Direct analysis of the mini-exon donor RNA of *Trypanosoma brucei*: detection of a novel cap structure also present in messenger RNA

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ABSTRACT

The mini-exon, a short segment found at the 5' end of trypanosome mRNAs, is contributed by a small RNA, the mini-exon donor (medRNA). *In vivo* ³²P-labeled medRNA, a set of smaller RNAs related to it, and mRNA, were purified from *Trypanosoma brucei* by hybrid selection and gel electrophoresis. Using RNA fingerprinting and sequencing techniques, mini-exon oligonucleotides were identified and characterized. We detected a novel 5' terminal capped oligonucleotide present in both medRNA and mRNA. This structure contained m⁷G and at least four modified nucleotides, not identified previously. If the *T. brucei* mini-exon has exactly four transcribed nucleotides upstream from its originally designated 5' end, it would begin with the sequence: m⁷GpppA*A*C*U*AA*CG (asterisks denote modification) and medRNA would be 140 nucleotides long, excluding the m⁷G residue. The mini-exon contains, and retains during its transfer to mRNA, a novel 5' terminal structure whose presence could confer unique functional attributes.

INTRODUCTION

Messenger RNA synthesis in trypanosomes and other parasitic kinetoplastids involves novel processes. Some form of discontinuous synthesis (reviewed in ref. 1) is needed for 5' end formation. Other cases of discontinuous mRNA synthesis, either by priming or trans-splicing, have been described in viral, organellar and cellular systems (2-5). All mRNAs in *Trypanosoma brucei* examined thus far begin with an identical leader sequence called the mini-exon (6) or spliced leader (7). An oligonucleotide complementary to the mini-exon causes hybrid arrest of most trypanosome mRNA translation *in vitro* (8,9). Indirect analyses of trypanosome RNA populations using Northern blotting and primer extension techniques suggested the existence of an abundant RNA, about 136 nucleotides long, having the mini-exon at its 5' end (10-13). This RNA is likely to be the primary source of the mini-exon found on mRNA molecules. In this study, it is termed the mini-exon donor RNA (medRNA). In *T. brucei*, medRNA is encoded primarily in 1.35 kbp tandem repeats (14,15), which are unlinked to any known structural genes. In several cases, DNA encoding a particular mRNA has been mapped to a chromosome with no medRNA coding sequences, suggesting the obligatory transfer of the mini-exon from medRNA to mRNA. Recent pulse-labeling studies

using [^3H]RNA precursors have shown that medRNA may have a much shorter half-life than mRNA, in keeping with its role as an RNA donor (16,17). Despite efforts to characterize it, the mechanism by which the mini-exon becomes part of mRNA is not understood. The mini-exon may prime transcription of structural genes, or it may be added by trans-splicing.

A variety of studies on eukaryotic mRNA biosynthesis reveal unexpected complexities in which intermediates and modified structures arise whose characterization requires direct analysis of RNAs synthesized *in vivo*. For example, the characterization of 5' terminal cap structures (18-20) was necessary for subsequent studies on the requirements for such structures in mRNA splicing (21,22). In addition, the discovery of unusual 2' nucleotide groups in populations of mRNA precursor molecules (23) helped to reveal the lariat structures characteristic of splicing intermediates (21,24). Since such studies show that many RNA structures have no counterparts in DNA, DNA sequencing studies may provide only partial information about RNA structure.

So far, no direct studies of trypanosome mRNA structure have been reported. medRNA was initially identified by indirect methods (10-13). Studies using chemical decapping of unlabeled RNA populations containing medRNA and mRNA have suggested that both may contain a cap structure (25,26). Furthermore, recent work using Northern blots and S1 analyses has suggested that a segment of medRNA may be joined by a 2'-5' linkage to high molecular weight RNA molecules (17,27,28). In this communication, RNA fingerprinting and sequencing studies of [^{32}P]RNA populations isolated from the procyclic form of *T. brucei* will be presented, the sequence context of the mini-exon portion of medRNA and related molecules (including mRNA) established, and properties of a unique, highly modified 5' terminal cap structure described.

MATERIALS AND METHODS

Trypanosome labeling and RNA isolation

This study used a cloned line of *T. brucei* strain 427, variant 117, which had been transformed by G. Lamont to the insect form (procyclics) *in vitro* (29). The procyclics were maintained in a CO_2 incubator at 27° C in SDM-79 (30) containing 10% fetal calf serum, 7.5 mg/l heme, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The medium of stationary phase procyclics (at approximately 2×10^7 trypanosomes per ml) was changed to low phosphate (0.2 mM) SDM-79 made with dialyzed serum (Gibco). After 2 days, [^{32}P]orthophosphate (NEN) was added to 0.5-1 mCi/ml. Nucleic acids were isolated after 20-24 hrs of additional incubation.

Glassware for RNA purification was siliconized and baked. Plasticware was treated with diethylpyrocarbonate or autoclaved. After labeling, cells were pelleted at room temperature and then lysed in 0.8 ml (per 20 ml cell culture) of 6 M guanidinium isothiocyanate, 0.05 M Tris (pH 7.4), 0.01 M EDTA (pH 8.0), 2% SDS, 2% Triton X-

100, 2% Sarkosyl and 50 $\mu\text{g}/\text{ml}$ tRNA (Sigma). The lysate was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) containing 0.1% 8-hydroxyquinoline and re-extracted until the aqueous phase was clear. After ethanol precipitation, the nucleic acids were loaded onto a CF11 cellulose (Whatman) column (31) in 0.2 M NaCl, 0.1 M Tris (pH 7.4), 0.2 mM EDTA, and 50% ethanol, rinsed 10 times in the loading solution, rinsed with 100% ethanol, eluted in water and lyophilized. Typically, $\sim 3 \times 10^9$ dpm of nucleic acids, at $\sim 10^7$ dpm per μg , were obtained from $\sim 5 \times 10^8$ trypanosomes. Estimates of specific activity were based on the assumption that recovery of RNA from labeled and unlabeled trypanosomes was similar.

