second-dimension solvent 0.1 M sodium phosphate [pH 6.8]–ammonium sulfate–*n*-propanol (100:60:2, vol/wt/vol). The positions of nonradioactive markers (visualized by UV irradiation) are indicated. 1 and 2, first and second dimensions.

UMP derivatives were present, indicating that these T_2-resistant species were primarily cap 1 structures. This conclusion is in agreement with the results of primer extension of these SL RNAs (Fig. 3), which indicate that when avian myeloblastosis virus reverse transcriptase is not stopped opposite the fifth nucleotide of the SL RNA, it proceeds to a position opposite either the first or the second nucleotide. It appears from these experiments that methylation of the second, third, and fourth SL RNA nucleotides is coordinated, as only molecules that contain methyl groups on all of these residues, or on none of them, were detected.

The major T_2-resistant structure present in SL RNAs from trypanosomes treated with sinefungin is a species which migrates more slowly than the fully modified cap structure under the conditions of PEI cellulose TLC (Fig. 4). A T_2-resistant species with a mobility identical to that of the fully modified cap 4 structure is also observed, but in a greatly reduced quantity. The nucleotide composition of the slowly migrating species was determined by two-dimensional cellulose TLC and is shown in Fig. 5B. Digestion of the species with pyrophosphatase and nuclease P1 released AMP, GMP, and P_i. Two minor products, which migrate very quickly in the first dimension, are likely to be undigested and/or incompletely digested compounds. These results are consistent with the hypothesis that the T_2-resistant species has the structure GpppAp and that the majority of SL RNAs in sinefungin-treated *T. brucei* are not 5′ methylated.

Poly(A)$^+$ RNAs synthesized in *T. brucei* in the presence of sinefungin contain only the fully modified cap structure, even though this structure is present on a minority of the cellular SL RNAs (Fig. 4). This results supports our hypothesis that the cap 4 structure is required for the SL RNA to be utilized as a substrate for *trans* splicing.

5′ SL RNA modifications are not required for SL RNP core particle formation. The SL RNA is present in the cell as part of a small RNP particle which resembles U snRNPs in that it contains a salt-resistant core (18). The effect of sinefungin on the assembly of this core SL RNP was examined by applying S150 extracts prepared simultaneously from cells grown in the absence of sinefungin and from cells grown in the presence of 2 μg of sinefungin per ml to Cs_2SO_4 density gradients. The distributions of the SL RNP and U2 snRNP particles within the gradients were determined by primer extension analysis. Products of the primer extension assays for fractions 3 (at the top of the gradients) through 10 (at the bottom of the gradients; the position of free RNAs) are shown in Fig. 6. No detectable extension products were observed in fractions 1 and 2. The results indicate that the majority of SL RNAs in extracts prepared from *T. brucei* grown either in the absence of sinefungin or in the presence of 2 μg of sinefungin per ml are distributed in the gradients as salt-resistant RNP particles. Furthermore, the distributions of fully modified and partially modified SL RNAs are identical within the gradient containing an extract of sinefungin-treated cells. These results indicate that formation of the Cs_2SO_4-resistant SL RNP is not dependent on the presence of a 5′ cap 4 structure. The U2 snRNA is also distributed in a similar manner in the gradients containing extracts of untreated and sinefungin-treated trypanosomes. However, unlike the distribution of the SL RNA, which forms a sharp peak, the U2 snRNA distribution trails towards the bottoms of the gradients. Similar results with the U2 snRNA have been previously reported and suggest that this RNP particle is less stable than the SL RNP under stringent ionic conditions (8).

