

Trypanosome mRNAs have unusual “cap 4” structures acquired by addition of a spliced leader

(mRNA methylation/trans-splicing)

KAREN L. PERRY*, KENNETH P. WATKINS*, AND NINA AGABIAN*

School of Public Health and Naval Biosciences Laboratory, University of California, Berkeley, Berkeley, CA 94720

Communicated by Charles Yanofsky, June 5, 1987 (received for review November 19, 1986)

ABSTRACT A single capped oligonucleotide is released from *Trypanosoma brucei* poly(A)⁺ RNA upon digestion with RNase T2. This observation supports the hypothesis that all *T. brucei* mRNAs share a common leader sequence. Digestion of the T2-resistant species with nucleotide pyrophosphatase shows that the capping nucleotide is 7-methylguanosine 5'-monophosphate (pm⁷G). Additional characterization of the T2-resistant fragment indicates that modifications are present on the first four transcribed nucleotides; the 5' termini of *T. brucei* mRNAs can, therefore, be described as “cap 4” structures. Identical 5'-cap structures are found on the *T. brucei* spliced leader (SL) RNA; an observation compatible with the hypothesis that the small SL RNA acts as a donor of the SL for the mRNA. However, we find that within a population of purified SL RNAs are species that are capped but incompletely modified. The presence of these unmodified and partially modified species allowed us to analyze the 5' sequence of the SL RNA transcript. The results indicate that transcription begins four nucleotides upstream of the reported 5' end. Therefore, the *T. brucei* SL transcript is actually 39 rather than 35 nucleotides long. We have also analyzed the capped oligonucleotide of a distantly related Trypanosomatid, *Leptomonas collosoma* and find it to be identical to that of *T. brucei*. The potential significance of these results is discussed in light of observations of trypanosome gene expression.

One of the unusual aspects of the molecular biology of the Trypanosomatidae is that the mRNAs of these parasitic protozoa contain a common leader sequence (reviewed in ref. 1). This phenomenon was first described and has been most extensively studied in the African trypanosome, *Trypanosoma brucei*. All *T. brucei* mRNAs that have been examined share a 5'-terminal sequence that was reported to be 35 nucleotides (1). This sequence has been termed the spliced leader (SL) because it is encoded separately in the genome, in a 1.4-kilobase repeat unit that is present in ≈200 tandemly arranged copies (2, 3). A 135- to 141-nucleotide transcript of the 1.4-kilobase repeat unit, termed the SL RNA, has the leader sequence at its 5' end and may act as a donor of the SL to mRNAs (4–6). Several models have been presented to describe the process by which the SL is joined to the 5' ends of the mRNAs (4, 6). These models suggest either that addition of the SL to pre-mRNAs is post-transcriptional and requires RNA splicing or that the SL RNA serves as a primer for mRNA transcription. Murphy *et al.* (7) and Sutton and Boothroyd (8) have presented data that support the former hypothesis and suggest that the mechanism by which the SL and mRNA transcripts are joined is a trans-splicing reaction.

Evidence has indicated (9, 10) that the SL RNA of *T. brucei* contains a 5'-terminal modification with properties similar to those of a cap structure. Caps have been detected on all

eukaryotic cellular mRNAs analyzed to date as well as on many small nuclear RNAs and are intimately involved in several aspects of RNA metabolism (11–13). Because of the significant role that the SL plays in trypanosome gene expression, we considered it essential to examine more precisely the nature of its 5'-terminal modification(s).

In this paper we analyze and compare the cap structures on the SL RNA and poly(A)⁺ RNAs of *T. brucei*. We find that these RNA species have identical 5' termini containing a 7-methylguanosine (m⁷G) cap and additional modifications on the first four nucleotides. These “cap 4” structures are the most highly modified 5' termini that have been described on eukaryotic mRNAs. Identical modifications were found on the poly(A)⁺ RNA of *Leptomonas collosoma*, an insect trypanosomatid that is only distantly related to *T. brucei*. These and other observations suggest that the SL cap structures that we have characterized may have been conserved throughout the evolution of the Trypanosomatidae.

