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# Mature mRNAs of *Trypanosoma brucei* possess a 5′ cap acquired by discontinuous RNA synthesis

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## ABSTRACT

Mature mRNAs of Trypanosoma brucei have a common 5' terminal sequence of 35 nucleotides. This is acquired by an unknown mechanism from the 5' end of a separately transcribed precursor RNA of about 140 nt called the mini-exon-derived RNA or medRNA. We have investigated the nature of the 5' ends of mature mRNAs and of the medRNA by chemical decapping and enzymic recapping. We infer that a 5' cap is present on both of these RNAs and conclude that the mini-exon-derived RNA donates its 5' cap along with the mini-exon sequence to the pre-mRNA. Using nuclear run-on experiments we show that medRNA synthesis is much more sensitive to α-amanitin than 5S RNA synthesis and only slightly less sensitive than tubulin gene transcription. This result, together with the presence of a cap at the 5' end of the medRNA indicates that the mini-exon is transcribed by an RNA polymerase II type enzyme. Our experiments also confirm the existence of a second minor medRNA of about 125 nt and show the presence of other small capped RNAs possibly analogous to the small nuclear RNAs of other organisms.

## INTRODUCTION

Trypanosoma brucei belongs to the parasitic protozoan family of Trypanosomatidae. This family contains members of interest for their medical importance and biochemical peculiarities (1-5), which include the presence of a common 35 nucleotide sequence at the 5' end of all mRNAs studied (6,7). The 35 nucleotides are encoded by a separate mini-exon, which is part of a 1.35 kb repeat, present in about 200 copies arranged in clusters in the T. brucei genome (8-13). Analysis of stable RNA (9,13,15) and nascent RNA (15) showed that the 1.35 kb repeat is transcribed into an RNA of about 140 nucleotides referred to as the mini-exon-derived RNA or medRNA, which has the mini-exon sequence at its 5' end (see Fig. 1).

Several independent lines of evidence indicate that, after transcription of the medRNA has been completed, its mini-exon sequence is joined to the main body of trypanosome mRNAs in a second unconnected event (9,13,15-17).

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Three basic mechanisms for this discontinuous synthesis of mRNAs can be envisaged:

1. Priming of mRNA synthesis by a) the mini-exon sequence or b) the medRNA followed by splicing.
2. RNA ligation followed by normal splicing.
3. A novel bimolecular splicing event (transsplicing).

Only one version of mechanism 1 - a jumping polymerase - has been excluded by nuclear run-on experiments showing that mini-exon transcription is far more sensitive to inhibition by α-amanitin than transcription of the genes for Variant Surface Glycoproteins (VSGs) (16).

The transcription experiments with isolated nuclei gave no conclusive information on the polymerase responsible for medRNA synthesis (16). Initial results indicated that synthesis of medRNA is quite less sensitive to α-amanitin than synthesis of precursor of tubulin mRNA (16). The mini-exon repeat lacks conserved sequences resembling pol II or pol III promoter signals in other eukaryotes, but the equivalent of these signals in trypanosomes is still unknown. In this paper we address the question of the polymerase responsible for medRNA synthesis in more detail. We have quantitated the sensitivity to α-amanitin of transcription of genes assumed to be transcribed by RNA pol I (ribosomal repeat), RNA pol II (tubulin genes) and RNA pol III (5S RNA gene) and compared these to the sensitivity of medRNA transcription.

Another way to distinguish pol II and pol III transcripts is by the nature of their 5' ends. RNA polymerase II, which is responsible for mRNA and small nuclear RNA (SnRNA) transcription, yields 5' capped transcripts (18-20), while polymerase III yields primary transcripts with a 5' triphosphate. Since the 5' end of mature trypanosome mRNAs is presumed to be derived from the medRNA, we have investigated whether mature mRNAs of _T. brucei_ contain a 5' cap and if so, whether the cap is already present on the medRNA.

The 5' termini of individual transcripts are usually determined by chromographical analysis or fingerprinting of digested RNA synthesized _in vitro_ or of RNA labelled _in vivo_ and then highly purified (see ref. 18 for an overview). A homologous _in vitro_ transcription system for trypanosomes is not yet available and _in vivo_ labelling is inefficient in _T. brucei_. To circumvent these problems we have used an indirect approach for the analysis of trypanosome RNA 5' termini.

