The EMBO Journal vol.7 no.8 pp.2539 - 2545, 1988

Physical identification of branched intron side-products of splicing in *Trypanosoma brucei*

David Ralph[1], Jin Huang and Lex H.T.Van der Ploeg

Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

[1]Present address: Department of Biochemistry, University of Massachusetts Medical School, Worcester, MA 01655, USA

Communicated by H.Eisen

Every mRNA in trypanosomes consists of two exons, a common 5′ capped mini-exon or spliced leader and a coding-exon. All evidence suggests that the exons are joined by *trans*-splicing of two individual precursor RNAs, the mini-exon donor RNA or spliced leader precursor RNA (medRNA) and the pre-mRNA. We studied intermediates of the splicing reaction using denaturing two-dimensional PAGE and structurally identified a group of small (~180–300 nt) non-polyadenylated, Y-shaped branched RNAs. The branched Y-shaped RNAs contain the 105 nt medRNA derived intron, joined in a 2′–5′ phosphodiester bond to small heterogeneously sized RNAs. These non-polyadenylated branched Y-shaped RNA molecules are analogous to the lariat shaped introns of higher eukaryotes and presumably represent the released intron-like by-products of a *trans*-splicing reaction which joins the mini-exon and the major coding-exon.

Key words: branched RNA/discontinuous transcription/*trans*-splicing/*Trypanosoma brucei*

Introduction

Every mRNA identified so far in trypanosomes consists of two exons, a common 5′ mini-exon or spliced leader and a protein coding exon. These exons are transcribed from separate genes and are joined at a later stage (for review see Borst, 1986; Van der Ploeg, 1986). The 5′ common mini-exon was first analyzed by primer extension and estimated to be 35 nt (Van der Ploeg *et al.*, 1982; Boothroyd and Cross, 1982). However, direct sequence analysis of the mini-exon RNA showed that another four highly modified nucleotides, not identifiable by primer extension, exist at the immediate 5′ end of the mini-exon, which therefore measures 39 nt (Freistadt *et al.*, 1987).

An abundance of evidence suggests that the joining of mini-exon and coding-exon occurs through the *trans*-splicing of independently transcribed precursor RNAs. First, the capped mini-exon has been shown to be derived from a 200-fold repetitive gene whose transcription generates a 140 nt mini-exon donor (medRNA) or spliced leader precursor RNA (De Lange *et al.*, 1983; Nelson *et al.*, 1983; Kooter *et al.*, 1984; Campbell *et al.*, 1984). Second, the tandem arrays of medRNA genes and several protein coding genes have been shown to be located on different chromosomes. These genes must therefore be transcribed separately, and the joining of the exons presumably occurs at a post-transcriptional level (Van der Ploeg *et al.*, 1984; Guyaux *et al.*, 1985). Third, some protein coding genes in trypanosomes are transcribed by RNA polymerases that are insensitive to alpha-amanitin, while the medRNA genes are transcribed by RNA polymerases that are sensitive to alpha-amanitin (Kooter and Borst, 1984). These data suggest that even different RNA polymerases can generate the individual precursor RNAs (Shea *et al.*, 1987). Fourth, the presence of the canonical splice-donor, splice-acceptor sequences (GT–AG; Mount, 1982) at the exon–intron boundary (of the medRNA) and the intron–exon boundary (of the coding transcript) implies that the exons are joined by RNA splicing, as if the RNAs were part of a single pre-mRNA in which the exons are separated by a co-linear intron (see Figure 1). Fifth, evidence against priming-ligation models for mini-exon addition has been obtained from the determination of the half-life of putative high mol. wt splice intermediates, which appears too short to support the priming/ligation models (Laird *et al.*, 1987). Finally, evidence in support of *trans*-splicing has been obtained from the analysis of predicted splicing intermediates. Splicing of exons that are joined in *cis* involves the formation of a branched lariat intron which is released, as a by-product of the splice reaction (Ruskin *et al.*, 1984; Zeitlin and Efstratiadis, 1984). In *trans*-splicing, however, separate transcripts containing the 5′ and 3′ exons are each flanked by intron-like sequences (Figure 1). The medRNA derived 5′ half-intron, which only contains the GT consensus sequence of the splice donor site, and the pre-mRNA derived 3′ half-intron, which only contains the AG consensus sequence of the splice-acceptor site, will be referred to as the 5′ donor- and 3′ acceptor-introns, respectively. Predicted intermediates and by-products of a *trans*-splicing reaction that is mechanistically similar to *cis*-splicing are: branched polyadenylated pre-mRNAs, containing the 3′ acceptor-intron, 3′ exon and, linked in a 2′–5′ phosphodiester bond, the 105 nt medRNA derived 5′ donor-intron; and the released donor- and acceptor-introns joined in a branched Y-shaped RNA molecule (see Figure 1). The latter should accumulate as side products of the splicing reaction. Indirect evidence obtained by Murphy *et al.* (1986) and Sutton and Boothroyd (1986) suggested the presence of the polyadenylated branched Y-shaped pre-mRNAs. These investigators showed that after de-branching of putative polyadenylated pre-mRNAs a free 105 nt RNA molecule accumulates. In order to define the mechanism of *trans*-splicing in *Trypanosoma brucei* we determined whether or not free branched Y-shaped donor- and acceptor-intron by-products of the splicing reaction exist and we wished to physically identify and prove the existence of Y-shaped (lariat-like) RNA molecules (Figure 1).