Hybrid selection

The plasmid pMX117.13 (constructed by P. Hevezi), a genomic clone of the *T. brucei* mini-exon repeat unit, was used for hybrid selection. It contains two tandem copies of the unit length (1.35 kbp) (14,15) Sau3AI fragment inserted into the BamHI site of pAT153, a derivative of pBR322. Linearized plasmid DNA was boiled in 0.1 N NaOH for 15 min, neutralized, and diluted into cold 2 M NaCl. The DNA was bound to nitrocellulose at not greater than 50 $\mu\text{g}/\text{cm}^2$, baked for 2 hrs, and pre-hybridized in the hybridization solution, 50% formamide, 0.6 M NaCl, 0.01 M EDTA (pH 8.0), 0.02 M TES (pH 7.4, Sigma), 0.2% SDS, 1 mg/ml poly rA (Sigma), and 0.2 mg/ml tRNA for 2-16 hrs at 15° C. A 5-10 fold molar excess of DNA to expected RNA was used. The RNA was selected for two days at 15° C in a minimal volume (0.125 ml per cm^2 of nitrocellulose) of fresh hybridization solution. The filters were rinsed in 50% formamide, 0.6 M NaCl, 0.01 M EDTA (pH 8.0), 0.02 M TES, (pH 7.4), 0.2% SDS, 0.1 mg/ml poly rA and 10 $\mu\text{g}/\text{ml}$ tRNA at 15° C. RNA was eluted 3 times for 10 min each at 90° C, in a minimal volume of 99% formamide, 1% SDS, and 50 $\mu\text{g}/\text{ml}$ tRNA. The pooled eluates were ethanol precipitated and chromatographed on CF11 cellulose.

Oligo(dT) selection

Oligo(dT) cellulose (Collaborative Research) was washed with 10 column volumes of 0.01 M Tris (pH 7.4), 0.001 M EDTA and 0.1% SDS. It was then equilibrated with 10 column volumes of 0.4 M NaCl, 0.01 M Tris (pH 7.4), 0.001 M EDTA and 0.1% SDS containing 500 μg of yeast tRNA (32). The RNA was heated to 65° C for 5 min, brought to 0.4 M NaCl and loaded onto the column. The flow-through fraction was reappplied. After 10 rinses with the binding buffer, the poly(A)⁺ RNA was eluted with the wash buffer at 37° C. The RNA was then chromatographed on CF11 cellulose and lyophilized.

Sucrose gradients

In vivo labeled [³²P]RNA in 0.01 M Tris (pH 7.5), 0.01 M EDTA and 0.2% SDS was layered onto 5 ml 15% to 30% sucrose gradients in the same buffer containing 0.1 M NaCl (33). Gradients were centrifuged for 5 hrs at 45,000 rpm in an SW50.1 rotor in a Beckman L2-65B centrifuge. Three-drop fractions were collected and counted. High molecular weight fractions (greater than 700 nucleotides) were pooled, precipitated two times and fingerprinted.

RNA elution from gels

RNA was extracted by soaking excised gel bands in a minimal volume (usually 0.1 ml) of 0.2 M NaCl, 0.1 M Tris (pH 7.4), 0.002 M EDTA (pH 8.0), 0.2% SDS and 50 $\mu\text{g/ml}$ tRNA at 65° C four times for 30 min each. The pooled RNA was then chromatographed on CF11 cellulose. Typically, $\sim 6 \times 10^4$ dpm of labeled medRNA were obtained.

RNA fingerprinting and secondary analysis

RNA samples, with 10 μg of *E. coli* tRNA, were incubated at 37° C in 2 μl of 0.01 M Tris (pH 7.6), 0.001 M EDTA, containing 1 mg/ml RNase T1 (Sankyo, Calbiochem), for 45 min, or RNase A (Worthington) (0.25-1.0 mg/ml) for 15-45 min. Oligonucleotides were fractionated in the first dimension in 10% polyacrylamide gels containing 6 M urea and 0.025 M citric acid (pH 3.5) (34) or on cellulose acetate strips and then were blotted onto DEAE cellulose plates (Brinkmann) and fractionated in the second dimension by ascending homochromatography (35).

Oligonucleotides were eluted from fingerprints and analyzed further as described previously (2). Nuclease P1 (Calbiochem) standard conditions were 1 mg/ml in 0.01 M sodium acetate (pH 6.0) with 10 μg tRNA. For complete digestion of the cap structure, nuclease P1 conditions were 50 μg of nuclease per reaction in 0.005 M sodium acetate (pH 5.2) for 2 hrs with no tRNA present. Other enzymatic conditions were: calf alkaline phosphatase (Boehringer Mannheim) in 0-17% glycerol, 0.05 M Tris (pH 8.0) and 0.25 M NaCl; and nucleotide pyrophosphatase (Sigma), 0.002 M ATP, 0.02 M Tris (pH 7.5) and 0.001 M MgCl_2 for 2 hrs at 37° C.