Total or poly$(A)^+$ RNA was capped with ($\alpha$-$^{32}$P)GTP using Vaccinia virus guanylyltransferase with or without prior decapping. The labelled RNA was

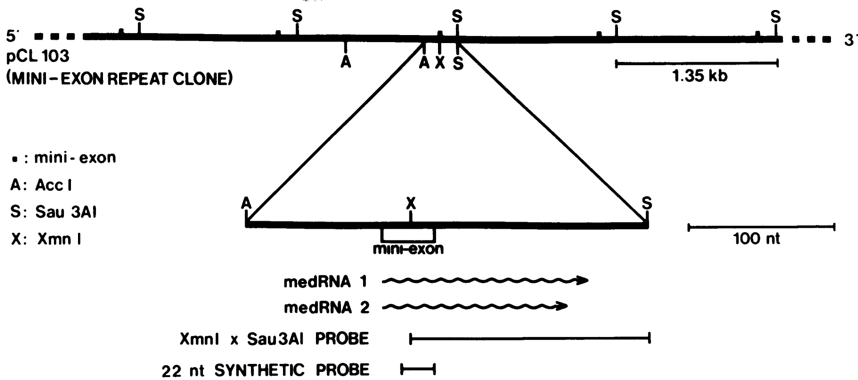


Figure 1. Structure and expression of the mini-exon repeat. A simple map of several consecutive mini-exon repeats; Part of the insert of pCL103 is shown, which contains about 15 such repeats cloned as a partial Mbo I fragment in the Bam HI site of pAT153. The enlarged section of the repeat shows the regions transcribed as medRNA (see text) and the regions covered by the probes used in the experiments shown in Figures 3 and 5.

then used as a probe on dot blots of gene clones or analyzed by polyacrylamide gel electrophoresis. By comparing the results of the capping with and without prior decapping we were able to discriminate between 5' capped termini, di- or triphosphate 5' termini and monophosphate or hydroxyl 5' termini (see Table 1).

## METHODS AND MATERIALS

Trypanosomes. The trypanosomes used in this study belonged to the 221a variant antigen type of the *Trypanosoma brucei* 427 stock described by Cross (23). Growth and isolation of trypanosomes was performed as described by Fairlamb *et al.* (24).

Recombinant plasmids. The DNAs used in the experiment shown in Figure 2 are the following: 1: λ DNA, 2: TcV 221.5 (a VSG 221 cDNA clone (25)), 3: TcV 1.8 (a VSG 1.8 cDNA clone (26)), 4: pTbαβT-1 (an α + β tubulin genomic clone (27)), 5: pCL103 (a mini-exon repeat clone; see Figure 1), 6: plasmid vector pAT153. The plasmids used in the nuclear run-on experiment shown in Figure 4 are the following: VSG cDNA: TcV221.5 (see above), Tubulin: pTbαβT-1 (see above), for the mini-exon repeat: pCL102, which is similar to pCL103 in Figure 1 (see also ref. 15 and 16), for the ribosomal repeat: pR4 (see ref. 16), and for 5S RNA: pTb5S-1 which contains a Mbo I partial digestion fragment spanning five 5S repeat units, each 760 bp in length. This last clone was obtained from a clone bank of *T. brucei*, strain 427, with a 5S RNA probe, isolated from a polyacrylamide gel, similar to the one shown in Fig. 3A, and end-labelled with polynucleotide kinase.

Isolation, gel electrophoresis and blotting of RNA. RNA was isolated as described by Kooter *et al.* (28). Glyoxylated RNA was size-fractionated and blotted according to the procedure of Thomas (29). 7M urea polyacrylamide gels were electroblotted onto GeneScreen Plus (NEN) (Figure 3) or onto

Table 1. Effects of the capping and decapping treatments on RNA (see text).

| DECAPPING REMOVES THE 5' CAP AND THE 3' TERMINAL NUCLEOSIDE | 5' MONOPHOSHATE (PROCESSED rRNA AND tRNA): 5'pN | 5' DI AND TRI PHOSPHATES (5S RNA): 5' ppN AND 5' pppN | 5' CAP (mRNA AND SnRNA): 5' XpppN |
|---|---|---|---|
| $G^{32}ppp$ CAPPING | 5' pN ΔMW= 0 | 5' $G^{32}pppN$ ΔMW=+1 | 5' XpppN ΔMW= 0 |
| DECAPPING | 5' pN ΔMW=-1 | 5' (p)ppN ΔMW=-1 | 5' pppN ΔMW=-2 |
| DECAPPING & $G^{32}ppp$ RECAPPING | 5' pN ΔMW=-1 | 5' $G^{32}pppN$ ΔMW= 0 | 5' $G^{32}pppN$ ΔMW=-1 |

The 5' ends of RNA molecules are shown schematically. The capping treatment with Vaccinia virus guanylyltransferase requires a di- or triphosphate 5' end. The decapping treatment specifically removes nucleosides with a 2' and 3' hydroxyl group such as in the 3' terminal nucleotide and in a 5' terminal cap (21). Changes in molecular weight due to removal of a 3' nucleoside and/or addition of a 5' cap are given. N is the first transcribed nucleotide. X is any given 5'ppp5' attached cap structure. The primary transcripts of all polymerases possess a 5' triphosphate. However, the processed RNAs of pol I (rRNA) and of pol III (e.g. tRNA) transcripts generally contain a 5' monophosphate. The pol III-transcribed 5S RNA retains a triphosphate 5' end. Pol II transcripts are capped during transcription (22).