We show here that a small group of non-polyadenylated branched Y-shaped RNAs exist in trypanosomes. The free

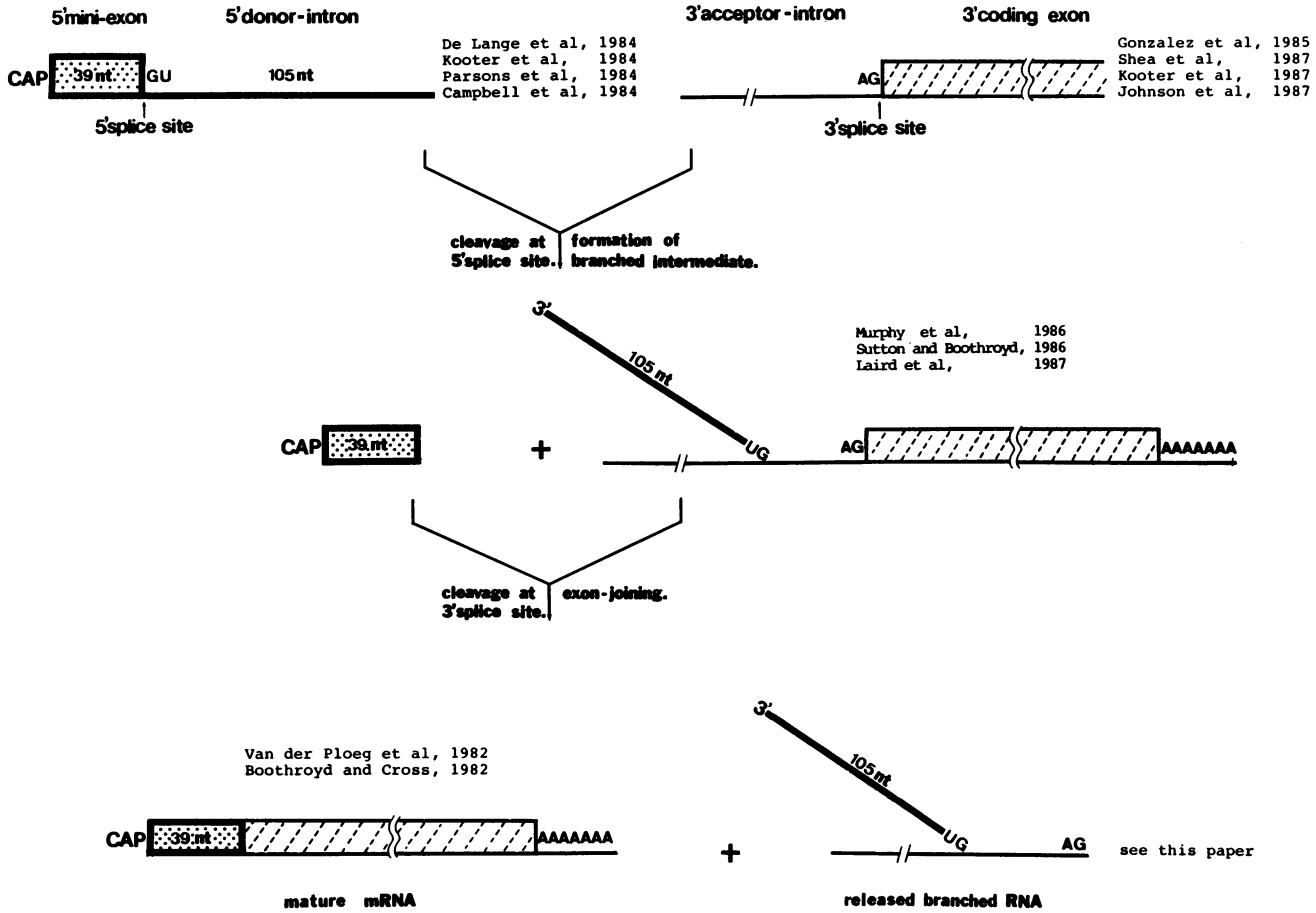

Fig. 1. Schematic representation of intermediates involved in *trans*-splicing of pre-mRNA in *T.brucei*. Mini-exon and coding-exon joining in *T.brucei* is mediated by RNA splicing of two separately transcribed RNA precursors (the medRNA indicated on the left and the pre-mRNA with the protein coding-exon indicated on the right hand side). Cleavage at the 5′ splice site of the medRNA (left hand top panel) generates a 39 nt 5′ mini-exon (represented by the dotted boxes) and releases the 105 nt 5′ donor-intron. Branched splicing intermediates that are generated presumably contain the entire 105mer 5′ donor-intron joined in a 2′−5′ phosphodiester bond to polyadenylated pre-mRNAs that contain the coding exon (middle panel). GU and AG represent the first two nucleotides of the medRNA derived 5′ donor-intron and the last two nucleotides of the 3′ acceptor-intron that precedes the coding-exon respectively.

structured RNA molecules are small and consist of the 5′ donor-intron, joined to short (~80−200 nt) RNAs via a 2′−5′ phosphodiester bond. These short non-polyadenylated RNAs are presumably the remainders of the 3′ acceptor-introns of the protein coding pre-mRNAs, or the remainders of the intergenic regions of the polycistronic trypanosome pre-mRNAs.

Results

Identification of structured RNAs

We wished to physically identify and thus prove the existence of branched Y-shaped RNA molecules in trypanosomes. To this end trypanosome RNA was size fractionated using two-dimensional (2-D) denaturing polyacrylamide gel electrophoresis (PAGE). The two dimensions were 4% and 8% polyacrylamide gels, respectively. Linear RNA molecules are separated entirely on the basis of their