Digestion products were analyzed by high voltage electrophoresis on 3MM or 540 paper (Whatman), or DE 81 DEAE paper (Whatman), in pyridine acetate buffer (pH 3.5); or on DEAE paper in 2.5% formic acid, 8.7% acetic acid, (pH 1.9); or by thin-layer chromatography on 20 x 20 cm^2 cellulose sheets (Kodak) developed in isobutyric acid (Sigma) and 0.5 M NH_4OH , at a ratio of 10:6. Cap markers ($\text{m}^7\text{GpppA}_{\text{OH}}$, m^7GpppAm , and $\text{m}^7\text{GpppG}_{\text{OH}}$) were purchased from Pharmacia. RNA products in this text have 5' hydroxyl and 3' phosphate termini unless otherwise noted, except, for simplicity, the 5' nucleoside monophosphates are designated pN (rather than pN_{OH}).

RESULTS

In vivo labeling and gel electrophoresis of hybrid-selected RNAs

Procyclic culture forms yielded up to 100-fold more acid-precipitable radioactivity per trypanosome than bloodstream forms during initial experiments. For this reason, RNA from procyclic forms was used in subsequent studies. Hybrid selection followed by preparative gel electrophoresis was used to purify specific RNAs. The DNA used for hybrid selection was a double-stranded plasmid containing the entire genomic repeat unit encoding medRNA. It typically retained about 1% of the *in vivo* labeled nucleic acid.

Figure 1. Gel electrophoresis of [^{32}P]RNA from procyclic forms. *In vivo* labeled nucleic acids were fractionated in a 10% polyacrylamide gel containing 7 M urea before (lane a) and after (lane b) hybrid selection by DNA of a genomic clone encoding medRNA. RNAs were eluted from the indicated positions in the gel: area 1 (mRNA), band 2 (medRNA), band 3 (medRNA'), band 4 (RNA about 100 nucleotides long), band 5 (RNA about 70 nucleotides), and band 6 (RNA about 35 nucleotides). Lane (c) is a darker exposure of lane (b). Five small ribosomal RNAs in lane (a) are indicated by their size in nucleotides (36-39).

The major component (Figure 1, lane b, band 2) of the selected RNA, a species of about 140 nucleotides, was tentatively identified as medRNA. It co-migrated with the expected medRNA band on Northern blots (not shown). Higher molecular weight

Figure 2. Fingerprint analysis of medRNA and other *in vivo* labeled RNAs. The RNase T1 fingerprint of medRNA (panel B) was compared to those of several RNAs eluted from the gel shown in Figure 1: medRNA' (panel C); mRNA, area 1, (panel D); ~35-nucleotide RNA, band 6, (panel F); ~100-nucleotide RNA, band 4, (panel G); and to the RNase T1 fingerprint of poly(A)⁺ RNA (panel H). The RNase A fingerprint of medRNA appears in panel (A) and that of the ~35-nucleotide RNA, band 6, is presented in panel (E). Spots containing oligonucleotides from the mini-exon are indicated by numbers and letters. The 3' terminal nucleotide of medRNA (spot 9) is also marked in the RNase T1 fingerprint (panel B). The arrowhead in panel (G) identifies the expected location of the oligonucleotide AAUCUG, which begins at about position 110 in medRNA (ref. 10) and is missing from the fingerprint of the ~100 nucleotide long RNA. The origin of the fingerprints is indicated by the arrows in panel (A).

RNAs (Figure 1, lane b, area 1), presumably mini-exon-containing mRNAs, were also present. Two species which co-migrated with two of the small rRNAs [170 and 215 nucleotides (36-38)] were also selected at low efficiency, but were not analyzed further. Computer analyses revealed that the sequences of the small rRNAs do not show extensive regions of homology to medRNA or its complement. The selected RNA was also enriched for an RNA slightly smaller than medRNA, designated medRNA' (Figure 1, lane b, band 3), and RNAs of about 100, 70, and 35 nucleotides (Figure 1, lane c, bands 4-6). RNAs were eluted from excised gel bands and analyzed further.

Identification of medRNA

RNA fingerprinting and secondary analysis were used to compare the oligonucleotides comprising medRNA to those predicted by sequence analysis of medRNA genes. Complete RNase A (Figure 2A) and RNase T1 (Figure 2B) digests of this RNA were fingerprinted. For secondary analysis, oligonucleotides were eluted from fingerprints, treated with RNases of known specificity and the products identified by high voltage electrophoresis. The fingerprint of medRNA' was nearly identical to that of medRNA (compare Figures 2B and 2C). Data from oligonucleotides common to both RNAs were pooled. Since these two RNAs differed only at their 3' ends (see below), pooling provided sufficient material for detailed characterization of the remainder of the molecule and, in particular, for analysis of oligonucleotides from the mini-exon portion.

RNase T1 fingerprint spots 2, 3, 7, and 10 and RNase A fingerprint spots D, E, I, K, L and M from medRNA (Figure 2, panels A and B) and medRNA' (Figure 2C) contained oligonucleotides from the mini-exon portion of these RNAs (Table 1). The two prominent RNase T1-resistant spots, 2 and 3, provided a marker for the mini-exon. Spot 2 consisted of two co-migrating decanucleotides, 2a and 2b (both from the mini-exon), of identical nucleotide composition but different sequence. The 5' terminal oligonucleotide AACG, predicted by several indirect studies of medRNA (10-13), was not found. Secondary analysis suggested that oligonucleotides 3 and D uniquely contained the capped 5' terminus.