Zeta-Probe (Bio-Rad) (Figure 4A) using procedures specified by the manufacturers. Hybridizations with the synthetic 22 nucleotide probe were performed as described by De Lange et al. (11). Post-hybridization washes in these experiments were in 3 x SSC at 30°C. Hybridizations with the Xmn I x Sau 3A1 probe were washed to a stringency of 0.1 x SSC, 65°C.

Decapping of RNA. Decapping of RNA was achieved by periodate oxidation and aniline cleavage (21) as follows. 20 μg of total or poly(A)$^+$ RNA was incubated for 30 minutes at 0°C in 50 μl 100 mM Sodium acetate (NaOAc), 0.4 mM $NaIO_4$, pH 5.3, and subsequently precipitated with 2.5 volumes ethanol and washed once with 70% ethanol. The RNA pellet was then dissolved in 20 μl 10 mM NaOAc, 300 mM redistilled aniline, pH 5.3, and incubated for 3 hrs at room temperature. NaOAc was then added to a final concentration of 200 mM and the RNA was ethanol precipitated and washed twice. This treatment specifically removes nucleosides with a 2' and 3' hydroxyl group such as in the 3' terminal nucleotide and in a 5' terminal cap (21).

Capping of RNA. RNA was capped by incubating 20 μg total or poly(A)$^+$ RNA for 45 minutes at 37°C in 25 μl 250-750 μCi (α-$^{32}$P)GTP (410 Ci/mmol), 10-25 units Vaccinia virus guanylyltransferase (BRL), 160 μM ATP, 20 μM unlabelled GTP, 2.5 mM DTT, 6 mM KCl, 1.25 mM $MgCl_2$, 50 mM Tris.HCl, pH 7.8. The reaction was stopped by adding 25 μl of 10 mM EDTA, 0.1% SDS, 10 mM Tris.HCl pH 7.3 and, after a phenol/chloroform/isoamylalcohol (25:24:1) extraction the RNA was passed through a Sephadex G50 column and ethanol precipitated.

Nuclear run-on assay. Synthesis of nascent RNA in isolated nuclei in the presence or absence of α-amanitin was essentially done as described by Kooter and Borst (16). In vitro elongated RNA was hybridized to filters onto which DNA was dotted as described (15). To quantitate the amount of hybridizing nascent RNA, the dots were cut out and counted in a Beckman LS 3145T liquid scintillation counter in Insta-Gel counting liquid (Packard).

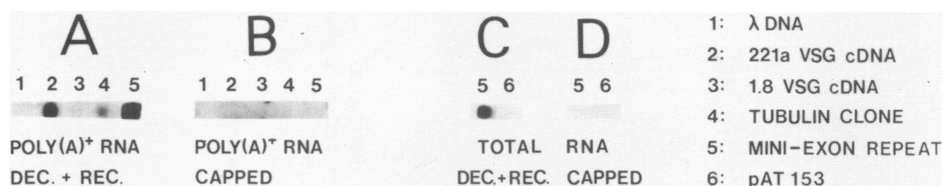


Figure 2. Dot blot analysis of capped and decapped-recapped 221a RNA. 3 µg of the DNAs indicated was denatured and spotted onto twin sets of nitrocellulose filters and hybridized with: A) decapped, $^{32}$P recapped poly(A)$^{+}$ RNA; B) $^{32}$P capped poly(A)$^{+}$ RNA; C) decapped, $^{32}$P recapped total RNA or D) $^{32}$P capped total RNA. The filters in panels A and B were washed down to 3 x SSC, 65°C; the filters in panels C and D to 0.1 x SSC, 65°C. The dotted DNAs are specified in detail in Methods and Materials. All DNAs, except for no. 5, lack mini-exon sequences. The dot no. 5 in panel A is a three times shorter exposure than the other dots in panels A and B.

## RESULTS

### Indirect analysis of the 5' termini of medRNA and mRNAs.

The presence of a cap at the 5' end of specific mRNAs was investigated by using poly(A)$^{+}$ RNA, which was both decapped and $^{32}$P recapped (Fig. 2A) or only $^{32}$P capped (Fig. 2B), as probes on identical dot blots. A similar experiment was done with total RNA to investigate the presence of a cap at the 5' end of medRNA (Fig. 2C and 2D).

Figures 2A and 2B show that the labelled GTP is added to 221 VSG mRNA and tubulin mRNA only if the RNA has undergone a prior decapping treatment. The difference in label intensity reflects the different levels of VSG and tubulin mRNAs in stable RNA. The barely detectable tubulin signal shows the limits of this technique. The specificity of the hybridization is illustrated by the lack of hybridization of the 1.8 VSG cDNA clone with the labelled RNA from variant 221, in which the 1.8 gene is not expressed. In view of the nature of the decapping treatment (see ref. 21 and Methods and Materials) and of the substrate requirement of the capping enzyme (a di- or triphosphate 5' terminus (30)), we conclude that both VSG mRNA and tubulin mRNA contain a 5' cap or cap-like structure.