MATERIALS AND METHODS

Radioactive Labeling of Trypanosomes. Variant 1.1 of the IsTat serodeme (*T. brucei* stock EATRO 164) has been described (14). For the *in vitro* labeling of procyclic forms, cells, suspended in phosphate-free BSM (15) to a final density of 2×10^7 cells per ml, were incubated for 3 hr at 26°C. [³²P]orthophosphate was added to a concentration of 0.1 mCi/ml (1 Ci = 37 GBq), and incubation was continued overnight. For the *in vivo* labeling of *L. collosoma* (American Type Culture Collection catalog no. 30261), cells, suspended in Dulbecco's modified Eagle's medium high-glucose/phosphate-free/Hepes (Irvine), were incubated for 1 hr at 26°C. [³²P]orthophosphate was added to a final concentration of 0.125 mCi/ml, and incubation was continued for 3 to 4 hr. Purification of total RNA was by the guanidinium/“hot” phenol method as described by Maniatis *et al.* (16).

Cap Analyses. Labeled RNAs (either poly(A)⁺ or SL RNAs) were digested to completion with RNase T2 in 50 mM sodium 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 18 hr in 2 M pyridinium formate (pH 3.4) (17). Cap spots were located by autoradiography and eluted with 2 M triethylammonium bicarbonate (17).

Analysis of the T2-resistant oligonucleotides was by digestion with either nucleotide pyrophosphatase (in 10 mM Tris-HCl, pH 7.6/10 mM MgCl₂ for 1 hr at 37°C), nuclease P1 (in 50 mM sodium acetate, pH 5.2, for 1 hr at 37°C), or a combination of the enzymes. The digestion products were chromatographed on cellulose thin layer plates using solvent

Abbreviations: SL, spliced leader; pm⁷G, 7-methylguanosine 5'-monophosphate; m⁷G, 7-methylguanosine.

*Present address: Intercampus Program, Molecular Parasitology, University of California, Laurel Heights, Box 1204, Suite 150, San Francisco, CA 94143.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

A [isobutyric acid/concentrated NH₄OH/H₂O, 66:1:33 (vol/vol)], solvent B [*t*-butanol/concentrated HCl/H₂O, 70:15:15 (vol/vol)], and solvent C [0.1 M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol, 100:60:2 (vol/wt/vol)] (17).

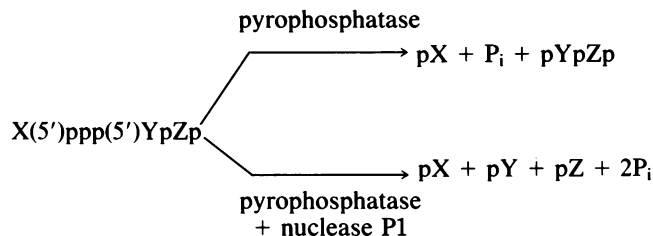
5'-End-Labeling of RNAs. SL RNA hybrid selected from RNA prepared from procyclic and bloodstream-form cells was treated with tobacco acid pyrophosphatase (50 mM sodium acetate, pH 5.2/1 mM dithiothreitol/0.1 mM EDTA for 30 min at 37°C) and bacterial alkaline phosphatase (50 mM Tris-HCl, pH 8.0, for 30 min at 45°C with 1 unit of bacterial alkaline phosphatase). End-labeling was carried out in a solution of 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 5 mM dithiothreitol, 50 pmol of [γ -³²P]ATP (3000 Ci/mmol), and 10 units of polynucleotide kinase for 30 min at 30°C. To obtain ³²P-labeled 2'-*O*-methyl-N⁶-methyladenosine 5'-monophosphate (pm⁶Am) for use as a marker, globin mRNA (Bethesda Research Laboratories) was similarly treated.

RESULTS

mRNA Cap Structure. The objective of this work was to characterize and compare the cap structures of *T. brucei* poly(A)⁺ and SL RNAs in order to lay the groundwork for a study of the function of the SL. We began our determination of the SL cap structure by working with poly(A)⁺ RNA, as it could be obtained in greater quantities than the SL RNA. Poly(A)⁺ RNAs from the procyclic form of *T. brucei* uniformly labeled *in vivo* with ³²P were digested to completion with RNase T2, producing 3'-mononucleotides and T2-resistant species. These digestion products were separated on the basis of charge by PEI cellulose TLC. Fig. 1, lane 1, shows that *T. brucei* poly(A)⁺ RNAs that have been twice purified by oligo(dT)-cellulose chromatography contain a

slowly migrating, T2-resistant structure(s). This was shown to consist of a single, predominant species following its elution from the thin layer plate and subsequent analysis by two-dimensional chromatography (Fig. 2A). Because RNase T2 cannot hydrolyze pyrophosphate bonds or 5' bonds adjacent to a 2'-*O* modification, this T2-resistant structure is presumably an oligonucleotide with internal ribose modifications and/or a cap.