Supporting the interpretation that spot 3 is a capped oligonucleotide with the sequence AACG at its 3' end, RNase A digestion of spot 3 released G and a binary spot with components too slow in electrophoretic mobility to be any conventional products (of combined RNase T1 and RNase A digestion) from anywhere in the sequence predicted for medRNA (Figure 3, lane c; Table 1). The novel products co-migrated with those of spot D, which resolved into two components during electrophoresis on DEAE paper at pH 1.9 (Figure 3, lane d). These results, and additional data presented in Table 1, demonstrated that the only difference between spot 3 (from the RNase T1 fingerprint) and spot D (from the RNase A fingerprint) is the presence of a 3' terminal G residue on spot 3.

Throughout this study, cap-containing oligonucleotides eluted from fingerprints separated into two components during DEAE paper high voltage electrophoresis. Only one form of the cap was detected when the fingerprinting step was omitted. Further analysis indicated that the faster member of the binary spot is the authentic form of the capped oligonucleotide (because it contains m⁷G; see below) while the slower member contains an artifactual derivative. At neutral or alkaline pH, m⁷G is susceptible to ring-opening which results in a loss of positive charge (13). This reaction could explain why a single homochromatographic spot was electrophoretically heterogeneous.

Digestion of spot 3 with RNase U2 released several species: a slow moving binary spot plus ACG and CG (Table 1). Since RNase U2 is specific for purines (and therefore cleaves after A residues in oligonucleotides from RNase T1 fingerprints), the com-

Table 1. Secondary Analysis of Selected Oligonucleotides

Fingerprint Spot	RNase T2 Products	Pancreatic RNase A Products	RNase T1 Products	RNase U2 Products	Proposed Sequence
<u>RNase T1 Products of medRNA and medRNA'</u>					
2	C,A,G, <u>U</u>	AG,AU,AC,G,C,U	---	UUG,UUA,CUA,UA,G	(2a) CUAUUUUUAG (2b) UACUAUUUUG
3	C,A,X ^a /G	X ^a ,G	---	X ^a ,ACG ^b ,CG	CapAACG
7	C, <u>A</u> ,G	AAC,AG	---	CA,A,G	AACAG
9 ^d	<u>C</u> ,A,U	AC,C,U	---	[C ₄ ,U],A	ACCUCC _{OH} ^d
10	C,G, <u>U</u>	G,C, <u>U</u>	---	unchanged	UUUCUG
<u>Pancreatic RNase A Products of medRNA and medRNA'</u>					
D	C,A,X ^a	---	unchanged	X ^a ,AC ^b , (A/C)	CapAAC
E	C, <u>A</u> ,G	---	AAC,AG,AA ^c ,C	G,A/C	AGAAC
I	A,G,U	---	AG, (AU), (G),U	A,G,U	AGU, (GAU) ^e
K	G,U	---	G,U	G,U	GU
L	A,U	---	unchanged	A,U	AU
M	C,G	---	G,C	G,A/C	GC
<u>RNase T1 Products of Band 6 RNA</u>					
2	C,A,G, <u>U</u>	AG,AU	---	UUA,CUA,G	CUAUUUUUAG
3	C,A,X ^a /G	X ^a	---	X ^a	CapAACG
7	C,A,G	(AAC),AG	---	CA	AACAG
10	C,U	U	---	unchanged	UUUCUG
26	C,A,U	AU,AC,U	---	CUA,UA	UACUAUAU _{OH} ^f

Table 1. Secondary Analysis of Selected Oligonucleotides. Oligonucleotide designations refer to the fingerprint spots marked in Figure 2. Conditions for secondary enzymatic digestions are given in Materials and Methods. Products were fractionated by high voltage electrophoresis on either Whatman 3MM or 540 paper, pH 3.5 (RNase T2 digests); DEAE paper, pH 3.5 (RNase A and RNase T1 digests); or DEAE paper, pH 1.9 (RNase U2 digests). The specificities of RNases which cleave via 2', 3' cyclic intermediates to yield 3' phosphorylated termini are as follows: RNase T1 cleaves after G residues, RNase A cleaves after C and U residues, RNase U2 cleaves after A and G residues, RNase T2 cleaves after all conventional nucleotides. Species indicated by underlining were obtained in relatively high yield; parentheses, a very low yield. Species separated by a slash co-migrate on the analytical system used and thus cannot be distinguished. Brackets indicate the likely base composition, but not sequence, of a digestion product. The "proposed sequence" for each oligonucleotide is based on: (i) its position in the fingerprint and the identity of its secondary products, (ii) a homol-

ogy with the DNA genomic sequence of ref. 10 and (iii) previous studies of medRNA indicating its approximate length (10-13) and the likelihood of a 5' terminal cap (25,26).

^a "X" represents a variety of capped secondary products, whose common structural elements are described in the text.

^b medRNA and medRNA' spots 3 and D contain a bond which is partially resistant to cleavage by RNase U2, leading to the incomplete digestion products ACG and AC, respectively.

^c Under the conditions used here, RNase T1 cleaves after certain A residues (unpublished observations).

^d Spot 9, the 3' terminal oligonucleotide of medRNA, is predicted by the DNA sequencing of ref. 10 to contain RNA homologous to the DNA sequence ACCTCCACTC... The assigned sequence was chosen because the data show that (i) the intensity of AC in the RNase A digest of spot 9 relative to C and U suggests only one copy of AC, thus limiting the sequence to ACCUCCA or something shorter; (ii) the lack of A or G released by RNase A limits the sequence to ACCUCCA_{OH} or shorter; and (iii) the relative intensities of C and U following RNase T2 treatment and the mobilities of the RNase U2 products together suggest the sequence ACCUCC_{OH}, although ACCUCC is not ruled out.