The same can be concluded for medRNA as can be seen in Figure 2C and 2D. Hybridization to the mini-exon repeat clone pCL103 at the washing stringency of 0.1 x SSC, 65°C used in this experiment is specific for medRNA.

In contrast, the strong signal of the mini-exon-repeat spot in Figure 2A is due to the cumulative label of all mini-exon sequences with a 5' cap since at the washing stringency of 3 x SSC, 65°C employed, the mini-exon

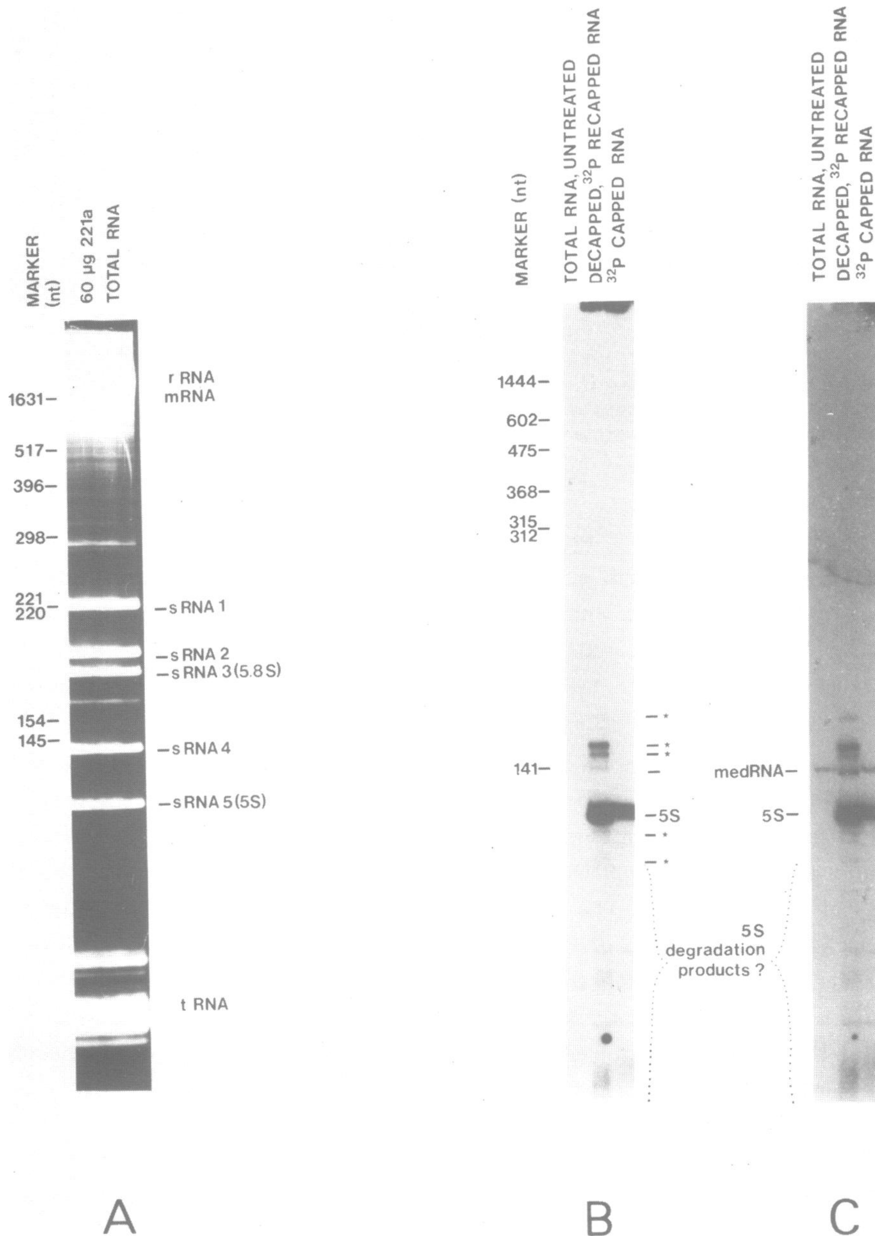


**Figure 3:** Polyacrylamide gel electrophoresis and hybridization of capped, decapped-recapped and untreated RNA. Panel A shows an ethidium bromide stain of 60 µg of total 221a RNA size-fractionated on a 7 M urea, 8% polyacrylamide gel. The marker is a denatured Hinf I digest of pAT153. The

small RNA desigations are taken from ref. 32. Panel B shows a 7M urea, 6% polyacrylamide gel of the RNAs specified which was electroblotted and then autoradiographed. First lane: 4 µg unlabelled total 221 RNA. Second lane: 12 µg decapped, $^{32}P$ recapped total 221 RNA. Third lane: 8 µg $^{32}P$ capped total 221 RNA. Undefined small capped RNAs are indicated with an asterisk. The marker used was a denatured kinase-labelled Taq I digest of pAT153. The autoradiogram in Panel C shows a hybridization of the same blot with the nick translated 161 bp Xmn I x Sau 3A1 fragment of pCL103 (a mini-exon repeat clone; see Fig. 1) washed to a stringency of 0.1 x SSC, 65°C. Therefore the signals in panel C are the cumulative result of the capping treatments and of the hybridization with the mini-exon repeat probe. At the washing stringency used, this probe does not hybridize with mRNAs.

sequences of both mRNAs and of the medRNA itself, which lacks a poly(A) tail but can contaminate poly(A)-enriched RNA, could form stable hybrids with the mini-exon repeat.