DISCUSSION

Results presented here indicate that *trans* splicing in procyclic-form *T. brucei* is inhibited by the antibiotic sinefungin. A fourfold or greater decrease in the concentration of branched intermediates was observed within 15 min of addition of the drug to the culture, and an accumulation of incompletely processed pre-mRNAs was observed within 1/2 h. Concomitant with the disruption of *trans* splicing was an inhibition of 5′ methylation of the SL RNA. The majority of SL RNA molecules that were synthesized in the presence of the drug completely lacked 5′ methyl groups and contained only a G cap. Only a small minority of the newly synthesized SL RNAs were completely 5′ methylated. Nevertheless, poly(A)$^+$ RNAs synthesized during incubation of trypanosomes in sinefungin contained only the fully methylated cap 4 structures. These results are consistent with the interpretation that the 5′ cap methylations are required for the SL RNA to be utilized as a substrate for *trans* splicing and that, therefore, the effect of sinefungin on SL RNA modification and its effect on *trans* splicing are correlated.

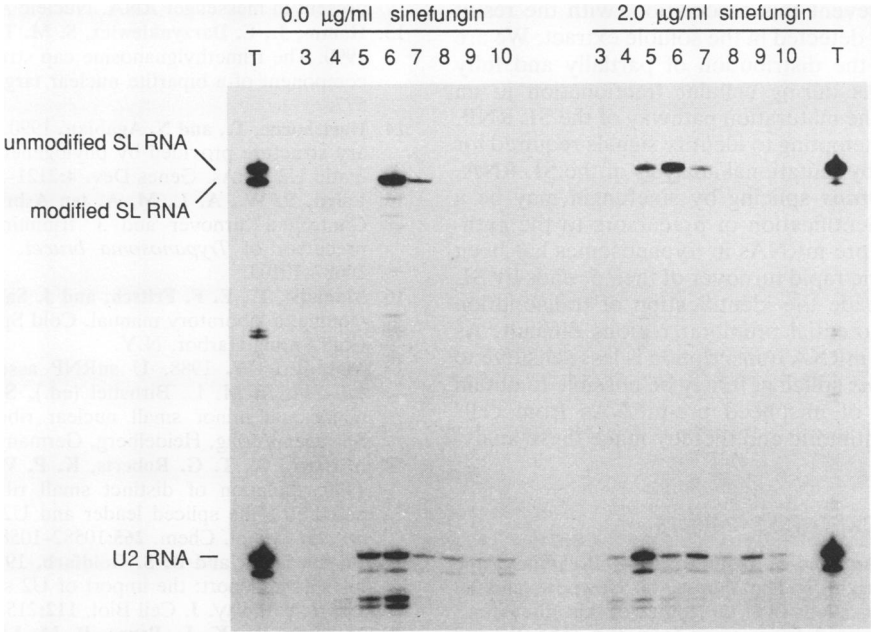

FIG. 6. Primer extension analyses of Cs_2SO_4 equilibrium density gradient fractions using oligonucleotide primers complementary to the SL RNA and to the U2 snRNA. Assays were performed with total RNA and RNA prepared from gradient fractions 3 through 10 of trypanosomes grown in the absence of sinefungin and of trypanosomes grown for 1/2 h in media containing 2 μg of sinefungin per ml. Positions of products that result from extension of the SL RNA primer hybridized either to SL RNA that is unmodified by methylation or partially methylated or to fully modified SL RNA and positions of products resulting from extension of the U2 snRNA primer are indicated on the left.

This hypothesis is supported by the recent finding that in a system of permeabilized *T. brucei* cells, *trans* splicing is also inhibited by conditions that prevent SL RNA methylation (35).

Our experiments do not address the question of whether the disruption of *trans* splicing by sinefungin is a result only of the inhibition of SL RNA methylation or whether other factors are influenced by the antibiotic. It is likely that upon prolonged exposure to the drug, the synthesis of other reaction components, such as the trimethylated U snRNAs, will also be disrupted. However, the very rapid inhibition of *trans* splicing that is observed upon addition of sinefungin to procyclic-form trypanosomes suggests that the primary effect is on a molecule with a high rate of turnover. As its half-life is approximately 4 min (15), the SL RNA is a likely candidate for such a molecule.