^e The minor amount of GAU present in spot I is from an unknown source, possibly a variant form of medRNA.

^f Spot 26 is related to spot 2b, which has the sequence UACUAUAUUG. Its release of the RNase U2 products CUA and UA, along with the RNase A products AC and AU, show that its sequence must comprise at least UACUAU. The lack of G in the RNase A products and of UUG in the RNase U2 products suggest that its longest extent could only comprise UACUAUAUU. The proposed sequence was chosen because (i) the relative intensity of UA in the RNase U2 products suggests that two moles are present, relative to CUA, thus extending the sequence at least to UACUAUA; (ii) the relative intensity of AU in the RNase A products suggests that two copies are not present, limiting the sequence to UACUAUAU_{OH} or something shorter; and (iii) the mobility of spot 26 on the RNase T1 fingerprint suggests that it has the sequence UACUAUAU_{OH}, although UACUAUA (and UACUAUAUU) are not ruled out.

bined data indicated that spot 3 has the sequence AACG at its 3' end. The release of A*CG (asterisk denotes modification) and CG by RNase U2 indicated that the 3' portion of spot 3 may contain a modified A residue. Since the RNase U2-resistant bond was susceptible to cleavage by RNase T2 (Table 1, note the A released by RNase T2), it is not blocked by 2'-O-methylation, a modification which confers resistance to all RNases requiring a free 2' hydroxyl group for cleavage. Since this bond is contained in a consensus sequence, A(m⁶)AC, for N⁶-methylation (40), the partial resistance may be caused by this modification. Although the extent of resistance was consistent from batch to batch, these experiments cannot determine whether medRNA is partially modified in this position or whether it is fully modified, but partially resistant to RNase U2. Analysis of the 5' cap-containing portion of spots 3 and D appears below.

In order to establish the overall length of medRNA, its 3' terminal oligonucleotide, spot 9, was examined (Figure 2B). Oligonucleotide 9 is likely to have the

Figure 3. Nuclease-resistant products of medRNA and mRNA capped oligonucleotides. The capped oligonucleotide, spot 3, was separately eluted from RNase T1 fingerprints of medRNA and mRNA and then either analyzed directly (lanes b and e, respectively) or treated with nucleases and fractionated by high voltage electrophoresis on DEAE paper, pH 1.9. The RNase A-resistant products are shown in lane (c) (medRNA) and lane (f) (mRNA). Spot D, from the RNase A fingerprint of medRNA is presented in lane (d). RNase T2 digestion of medRNA spot 3 yielded a binary spot (lane a), whose faster component co-migrated with the RNase T2-resistant oligonucleotide from the mRNA analog of spot D (lane g). The positions of oligonucleotide and cap markers are indicated. "O" identifies the origin of electrophoresis, "XC", the position of xylene cyanol.

sequence ACCUCC_{OH} (Table 1; see footnote d). It was not a clearly defined fingerprint spot, probably because a 3' hydroxyl group tends to reduce the precision of homochromatographic focusing. The fingerprint of medRNA' lacked spot 9. medRNA' is likely to be 6-10 nucleotides shorter than medRNA, as also suggested by gel mobility (see Figure 1). A map of medRNA showing the positions of oligonucleotides from the mini-exon portion and from the 3' end is presented in Figure 4.

Fingerprint analyses of other small RNAs containing the mini-exon

The medRNA fingerprint provided a guide for identification of related RNAs. When hybrid-selected RNAs of about 100 nucleotides (Figure 1, band 4) and about 70 nucleotides (Figure 1, band 5) were analyzed by fingerprinting (Figure 2G and data not shown, respectively), they were found to contain only sequences from the 5' end of medRNA. In particular, the fingerprint of RNA from band 4 lacked the oligonucleotide AAUCUG, a marker for the 3' portion of medRNA.

The RNase T1 fingerprint (Figure 2F) of an RNA of about 35 nucleotides (Figure 1, band 6) consisted entirely of mini-exon spots 2, 3, 7, 10 and a novel spot, 26.

Figure 4. Oligonucleotide map for the mini-exon portion and 3' terminus of medRNA. The oligonucleotides identified by numbers (RNase T1 products) and letters (RNase A products) are analyzed in Table 1. The DNA sequence is from ref. 10. The asterisk marks a partially RNase U2-resistant bond.

Oligonucleotides 3, 7 and 10 (Table 1) were identical to the analogous oligonucleotides from the medRNA. However, spot 2 from this RNA yielded only the products from medRNA oligonucleotide 2a. Oligonucleotide 2b yielded secondary digestion products consistent with the sequence UACUAUAU_{OH} (Table 1; see footnote f). This suggested that oligonucleotide 2b was a version of oligonucleotide 2b lacking 2 nucleotides from its 3' end. The RNase A fingerprint (Figure 2E) contained only spots with components from the mini-exon. The combined results supported the interpretation that the RNA in band 6 consists of a truncated version of the mini-exon lacking 2 nucleotides from its 3' end.

Comparison of medRNA and mRNA mini-exon-specific oligonucleotides

Oligonucleotides from the mini-exon portion of medRNA and mRNA molecules were compared. The mini-exon-specific spots 2 and 3 were easily detected in RNase T1 fingerprints of *T. brucei* mRNA prepared by two different approaches (Figure 2D, 2H): hybrid-selection followed by elution from the higher molecular weight region of preparative gels (Figure 1, area 1) or oligo(dT) selection.