Polyacrylamide gel analysis of capped RNA and of decapped-recapped RNA.

To further substantiate the results for medRNA, untreated, decapped-recapped and capped preparations of total 221a RNA were analyzed by polyacrylamide gel electrophoresis.

An ethidium bromide stained polyacrylamide gel of 60 µg of total 221a RNA is shown in Figure 3A to illustrate the complexity of the population of small RNAs of T. brucei . The major RNAs in the size range of 100-250 nt are ribosomal RNAs and are probably derived from a precursor RNA which also yields the large rRNAs (32). Small RNAs 3 and 5 have been partially sequenced and have been identified as 5.8S RNA and 5S RNA respectively (32).

Figure 3B shows an electroblot of a different gel of untreated, decapped-recapped and of capped total 221a RNA. There is one major RNA, which comigrates with 5S RNA that incorporates ($\alpha$-$^{32}P$)GTP without prior decapping, indicating that it has a 5' di- or triphosphate, in agreement with what is known of 5S RNA in other organisms. If the capping treatment is preceded by a decapping treatment, several additional bands appear (Fig. 3B, second lane). One of these migrates at the position of medRNA (140 nt), as shown by hybridization of the same blot with a medRNA-specific probe in Fig. 3C. The changes in molecular weight of RNA molecules following the capping or decapping treatments as described in Table 1 are responsible for the differences in migration of medRNA and 5S RNA observed in the different lanes of Figures 3B and 3C.

The experiment shown in Figure 3 confirms that the medRNA contains a 5' cap or cap-like structure. the absence of detectable mRNA in the second lane of panel B is probably due partly to the fact that much of the larger

RNA has remained in the slot and partly due to the inefficiency of labelling by this method.

Figure 3B also shows the presence of a number of other small capped transcripts (indicated with an asterisk) in addition to medRNA. They may be analogous to the small nuclear RNAs (SnRNAs) of other eukaryotes (33).

Some of the small RNAs below the 5S band in the last two lanes of Figures 3B and 3C, which apparently lack a 5' cap since they are labelled without prior decapping, do not show a difference in migration even though the RNA preparation in the second lane has undergone a decapping treatment which should remove a 3' nucleoside. This indicates that either the 3' nucleotide of these RNAs is insensitive to the decapping treatment or, more likely, that during or after the capping reaction, the 3' end of these molecules was cleaved off (degradation). If this is the case they are most likely degradation products of 5S RNA, as indicated in the figure.

α-Amanitin sensitivity of medRNA synthesis.

To examine the type of polymerase responsible for medRNA synthesis, we have done nuclear run-on experiments in the presence of various concentrations of α-amanitin, as described (16). The radioactively labelled nascent RNA was hybridized to filters, onto which an excess of DNA was spotted complementary to medRNA, tubulin RNA, VSG RNA, 5S RNA and rRNA. The hybridization was quantitated by counting the dots in a scintillation counter. The results, plotted as a function of the α-amanitin concentration are shown in Figure 4.

Transcription of medRNA is reduced to 50% of control at about 15 µg α-amanitin per ml, whereas the same degree of inhibition of 5S RNA synthesis requires about 150 µg α-amanitin per ml. This indicates that medRNA is not made by an RNA pol III type polymerase as is presumably the case for 5S RNA. Transcription of tubulin genes is highly sensitive to inhibition by α-amanitin as would be expected for RNA pol II transcription. The inhibition curve for medRNA resembles that of tubulin transcription but they differ significantly and reproducibly. Whether this difference is an artifact of the crude nuclear preparations used, which synthesize medRNA at a much higher rate than tubulin RNA, or whether the difference is due to the use of different polymerases for the synthesis of these RNAs is uncertain. Experiments with purified polymerases will be required to settle this question. Transcription of VSG genes and of rRNA is resistant to α-amanitin in isolated nuclei, as reported earlier (16).