The T2-resistant species was shown to be a capped oligonucleotide by subsequent digestion using the following scheme:



where X, Y, and Z are bases.

The T2-resistant species was shown to contain a pyrophosphate linkage by digestion with nucleotide pyrophosphatase followed by analysis of the products by two-dimensional cellulose TLC (Fig. 2B). The products of the reaction are pX, inorganic phosphate, and an oligonucleotide that migrates very slowly in the second dimension. A minor species, which migrates near the GMP standard, is not part of the cap structure as it is also seen in the minus-enzyme control (Fig. 2A). The cap nucleotide pX comigrates with an unlabeled pm⁷G standard in the solvent system shown and also when solvent A is used in the first dimension and solvent B in the second dimension (data not shown). *T. brucei* is, therefore, similar to all other eukaryotes studied in that its mRNA capping nucleotide is pm⁷G. Complete digestion of the T2-resistant species with nuclease P1 and nucleotide pyrophosphatase revealed that the capped fragment contains an unprecedented number of modified nucleotides. As shown in Fig. 2C, six nucleotides, including pm⁷G, are released upon digestion with these enzymes. One of the modified nucleotides migrates with unlabeled 2'-*O*-methylcytidine 5'-monophosphate (pCm), whereas another migrates with 2'-*O*-methyladenosine 5'-monophosphate (pAm). A third nucleotide comigrates with unmodified AMP, implying that the first unmodified nucleotide at the 5' end of the SL (the 3' end of the T2 fragment) is an adenosine residue. While the identities of the other two modified nucleotides have not yet been determined, some clues can be gleaned from an examination of the DNA sequence at the 5' end of the SL RNA gene. As we will discuss below, one of these species is a modified AMP; the other is a modified UMP. The order of the nucleotides within the fragment is also determined by the DNA sequence and will be described elsewhere.

Similar analyses to those shown in Figs. 1 and 2 were carried out with *in vivo*-labeled poly(A)⁺ RNA from *L. collosoma*. This RNA contains a single RNase T2-resistant species that migrates on PEI cellulose with a mobility similar to that of the *T. brucei* capped oligonucleotide (Fig. 1, lane 3). The analyses of this species indicate that the *L. collosoma* poly(A)⁺, capped oligonucleotide has the identical modified-base composition as does its counterpart in *T. brucei* (Fig. 2E).

SL RNA Cap Structures. It has been suggested that one potential role of the SL RNA in trypanosomes is to serve as a donor of the mRNA cap structure (7, 10). Substantiation of this hypothesis requires the demonstration that the base modifications found on poly(A)⁺ RNAs (or a subset thereof) are also present on the putative SL donor. To determine the

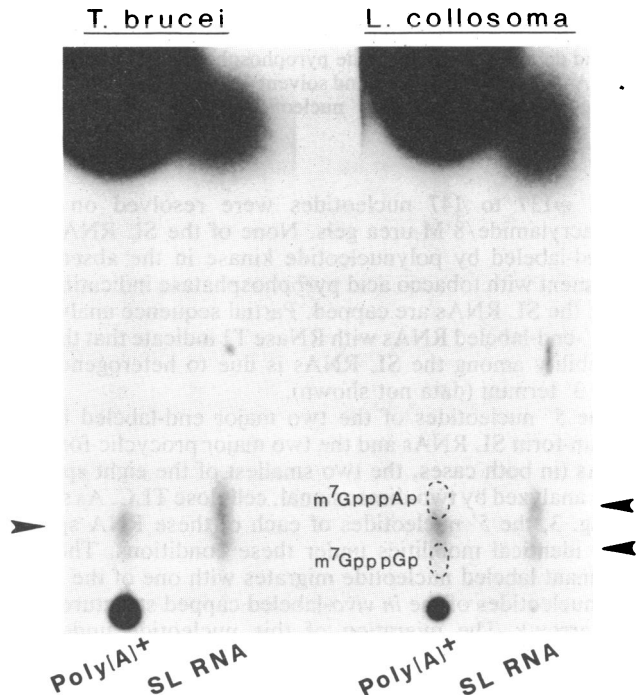


FIG. 1. Detection of T2-resistant structures. Poly(A)⁺ RNAs and SL RNAs labeled uniformly *in vivo* with ³²P were digested with RNase T2. The products were chromatographed on PEI cellulose thin layer plates using the solvent system of Silberklang *et al.* (17). The large spots near the top of the autoradiograph represent mononucleotides. Arrows mark the position of 5'-end structures. For comparison, positions of the T2-resistant cap structures of *Saccharomyces cerevisiae* poly(A)⁺ RNA are indicated with broken lines.