5′ RNA cap structures have been implicated as playing roles in several of the processes that are involved in nuclear *cis* splicing. The first of these roles is a direct requirement for an m^7G cap on substrate pre-mRNAs during their assembly into active splicing complexes. The need for a cap in this processing step appears to be a highly specific one rather than simply being a protective effect against nucleolytic degradation and may involve recognition by binding proteins (26). It seems likely that the m^7G cap of the SL RNA, and perhaps other 5′ modifications as well, is required during assembly of the *trans* spliceosome.

A second role of RNA cap structures in *cis* splicing is during the assembly and transport of the U snRNPs that are required for the reaction. In higher eukaryotes, it has been shown that the U1 through U5 snRNAs are transcribed initially with an m^7G cap and are subsequently transported to the cytoplasm where they are assembled into core U snRNPs (for reviews, see references 17 and 37). It is in the cell cytoplasm that the additional methyl groups of the TMG cap are transferred to the RNAs. It has recently been demonstrated that for the U1 and U2 snRNPs, these methylations are part of a bipartite signal which allows for transport of the particles back to the nucleus, where they become available to participate in splicing (10, 11, 13, 19). Perhaps 5′ modifications present on the SL RNA act in a manner analogous to the TMG methylations and play a role in maturation and/or transport of the SL RNP. This hypothesis is particularly intriguing because of the U snRNP-like nature of the SL RNP. It has been recently shown that the *T. brucei* SL RNP has a salt-stable core containing five low-molecular-weight polypeptides that are also common to the U2 and U4-U6 snRNPs (24). If, as in higher eukaryotes, the trypanosome core snRNPs (including that of the SL RNP) are formed in the cytoplasm, then subsequent methylation of the SL RNA may be required for transport of the particle back into the nucleus. It is somewhat perplexing, in light of the recent observation that the SL RNP contains core proteins, that it does not receive a TMG cap as does the SL RNA of nematodes. Perhaps in trypanosomes the signals for the U snRNA and SL RNA cap methylations reside either in additional, non-core binding proteins or in the nucleotide sequences of the RNAs.

The results of the density gradient fractionation of *T. brucei* extracts (Fig. 6) are interesting in that they reveal that while unmodified SL RNAs from cells treated with sinefungin were present in the gradients as salt-resistant particles, the majority of undermethylated SL RNAs from untreated procyclic-form cells were not detected in the gradient fractions. Although the latter SL RNAs may have been specifically degraded during preparation of the cellular extract, a more likely hypothesis is that they were removed from the extract during one of the early centrifugation steps, perhaps by an association with insoluble material. If this hypothesis is correct, then incubation of trypanosomes in the presence

of sinefungin may prevent this association, with the result that the SL RNPs are detected in the soluble extract. We are currently examining the distribution of partially and fully methylated SL RNAs during cellular fractionation in an attempt to elucidate the maturation pathway of the SL RNP. In addition, we are attempting to identify signals required for SL RNP maturation by mutational analysis of the SL RNA.

The inhibition of *trans* splicing by sinefungin may be a useful tool for the identification of precursors to the pathway. The analysis of pre-mRNAs in trypanosomes has been greatly hindered by the rapid turnover of their 5′ ends by SL addition. This has made the identification of transcription initiation sites and potential promoter regions difficult. As our data indicate that mRNA transcription is less sensitive to sinefungin than is *trans* splicing, it may be possible to obtain increased quantities of unspliced pre-mRNAs from cells incubated with the antibiotic and thereby make these analyses more attainable.

ACKNOWLEDGMENTS

We thank T. G. Roberts and T. White for critical reading of the manuscript and J. Dungan, T. Hartshorne, G. Newport, and K. Watkins for helpful suggestions over the course of this study.

This work was supported by a grant from the John D. and Catherine T. MacArthur Foundation and by National Institutes of Health grant AI212975. N.A. is a Burroughs Wellcome Scholar of Molecular Parasitology.

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