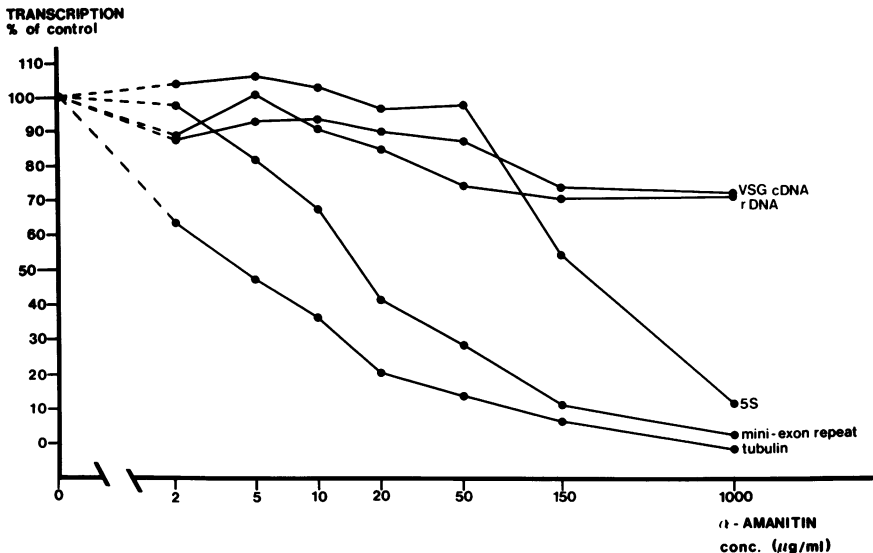


Figure 4. α-Amanitin sensitivity of transcription in nuclear run-ons. Radioactively labelled nascent RNA obtained from nuclear run-ons done at different concentrations of α-amanitin was hybridized to filters onto which 1 μg of the DNAs specified was spotted. (see Methods and materials for details on plasmids and procedures.) After hybridization, the filters were washed to 0.3 x SSC, 65°C, dried and counted in a liquid scintillation counter. The amount of hybridization to each DNA of nascent RNA synthesized in the absence of α-amanitin was set at 100%. The other values are plotted as percentages of this control. The values are a compilation of two independent experiments, each done in duplicate.

A second medRNA.

In S1 nuclease protection experiments, Kooter *et al*. (15) observed a minor mini-exon repeat transcript which ended at a position 14 nt upstream of the termination site of medRNA. Since this transcript was not observed in RNA blots (9,13,15), its artifactual generation by S1 nuclease could not be excluded. In some experiments analogous to the one shown in Fig. 3C (first lane), we noted a minor band of about 125 nt as is shown in Figure 5A. We infer that this band corresponds to the minor band found by Kooter *et al*.(15) in S1 experiments. As indicated in Fig. 1, this second medRNA presumably starts with the mini-exon sequence but ends before the major medRNA (see also ref. 15). Attempts to demonstrate the presence of a 5' cap on this RNA have not been successful, because of the low abundance of this RNA.

The medRNA downstream region is not detected in large RNA.

Previous experiments have shown that medRNA is made at a very high

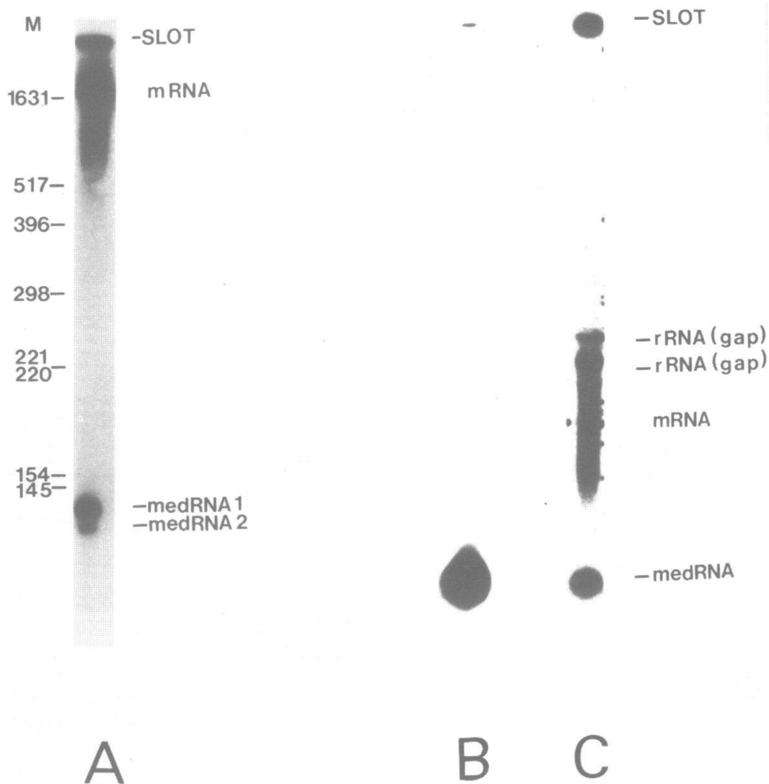


Figure 5. RNA blot analysis of total RNA. Panel A shows a 7M urea, 4% polyacrylamide gel of 10 µg of total 221a RNA, electroblotted and hybridized with a 5' end-labelled synthetic 22 nucleotide probe complementary to part of the mini-exon sequence (see Fig. 1 and ref. 11). The marker (M) is a denatured labelled Hinf I digest of pAT153. Panel B shows a 0.5% agarose gel of 25 µg of glyoxylated total 221a RNA, which was blotted, hybridized to the 161 bp nick translated Xmn I x Sau3A1 medRNA probe shown in Figure 1, and washed down to 0.1 x SSC, 65°C. Under these stringent conditions only mini-exon sequences flanked by the region found 3' of the mini-exon in the mini-exon repeat hybridize. This filter was rehybridized after the probe was removed by an overnight treatment with 0.1% SDS in water at 65°C with the synthetic 22-mer probe shown in Figure 1 and washed down to 3 x SSC, 30°C (Panel C). The gaps in the mRNA smear in panel C are due to saturation of the nitrocellulose filter during blotting by excessive amounts of rRNA.