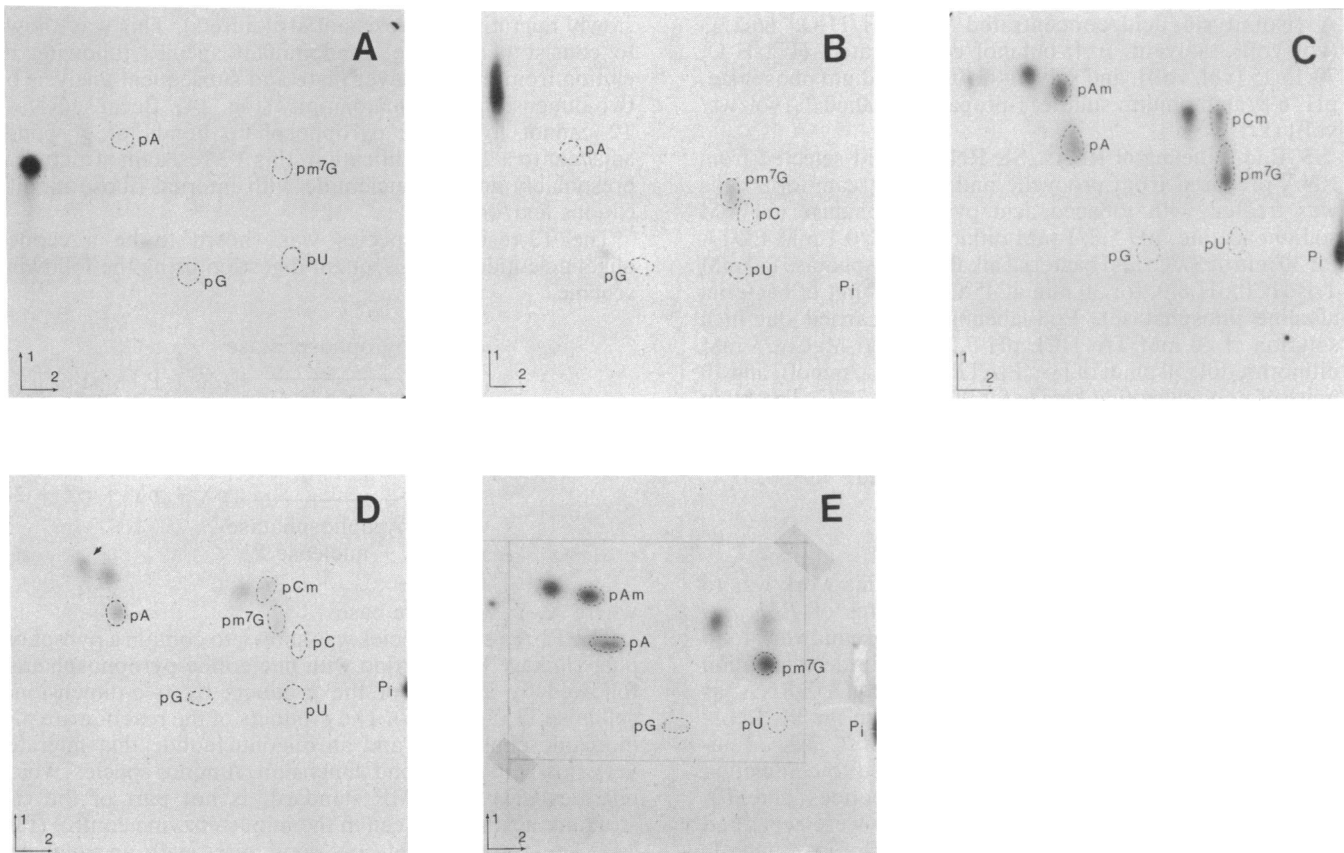


FIG. 2. Analysis of cap structures of *T. brucei* and *L. collosoma* poly(A)⁺ RNA and SL RNA. The T2-resistant cap structures from *T. brucei* poly(A)⁺ RNA labeled uniformly *in vivo* were eluted from PEI cellulose and not digested further (A), digested with nucleotide pyrophosphatase (B), or digested with nucleotide pyrophosphatase and nuclease P1 (C). (D) The T2-resistant cap structures from *T. brucei* SL RNA labeled uniformly *in vivo* were eluted from PEI cellulose and digested with nucleotide pyrophosphatase and nuclease P1. (E) The T2-resistant cap structures from *L. collosoma* poly(A)⁺ RNA were eluted from PEI cellulose and digested with nucleotide pyrophosphatase and nuclease P1. The products were chromatographed on cellulose thin layer plates using solvent A in the first dimension and solvent C in the second dimension. The positions of nonradioactive markers (visualized by UV) are indicated. The arrow in D indicates the 5' nucleotide of the SL RNA transcript as identified in Fig. 3.

SL RNA cap structure(s), *T. brucei* and *L. collosoma* SL RNAs labeled uniformly *in vivo* were isolated by hybridization selection and gel purification. Upon digestion with RNase T2, each SL RNA gave rise to two resistant structures that are barely resolved by PEI cellulose chromatography (Fig. 1). The mobilities of these T2-resistant structures are similar to that of the poly(A)⁺, capped oligonucleotide. When the faster migrating species from the *T. brucei* SL RNA was eluted from the thin layer plate and digested to completion with pyrophosphatase and nuclease P1, its component nucleotides were observed to be identical to those found in the poly(A)⁺, capped oligonucleotide (Fig. 2D). This observation supports the hypothesis that the SL RNA is the mRNA cap donor. Unfortunately, we have been unable to obtain enough of the more slowly migrating species to analyze its nucleotide composition. The ratio of the two T2-resistant species to each other and our ability to resolve them varied from experiment to experiment. This