To demonstrate that the mini-exon-specific oligonucleotides were covalently attached to mRNA and not derived from hydrogen-bonded medRNA, poly(A)⁺ RNA was boiled in water and fractionated on a sucrose gradient. In RNase T1 fingerprints of high molecular weight RNA (greater than 700 nucleotides), the intensity of spots 2 and 3 was not diminished relative to control fingerprints of unboiled high molecular weight poly(A)⁺ RNA (data not shown). Therefore, the RNase T1 fingerprint provided a way to purify spots 2 and 3 from mRNA. Oligonucleotides from the mini-exon could not be identified in RNase A fingerprints of poly(A)⁺ RNA, presumably because of co-migration with other oligonucleotides.

Secondary analysis was used for positive identification of the mRNA spots 2 and 3. Spot 2 from mRNA, like spot 2 from medRNA, yielded UUG, UUA, CUA and UA after RNase U2 treatment. A series of tests revealed no significant differences between medRNA spot 3 and mRNA spot 3, other than the expected higher level of contamination in the mRNA spot. These spots co-migrated during electrophoresis on DEAE paper, pH 1.9 (Figure 3, lanes b and e) and both yielded a slowly migrating binary spot upon digestion with RNase A (Figure 3, lanes c and f).

In order to remove contaminants from the mRNA capped oligonucleotide, mRNA spot 3 was treated with RNase A and the products fractionated by high voltage electrophoresis on DEAE paper, pH 1.9. The ability of this system to separate the authentic cap from its slower, modified form led to the isolation of an mRNA oligonucleotide, denoted mRNA spot D, which contained only the faster component. These findings showed that mini-exon sequences were detectable in heterogeneous populations of trypanosome mRNA whether the RNA was selected by its 5' end (the mini-exon) or its 3' end [poly(A)⁺ tails].

Characterization of the RNase T2-resistant cap structure

The 5' cap present on most eukaryotic mRNAs contains m⁷G linked by a unique 5'-5' triphosphate bond to the first encoded nucleotide. This bond is sensitive to cleavage by pyrophosphatases. The first and second encoded nucleotides of cap structures can also contain base and sugar modifications (18,41). Methylations at the 2' position of ribose render phosphodiester bonds resistant to RNases which cleave via a 2', 3' cyclic phosphate intermediate. Since RNase T2 lacks base specificity, but is unable to cleave 2'-O-methylated bonds, it has traditionally been used to produce 5' cap structures containing m⁷G and the first few modified nucleotides (18).

Treatment of medRNA spot 3 with RNase T2 produced mononucleotides and a binary spot (Figure 3, lane a) which co-migrated on DEAE paper, pH 1.9, with the binary spot produced by RNase U2. This mobility was too slow for any conventional multiphosphorylated mononucleotide (such as pppGp), strongly suggesting the presence of a cap structure. The RNase T2-resistant component of mRNA spot D (Figure 3, lane g) co-migrated with the analogous product obtained from medRNA spot 3. The mRNA and medRNA cap structures are thus indistinguishable by these methods.

In order to obtain a large quantity of the mRNA cap, poly(A)⁺ RNA was treated with RNase T2 and phosphatase and a resistant oligonucleotide, the mRNA cap structure, was directly purified by DEAE high voltage electrophoresis, pH 1.9. This oligonucleotide (Figure 5, lane d) co-migrated with the faster of the two RNase T2- and phosphatase-resistant oligonucleotides from medRNA spots 3 and D and with the comparable product of mRNA spot D. The absence of the slower member of the binary spot (when fingerprinting was omitted) was consistent with the interpretation that it was an artifactual derivative, as discussed above.

Analysis of the core cap structure

Nuclease P1 has classically been used to release core cap structures (consisting of m⁷GpppN_{OH} where N is the first encoded nucleotide) and modified nucleoside 5' monophosphates (pN*) from RNase T2-resistant capped oligonucleotides (18,41-43). In contrast to RNases which cleave via a 2', 3' cyclic intermediate, nuclease P1 can cleave bonds with 2'-O-methylations, although such cleavage may proceed at a reduced rate (44,45). A capped oligonucleotide with no modified nucleotides other than m⁷G yields the same core cap whether treated with RNase T2 plus phosphatase, or with nuclease P1.

Figure 5. Nuclease P1 treatment of capped oligonucleotides. Cap-containing oligonucleotides from medRNA or poly(A)⁺ RNA were digested with nuclease P1 and the products fractionated by electrophoresis on DEAE paper, pH 1.9. Under standard digestion conditions, nuclease P1 treatment of medRNA spots 3 (lane a) and D (lane b) and the mRNA analog of spot D (lane c) yielded a complex array of products. The purified RNase T2-resistant mRNA cap structure (lane d) was digested with nuclease P1 under standard (lane e) and complete (lane f) digestion conditions. Products included oligonucleotides (spot "i", as well as spots "ii" and "iii", which co-migrate on this system) and mononucleotides (designated pA*/pC* and pU* because they do not precisely co-migrate with conventional nucleotides). The positions of phosphate, mononucleotide, and cap markers are indicated in the figure. "O" identifies the origin of electrophoresis; "XC", the position of xylene cyanol.