rate in isolated nuclei (16). In some experiments hybridization of nascent RNA to mini-exon repeat DNA was even greater than to the complete rDNA repeat. On the other hand, the steady state level of medRNA is not very high: In the stained gel in Fig. 3A no major band is directly attributable to medRNA. It is possible that the medRNA band is obscured by the sRNA 4 band. The

modest level of medRNA as compared to small ribosomal RNAs is also evident from the low intensity of the medRNA compared to the 5S band in the cap-labelled RNA in Figure 3B. Therefore we infer that the medRNA has a high turn-over rate.

A high turn-over rate indicates either degradation of part of the generated medRNA population and/or a high rate of processing in the discontinuous synthesis of mRNA. The latter possibility would imply a high rate of synthesis of processing intermediates, which should be detectable in steady state RNA if the subsequent steps do not proceed at too high a rate. If medRNA were used as a primer for pre-mRNA synthesis or were ligated to pre-mRNAs prior to splicing, one would expect to see larger RNAs containing the medRNA sequence downstream of the splice donor site. Such precursors have not been observed in previous work (9,15), but the conditions used in these experiments were not aimed at detecting low levels of precursor RNAs. We have therefore repeated this experiment with a probe of high specific activity and with an extensive autoradiographic exposure. Figure 5B shows that there is indeed no detectable hybridization between the medRNA and the slot. The control lane shows that large RNA with the mini-exon sequence had been properly transferred (see Fig. 5C).

## DISCUSSION

We have used a new indirect method to analyze the presence of caps on trypanosome RNAs. The method allows the analysis of minor RNAs in complex mixtures and should be useful in other cases where pure RNAs cannot be readily obtained in highly labelled form. The method yields no information on the actual structure of the cap or on the possible methylation of adjacent nucleotides as observed in other eukaryotes (16,18). However, a standard cap seems likely, because trypanosome mRNAs are translated efficiently in heterologous translation systems (see 4). We cannot exclude the formal possibility that a fraction of the medRNA and/or mRNA has a monophosphate or hydroxyl 5' end. It is also possible that some RNAs are not fully capped *in vitro*, if the capping reaction is influenced by the secondary structure of the RNA. Denaturation of RNA by methylmercury hydroxide was not included in our experiments since we found an overall lower degree of capping following such a treatment probably due to inhibition of the capping enzyme by traces of remaining methylmercury hydroxide.

Despite these shortcomings, our results clearly show that the medRNA and several mRNAs in *T. brucei* contain a 5' cap or cap-like structure.

A 5' cap is generally recognized as a hallmark of RNA polymerase II transcription. The α-amanitin sensitivity of medRNA synthesis in isolated nuclei also resembles that of an RNA pol II. We infer from the presence of a cap at its 5' end and from the relatively high level of α-amanitin sensitivity of its synthesis that the medRNA is synthesized by a trypanosome polymerase analogous to RNA pol II of other eukaryotes rather than by an RNA pol III.

From our results it seems likely that trypanosome mRNAs obtain their cap from medRNA, but the mechanism remains a matter of conjecture. The existence of medRNA 2, which presumably lacks part of the 3' end of medRNA 1, suggests that the 3' end of medRNA is not essential for function, in agreement with its lack of conservation in other *Trypanosomatidae* (10,13). This is in contrast to what one would expect if medRNA acts as a primer for pre-mRNA transcription or if intact medRNA is linked to pre-mRNA by ligation.

An alternative is that the medRNA serves as a cutting tool to process long poly-cistronic pre-mRNAs to multiple mature transcripts by transsplicing. This would explain how the very long transcription unit of VSG genes (16, 34,35, J.K. and P.B., unpubl. obs.) would be able to yield multiple transcripts that contain a mini-exon sequence (T. De Lange and J.K., unpubl. obs.). Transsplicing would also account for the strong clustering of tubulin genes in *Trypanosoma brucei* (27,36) and the failure to find a region between genes in this cluster that is not transcribed in isolated nuclei (T. De Lange, pers. comm.).

None of these considerations is conclusive, however, and the elucidation of the actual mechanism of discontinuous mRNA synthesis in trypanosomes will probably require the development of homologous *in vitro* transcription and *in vitro* splicing systems.