mol. wt, regardless of the polyacrylamide concentration. The relative mobilities of linear RNAs are therefore unaffected by polyacrylamide concentration and the linear RNA molecules form a diagonal in these 2-D gels (Figure 2A). However, covalently structured RNAs such as circular RNA molecules (and lariats) and other structured RNA molecules (such as branched Y-shaped RNAs) will not migrate solely according to their size, but are retarded more severely due to their non-linear structure (Knapp *et al.*, 1979; Grabowski *et al.*, 1984; Zeitlin and Efstratiadis, 1984). This effect becomes more pronounced with increasing polyacrylamide concentration. Therefore, structured RNA molecules migrate much slower in 8% polyacrylamide than the linear RNAs with which they co-migrated in 4% polyacrylamide.

To identify structured RNA molecules that contain the medRNA derived 105 nt 5′ donor-intron, Northern blots of 2-D gels of total trypanosome RNA were hybridized with a 5′ mini-exon and a 5′ donor-intron specific probe, respectively (see Figure 2 for location of probes). Figure 2C shows the hybridization of the 2-D Northern blot with an oligonucleotide complementary to the 39 nt 5′ mini-exon. Only linear RNA molecules that migrate along the diagonal are detected. Relatively small (<800 nt) highly structured RNA molecules that contain the 39 nt mini-exon are therefore not detectable in *T.brucei* total RNA. One of the predictions about *trans*-splicing reactions is that branched Y-shaped polyadenylated pre-mRNAs as well as branched Y-shaped donor- and acceptor-introns should exist. These molecules should specifically hybridize with a 5′ donor-intron specific probe. We, therefore, hybridized the 2-D gels with an oligonucleotide specific for the 105 nt medRNA derived 5′ donor-intron (Figure 2B) and a 105mer 5′ donor-intron anti-sense