Under standard nuclease P1 digestion conditions (which were sufficient for complete cleavage of bonds between conventional, but not all modified, nucleotides) medRNA and mRNA capped oligonucleotides from fingerprints produced a similar distinctive set of oligonucleotides and mononucleotides (Figure 5, lanes a-c). The oligonucleotides were subsequently shown to be partial products. The highly characteristic pattern of products emphasizes the similarity between the capped oligonucleotides in

Figure 6. Components of the mRNA cap structure. Products from nuclease P1 digests of the cap structure were eluted from DEAE paper and analyzed further by electrophoresis on Whatman 3MM paper, pH 3.5. Lane (a) shows the analysis of spot "iii"; lane (b), the mononucleotides pA*/pC*; and lane (c), pU*. Spot "i" was digested to completion with nuclease P1; the two resulting products are presented in lane (d). The positions of mononucleotide and cap markers are indicated. "O" identifies the origin of electrophoresis; "XC", the position of xylene cyanol.

mRNA and medRNA. Because mRNA spot D contained only the authentic form of the capped oligonucleotide, its products did not include the slower-migrating artifactual derivative mentioned above (compare Figure 5 lanes a and b to lane c). Standard conditions for nuclease P1 digestion released products of the same mobility from the isolated RNase T2-resistant mRNA cap structure (Figure 5, lane e) as from mRNA spot D (Figure 5, lane c). Stronger nuclease P1 digestion conditions, developed to release the core cap, yielded a product, spot "iii," which migrated near core cap markers, and mononucleotides (Figure 5, lane f).

Spot "iii" was eluted, treated with nucleotide pyrophosphatase and analyzed by electrophoresis on Whatman 540 paper, pH 3.5. Products included pm⁷G, (modified) pA, and an unidentified phosphatase-sensitive species. The release of pm⁷G and pA,

combined with the following evidence, suggested that spot "iii" was the core cap with the sequence $m^7GpppA^*_{OH}$. Spot "iii" was resistant to phosphatase, co-migrated with the m^7GpppA_{OH} and $m^7GpppA(2'-O)m$ standards on Whatman 540 paper high voltage electrophoresis, pH 3.5 (Figure 6, lane a), and migrated close to, but not identically with these two markers in high voltage electrophoresis on DEAE paper at pH 1.9 (Figure 5, lane f). The lack of exact co-migration on DEAE paper indicated that the A residue may have two modifications, 2'-O-methylation and N⁶-methylation. The mononucleotides produced by the complete nuclease P1 digestion of the cap structure had mobilities consistent with pA, pC and pU on Whatman 540 paper high voltage electrophoresis, pH 3.5 (Figure 6, lanes b and c). This system is relatively insensitive to nucleotide modifications except those which alter the charge of the molecule (44). Their mobilities on DEAE high voltage electrophoresis, pH 1.9 (Figure 5, lane f) and thin layer chromatography (not shown) suggested the presence of modifications in addition to the 2'-O-methylations expected of RNase T2-resistant bonds.

Characterization of the nuclease P1 partial digestion products

When spot "i" from the partial nuclease P1 digest of the mRNA cap structure was redigested with nuclease P1 under the stronger digestion conditions and analyzed by Whatman 540 paper high voltage electrophoresis, pH 3.5, (Figure 6, lane d), the core cap and a (modified) pA were released, suggesting that the m^7G of the cap is linked directly to two modified A residues: $m^7GpppA^*A^*$. Since it was shown above that the RNase T1-resistant capped oligonucleotide terminated with the sequence AACG, it was important to see how these A residues related to the two A residues present in spot "i".

Analysis of spot "ii", a nuclease P1 partial digestion product, combined with information about the mononucleotides released by complete nuclease P1 digestion of the mRNA cap structure, indicated that at least 2 nucleotides (pC* and pU*) lie between the last nucleotide in spot "i" ($m^7GpppA^*A^*$) and the first nucleotide in the sequence AACG. Although spot "ii" co-migrated with the core cap (Figure 5, lanes e and f), several properties distinguished it from the core cap. Spot "ii" was sensitive to nuclease P1 and some, but not all, of its phosphate groups were accessible to phosphatase (data not shown). Experiments are underway to see if it contains the pC* and pU* present in the complete nuclease P1 digests of the mRNA cap structure and thus constitutes the linker between spot "i" and the sequence ACG.

DISCUSSION

Direct analysis was used to characterize two essential components of the trypanosome mRNA biosynthetic pathway: the mini-exon donor molecule, medRNA, and mature mini-exon-containing mRNA. A cap structure was purified from poly(A)⁺ mRNA by two methods and found to be indistinguishable from the medRNA cap. This major cap structure appears to contain four previously uncharacterized modified nucleotides, in addition to m^7G . Furthermore, to evaluate their potential for serving

as intermediates in mRNA formation, several additional small RNAs were also analyzed. An established line of procyclic cells was chosen for these studies because they incorporated inorganic phosphate into RNA more efficiently than bloodstream forms. It is likely that conclusions drawn from structural studies of RNA from procyclic cells will apply to the other forms as well, since data point to a conserved mechanism of mRNA biosynthesis in the various trypanosome forms (14,15).

The medRNA was purified by hybrid selection followed by gel electrophoresis and characterized by RNA fingerprinting. Several features of medRNA were identified: the 5' terminal cap structure, an additional modified residue (possibly m⁶A) which lies just downstream from the cap structure, and the 3' end of the molecule. Inspection of the medRNA fingerprints and a complete oligonucleotide analysis to be published elsewhere reveal that this RNA could be a direct transcript of only one (ref. 10) of the four cloned medRNA gene sequences (10-13) that have been published. The 3' terminal nucleotide of medRNA corresponds to the 136th nucleotide of the medRNA genomic sequence (10).