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REFERENCES

1. Bernards, A. (1984) Biochim. Biophys. Acta 824: 1-15.
2. Borst, P., Bernards, A., Van der Ploeg, L.H.T., Michels, P.A.M.,

Liu, A.Y.C., De Lange, T. and Kooter, J.M. (1983) Europ. J. Biochem. 137: 383-389.
3. De Lange, T. Int. Rev. Cytol. in press.
4. Englund, P.T., Hajduk, S.L. and Marini, J.C. (1982) Ann. Rev. Biochem. 51: 695-726.
5. Opperdoes, F.R. British Medical Bullitin, in press.
6. De Lange, T., Michels, P.A.M., Veerman, H.J.G., Cornelissen, A.W.C.A. and Borst, P. (1984) Nucl. Acids Res. 12: 3777-3790.
7. Parsons, M., Nelson, R.G., Watkins, K.P. and Agabian, N. (1984) Cell 38: 309-316
8 Borst, P., Bernards, A., Van der Ploeg, L.H.T., Michels, P.A.M., Liu, A.Y.C., De Lange, T., Sloof, P., Veeneman, G.H., Tromp, M.C. and Van Boom, J.H. (1983) in: Genetic Rearrangement, Chater, K.F., Cullis, C.A., Hopwood, D.A., Johnston, A.A.W.B. and Woolhouse, H.W. Eds., pp. 207-233, Croom Helm, London.
9. Campbell,D.A., Thorntcn, D.A. and Boothroyd, J.C. (1984) Nature 311: 350-355.
10. De Lange, T., Berkvens, T.M., Veerman, H.J.G., Frasch, A.C.C., Barry, D.J. and Borst, P. (1984) Nucl. Acids Res. 12: 4431-4443.
11. De Lange, T., Liu, A.Y.C., Van der Ploeg, L., Borst, P., Tromp, M.C. and Van Boom, J.H. (1983) Cell 34: 891-900.
12. Dorfman, D.M. and Donelson, J.E. (1984). Nucl. Acids Res. 12: 4907-4920.
13. Milhausen, M., Nelson, R.G., Sathers, S., Selkirk, M. and Agabian, N. (1984) Cell 38: 721-729.
14. Nelson, R.G., Parsons, M., Barr, P.J., Stuart, K., Selkirk, M. and Agabian, N. (1983) Cell 34: 901-909.
15. Kooter, J.M., De Lange, T. and Borst, P. (1984) EMBO J. 3: 2387-2392.
16. Kooter, J.M. and Borst, P. (1984) Nucl. Acids Res. 12: 9457-9472.
17. Van der Ploeg, L.H.T., Cornelissen, A.W.C.A., Michels, P.A.M. and Borst, P. (1984) Cell 39: 213-221.
18. Banerjee, A.K. (1980) Microbiol. Rev. 44: 175-205.
19. Filipowicz, W. (1978) FEBS Letters 96: 1-11.
20. Shatkin, A.J. (1976) Cell 9: 645-653.
21. Fraenkel-Conrat, H. and Steinschneider, A. (1967) Methods in Enzymol. 12B: 243- 246.
22. Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S. and Darnell, J.E.Jr. (1980) Cell 19: 69-78.
23. Cross, G.A.M. (1975) Parasitology 71: 393-417.
24. Fairlamb, A.H., Weislogel, P.O., Hoeijmakers, J.H.J. and Borst, P. (1978) J. Cell Biol. 76: 293-309.
25. Frasch, A.C.C., Borst, P. and Van den Burg, J. (1982) Gene 17: 197-211.
26. Michels, P.A.M., Van der Ploeg, L.H.T., Liu, A.Y.C. and Borst, P. (1984) EMBO J. 3: 1345-1351.
27. Thomashow, L.S., Milhausen, M., Rutter, W.J. and Agabian, N. (1983) Cell 32: 35-43.
28. Hoeijmakers, J.H.J., Borst, P., Van den Burg, J., Weissman, C. and Cross, G.A.M. (1980) Gene 8: 391-417.
29. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77: 5201.
30 Monroy, G., Spencer, E. and Hurwitz, J. (1978) J. Biol. Chem. 253: 4490-4498.
31. Cordingley, J.S. and Turner, M.J. (1980) Mol. Biochem. Parasit. 1: 91-96.
32. Hasan, G., Turner, M.J. and Cordingley, J.S. (1984) Gene 27: 75-86.

33. Busch, H., Reddy, R., Rothblum, L. and Choi, Y.C. (1982) Ann. Rev. Biochem. 51: 617-654.
34. Bernards, A., Kooter, J.M. and Borst, P. (1985) Mol. Cell Biol. 5:545-553.
35. De Lange, T. (1985) Thesis, University of Amsterdam, The Netherlands.
36. Seebeck, T., Whittaker, P.A., Imboden, M.A., Hardman, N. and Braun, R. (1983) Proc. Natl. Acad. Sci. USA 80: 4634-4638.