is in contrast to the poly(A)⁺, capped oligonucleotide, which always migrates as a single, sharp spot. An explanation for two (or more) T2-resistant species in the purified SL RNA preparation is that some of the members of the population may not be fully modified at the 5'-end. To test this hypothesis, SL RNAs purified from procyclic and bloodstream-form cells were subjected to RNA sequence analysis. The SL RNAs were 5'-end-labeled with polynucleotide kinase and [³²P]ATP following treatment with tobacco acid pyrophosphatase and bacterial alkaline phosphatase. At least eight end-labeled SL RNAs ranging in size

from ≈137 to 147 nucleotides were resolved on 6.0% polyacrylamide/8 M urea gels. None of the SL RNAs was 5'-end-labeled by polynucleotide kinase in the absence of treatment with tobacco acid pyrophosphatase indicating that all of the SL RNAs are capped. Partial sequence analysis of the 5'-end-labeled RNAs with RNase T1 indicate that the size variability among the SL RNAs is due to heterogeneity at their 3' termini (data not shown).

The 5' nucleotides of the two major end-labeled bloodstream-form SL RNAs and the two major procyclic-form SL RNAs (in both cases, the two smallest of the eight species) were analyzed by two-dimensional, cellulose TLC. As shown in Fig. 3, the 5' nucleotides of each of these RNA species have identical mobilities under these conditions. The predominant labeled nucleotide migrates with one of the modified nucleotides of the *in vivo*-labeled capped structure (Fig. 2D, arrow). The migration of this nucleotide under the solvent conditions used suggests that it is a modified AMP. We also detect small amounts of unmodified AMP on these chromatographs. These results are compatible with the interpretation that the SL RNAs of *T. brucei* have identical 5' sequences but that some of the RNAs are incompletely modified. The minor T2-resistant species that we observe in the *in vivo*-labeled SL RNA preparations are, therefore, most likely capped oligonucleotides that are 3' truncated because of the partial modification.