Several other RNA species smaller than medRNA were similarly purified. All were found to be 3'-truncated forms of medRNA. The functional significance of these species is unknown: they may be alternative mini-exon donors or they may be mechanistically irrelevant degradation products.

A series of experiments was carried out to compare the cap structure present in medRNA to that in mRNA. The cap-containing oligonucleotide was first identified in fingerprints of medRNA. Using this fingerprint as a guide, an analogous spot was purified from poly(A)⁺ RNA and found to contain an RNase A-resistant moiety matching the one from medRNA. The secondary products released by RNases U2 and T2 indicated that the RNase T1-resistant capped oligonucleotides from medRNA and mRNA have the same sequence, AACG, at their 3' ends. Furthermore, nuclease P1 products of the medRNA capped oligonucleotide were indistinguishable from those of the analogous mRNA oligonucleotide. The similarity between the medRNA cap and the mRNA cap provides strong evidence that the mini-exon is transferred intact and that many, if not all, of the nucleotide modifications in the cap occur prior to transfer.

In considering these data, it is important to evaluate the population of poly(A)⁺ RNA, since oligo(dT) selection, which was used to isolate poly(A)⁺ RNA, may enrich for species other than mRNA. For example, the oligo(dT)-selected RNA studied by Laird et al. (17) contained material in the 140-200 nucleotide size class which was thought to be either polyadenylated medRNA or an medRNA read-through transcript retained by sandwich hybridization of its U-rich tail. However, polyacrylamide gel analysis of our poly(A)⁺ RNA indicated that the vast majority was much longer than 200 nucleotides (unpublished observations). Furthermore, the fingerprints of poly(A)⁺ RNA purified by sucrose gradient centrifugation (to remove molecules less than 700 nucleotides in length, or fragments associated by hydrogen bonding) contained the same capped oligonucleotide present in the total poly(A)⁺ population. Thus, under the

labeling conditions used here, it is likely that mRNA was the major source of capped oligonucleotides in the oligo(dT)-selected population. Since the capped oligonucleotide from mRNA could be obtained in much greater amounts than that from medRNA, it was used for most of our detailed studies of the cap structure.

A tentative model of the *T. brucei* cap structure can be built from the data obtained in this study. From the 5' end, m⁷G is linked by a 5'-5' triphosphate bond to two modified adenosine residues. At least two nucleotides, C and U (both modified), separate these two adenosines from the first unmodified residue which is likely to be an A. This nucleotide would correspond to the first A of the sequence AACG (from the 3' portion of the RNase T1 spot 3; see Table 1) if no additional nucleotides are present. Thus, our data indicate that the mini-exon is at least four nucleotides longer than originally reported, a possibility also recently discussed by Murphy et al. (27). Complete sequence data for the cap structure are not yet available; however, if the sequence of nucleotides within the cap structure matches the medRNA genomic sequences, which are conserved in this region, the 5' terminal structure of the mini-exon is m⁷GpppA*A*C*U*AA*CG. Thus, medRNA is 140 nucleotides long, excluding the m⁷G. The first four nucleotides are probably 2'-O-methylated and may have additional modifications. The modifications explain why these nucleotides were not detected in previous indirect analyses. The sixth nucleotide is also modified, but not 2'-O-methylated. Since this sequence contains two m⁶A consensus sequences (40), the second and fourth A residues are likely to be N⁶-methylated.

A cap structure with four modified nucleotides adjacent to the m⁷G ("cap 4") is unique, since all cap structures characterized previously have zero (cap 0), one (cap 1) or two (cap 2) 2'-O-methylated nucleotides (18,41). While 2'-O-methylations in bulk mRNA (41) and specific messages (42,46) tend to be heterogeneous, our current data indicate that the trypanosome mRNA cap structure could be unusually homogeneous.

A cap structure has been shown to have a role in priming of influenza transcription (47,48), mRNA splicing (21,22) and possibly 3' mRNA processing (49). The novel mini-exon cap that we have described may be necessary for the discontinuous synthesis of trypanosome mRNA. A highly modified cap could distinguish trans- and cis-splicing pathways. The finding by Krause and Hirsh (5) of a mini-exon-like RNA segment at the 5' end of some but not all *C. elegans* mRNAs may present a way to test this hypothesis. It will also be important to determine whether the primary transcripts of structural genes, in trypanosomes and *C. elegans* are capped. In addition, if the novel cap is necessary for the biosynthesis or subsequent utilization of trypanosome mRNA, it may be a target for specific chemotherapy against these parasitic protozoa.

While complete understanding of this process may await the development of *in vitro* systems, both our observation of a small RNA comprising almost a complete free mini-exon and that of Laird et al. (17), who noted variable amounts of a similarly sized RNA, suggest that the medRNA cleavage step may be accessible to direct study in the near future. As also discussed by Laird et al. (17), the variable recovery of the 5' ter-

minal medRNA fragment from extracts of radio-labeled cells may indicate that cleavage is facilitated at the junction between the mini-exon segment and the remainder of medRNA. If the free mini-exon is subject to trimming, a slightly truncated version of the mini-exon, like the band 6 RNA analyzed here, could result. We conclude that direct analysis of medRNA metabolism using the capped oligonucleotide and other features described here will help to elucidate the role of mini-exon sequences *in vivo*.

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