5'-Sequence Analysis of the SL RNAs. Our characterizations of the *T. brucei* and *L. collosoma* capped structures indicate

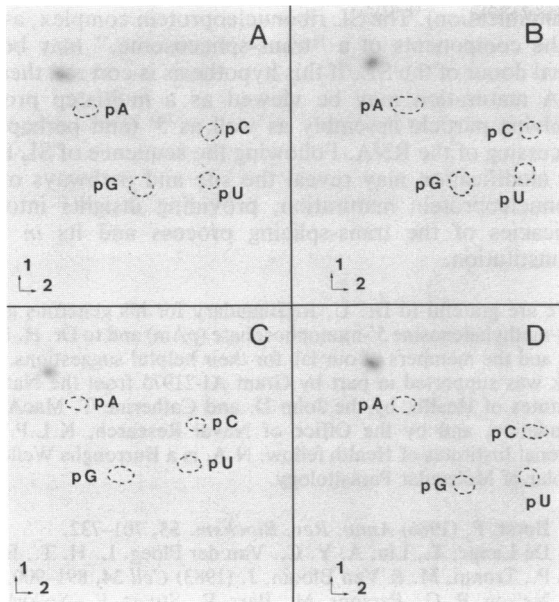


FIG. 3. Analysis of 5' nucleotides of SL RNAs end-labeled *in vitro*. Decapped, 5'-end-labeled SL RNAs were digested with phosphodiesterase I, and the products were separated by two-dimensional cellulose TLC. The radiolabeled nucleotides were visualized by autoradiography; nonradioactive markers were visualized by UV. A, Bloodstream-form SL RNA 2; B, bloodstream-form SL RNA 1; C, procyclic-form SL RNA 2; D, procyclic-form SL RNA 1.

that the composition of the first five transcribed nucleotides of their SLs are identical. This result contradicts determinations (6) of the *T. brucei* and *L. collosoma* SL RNA sequences (see Fig. 4B) and suggests that the methods used

to determine these sequences did not detect the 5'-modified nucleotides. This hypothesis has been verified by our analysis of the 5' sequence of the major bloodstream-form SL RNA. As shown in Fig. 4, the SL RNA extends four nucleotides beyond the reported 5' terminus. There is only limited alkaline hydrolysis of the SL RNA at these positions, consistent with our observation that the majority of SL RNAs contain 2'-O modifications of these nucleotides. Based on the intensity of the ladder, the first unmodified residue in this sequence appears to be the AMP at position +5, which agrees with our analysis of the 5'-capped, RNase T2-resistant oligonucleotide (Fig. 2C). These conclusions are also supported by primer-extension analyses of the SL RNA in which the extension products terminate with a strong stop opposite the unmodified A residue and with four weaker stops immediately beyond (opposite the four modified nucleotides) (ref. 7, K.P.W., unpublished results). Since pausing or termination of reverse transcriptase at, or immediately before, modified bases has been well documented (18-20), it is perhaps not surprising that the 5' end of the SL RNA was originally misassigned.

The 5' end of the *T. brucei* SL RNA transcript as determined above is indicated in Fig. 4B. The DNA sequence reveals that the 5'-transcribed nucleotide is a modified AMP. Because many of the mRNAs of higher eukaryotes and their viruses contain 2'-O-methyl-N⁶-methyladenosine 5'-monophosphate (pm⁶Am) at this position (11), we examined whether the 5'-transcribed nucleotide of the trypanosome SL might be similarly modified. ³²P-labeled pm⁶Am was obtained for use as a standard by 5'-end-labeling decapped rabbit globin mRNA and by digesting the RNA to completion with phosphodiesterase I. Our experiments indicate that the pm⁶Am does not comigrate with the SL 5'-transcribed nucleotide, suggesting that this nucleotide may be modified in

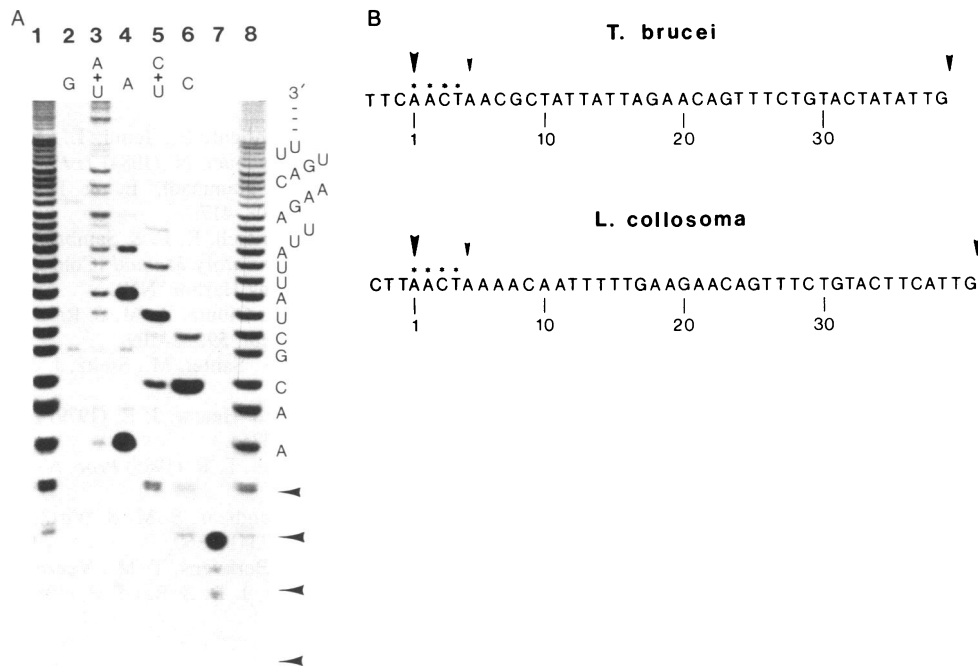


FIG. 4. RNA sequencing of the major, 5'-labeled bloodstream-form SL RNA. (A) Bloodstream-form SL RNA 1 was sequenced by partial hydrolysis with alkali (lanes 1 and 8), ribonucleases (lanes 2-6), and phosphodiesterase I (lane 7). Ribonuclease T1 is specific for guanosine residues, ribonuclease U₂ is specific for adenosine residues, ribonuclease Phy M is specific for adenosine and uridine residues, and *Bacillus cereus* ribonuclease is specific for cytidine and uridine residues. RNA sequencing reaction mixtures were analyzed on a 23% polyacrylamide/8 M urea gel in 90 mM Tris borate/2.5 mM EDTA. The arrows indicate the positions in the alkaline ladder of the first four transcribed nucleotides. The bands that represent the first two transcribed nucleotides are visualized only upon a longer exposure. (B) DNA sequences of the 5' regions of the *T. brucei* and *L. collosoma* SL RNA genes are shown. The large arrows indicate the 5' end of the *T. brucei* SL RNA transcript and the analogous position in the *L. collosoma* sequence. The arrows at positions +4 indicate the 5' ends that had been suggested (1). Arrows at the right indicate the splice junctions.

a manner that has not yet been seen among eukaryotic mRNA cap structures.

DISCUSSION

We have characterized the 5'-cap structures of both the poly(A)⁺ RNA and the SL RNA of *T. brucei*. If the SL RNA is indeed the donor of the SL sequence, one would predict that the SL RNA and the mature mRNAs would have similarly capped 5' ends. Our data indicate that these RNAs have identical 5' termini beginning with a m⁷G cap and followed by modifications on each of the first four transcribed nucleotides. The sequence of these nucleotides, as deduced from the DNA sequence, is m⁷G(5')ppp(5')A*mpAmpCmp-U*mp, where the asterisks represent additional unidentified modifications and m represents a 2'-O-methyl group. These results provide evidence that all *T. brucei* mRNAs share a common 5' sequence and clearly support the hypothesis that the donor of the leader sequence is the SL RNA.

The extent of modification of the *T. brucei* SL is extremely unusual; however, the chemical nature of the majority of the modifications is similar to that of mRNA modifications in other eukaryotes. The presence of a m⁷G cap is a nearly universal feature of eukaryotic mRNAs and is required for several aspects of their metabolism. Caps enhance mRNA translation and stability in higher eukaryotes (11) and are likely to fulfill similar functions in *T. brucei*. In addition, it has been shown (12, 13) that a m⁷G cap is required for the efficient splicing of pre-mRNAs of higher eukaryotes *in vitro*. As trans-splicing in *T. brucei* is, in some respects, mechanistically related to cis-splicing, the m⁷G cap of the SL RNA may be similarly required for its recognition as a splicing precursor.

Evidence that suggests that the modifications of the 5'-transcribed nucleotides also play an important role in the biology of the trypanosomes is provided by our finding that identical modifications are present on the SL of the insect trypanosomatid, *L. collosoma*. This result is particularly striking because *L. collosoma* and *T. brucei* are only distantly related. Although similar analyses have not yet been made with the SL RNAs of other species, DNA sequence information indicates that the four nucleotides that are modified in *T. brucei* and *L. collosoma* are highly conserved (6, 21, 22). These observations suggest that the modifications that we described above may have been retained throughout the evolution of the Trypanosomatidae and may play a significant role in SL function.

Our observation that all of the 5' modifications present on *T. brucei* poly(A)⁺ RNA are also present on the SL RNA argues against the hypothesis that these modifications are uniquely required for poly(A)⁺ RNA expression. Rather, the data suggest that the 5' modifications may be required for the activity of the SL RNA as a substrate in the trans-splicing reaction. This hypothesis is intriguing in light of other insights into the trans-splicing process and its comparison to cis-splicing. Of particular interest is the evidence that the SL RNA is part of a small, ribonucleoprotein complex that, in some respects, resembles the small nuclear ribonucleoproteins of higher eukaryotes (S. Michaeli and K.P.W., personal

communication). The SL ribonucleoprotein complex, as one of the components of a "trans-spliceosome," may be the actual donor of the SL. If this hypothesis is correct, then SL RNA maturation may be viewed as a multistep process involving particle assembly as well as 5' (and perhaps 3') processing of the RNA. Following the sequence of SL RNA cap modification may reveal the site and pathways of SL ribonucleoprotein maturation, providing insights into the intricacies of the trans-splicing process and its *in vitro* reconstitution.

We are grateful to Dr. U. RajBhandary for his generous gift of 2'-O-methyladenosine 5'-monophosphate (pAm) and to Dr. H. Kamen and the members of our lab for their helpful suggestions. This work was supported in part by Grant AI-21975 from the National Institutes of Health, by the John D. and Catherine T. MacArthur Foundation, and by the Office of Naval Research. K.L.P. is a National Institutes of Health fellow. N.A. is a Burroughs Wellcome Scholar of Molecular Parasitology.

1. Borst, P. (1986) *Annu. Rev. Biochem.* **55**, 701-732.
2. De Lange, T., Liu, A. Y. C., Van der Ploeg, L. H. T., Borst, P., Tromp, M. & Van Bloom, J. (1983) *Cell* **34**, 891-900.
3. Nelson, R. G., Parsons, M., Barr, P., Stuart, K., Selkirk, M. & Agabian, N. (1983) *Cell* **34**, 901-909.
4. Campbell, D. A., Thornton, D. A. & Boothroyd, J. C. (1984) *Nature (London)* **311**, 350-355.
5. Kooter, J. M., De Lange, T. & Borst, P. (1984) *EMBO J.* **3**, 2387-2392.
6. Milhausen, M., Nelson, R. G., Sather, S., Selkirk, M. & Agabian, N. (1984) *Cell* **38**, 721-729.
7. Murphy, W. J., Watkins, K. P. & Agabian, N. (1986) *Cell* **47**, 517-525.
8. Sutton, R. & Boothroyd, J. C. (1986) *Cell* **47**, 527-535.
9. Laird, P. W., Kooter, J. M., Loosbroek, N. & Borst, P. (1985) *Nucleic Acids Res.* **13**, 4253-4266.
10. Lenardo, M. J., Dorfman, D. M. & Donelson, J. E. (1985) *Mol. Cell. Biol.* **9**, 2487-2490.
11. Banerjee, A. K. (1980) *Microbiol. Rev.* **44**, 175-205.
12. Ederly, I. & Sonenberg, N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7590-7594.
13. Konarska, M. M., Padgett, R. A. & Sharp, P. A. (1984) *Cell* **38**, 731-736.
14. Stuart, K., Gobright, E., Jenni, L., Milhausen, M., Thomashow, L. & Agabian, N. (1984) *J. Parasitol.* **70**, 747-754.
15. Bienen, E. J., Hammadi, E. & Hill, G. C. (1981) *Exp. Parasitol.* **51**, 408-417.
16. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
17. Silberklang, M., Gillum, A. M. & RajBhandary, U. L. (1979) *Methods Enzymol.* **59**, 58-109.
18. Hagenbuchli, O., Santer, M., Steitz, J. & Mans, R. (1978) *Cell* **13**, 551-563.
19. Youvan, D. C. & Hearst, J. E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3751-3754.
20. Inoue, T. & Cech, T. R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 648-652.
21. Miller, S. I., Landfear, S. M. & Wirth, D. F. (1986) *Nucleic Acids Res.* **14**, 7341-7360.
22. De Lange, T., Berkvens, T. M., Veerman, H. J. G., Frasch, A. C. C., Barry, J. D. & Borst, P. (1984) *Nucleic Acids Res.* **12**, 4431-4443.