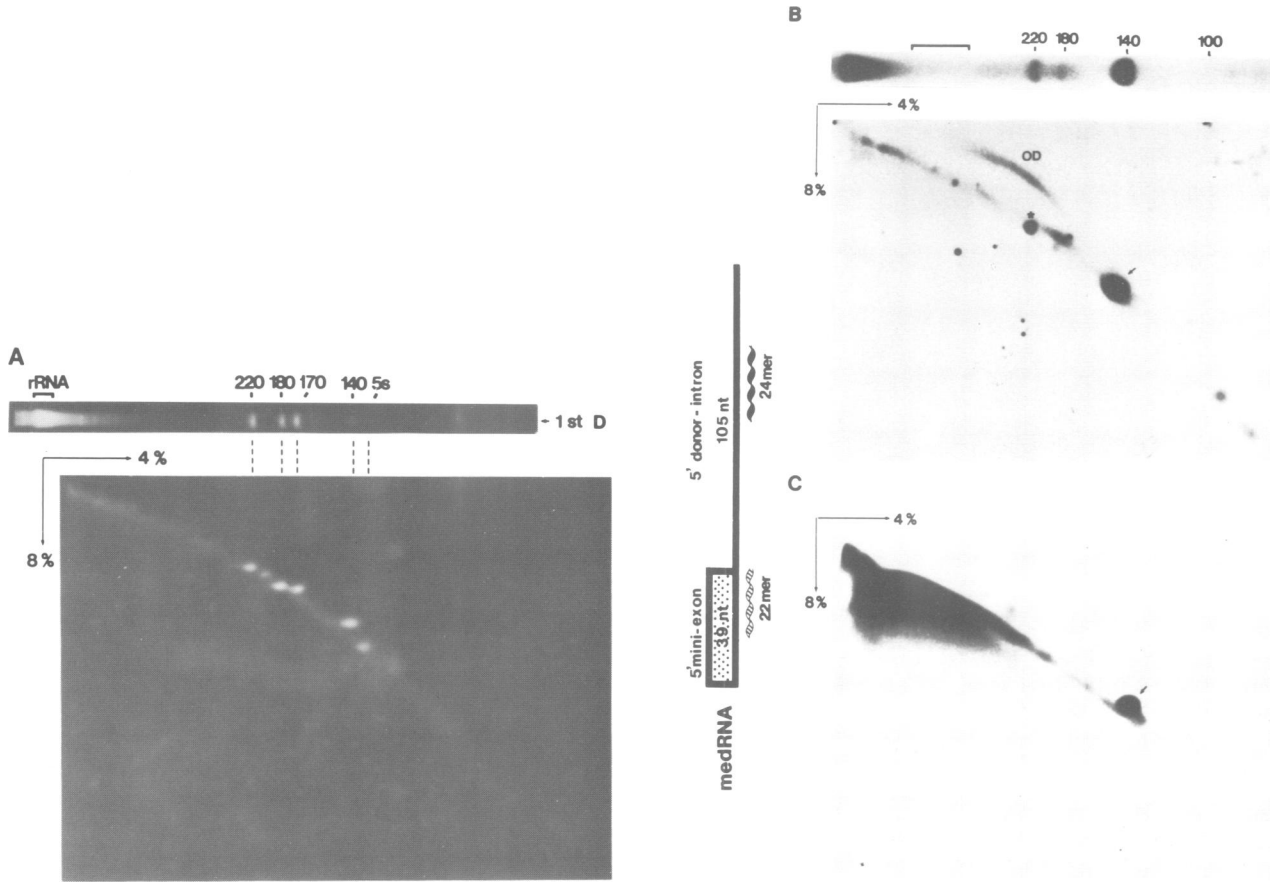

Fig. 2. Analysis of total trypanosome RNA by 2-D gel electrophoresis. The 2-D gel system used in the study consisted of 4% polyacrylamide/8.5 M urea and 8% polyacrylamide/8.5 M urea, first and second electrophoretic dimensions respectively. (**A**) Ethidium bromide staining pattern of the first dimension (4% polyacrylamide, top panel) and two dimensionally fractionated total trypanosome RNA (variant 118a, bottom panel). The mol. wts are based on the sizes of the small rRNA molecules. (**B**) Size fractionated total RNA was electro-blotted onto Genescreen Plus membranes. Hybridization with a 5′ donor-intron specific oligonucleotide (indicated by the black wavy line in the schematic representation of the medRNA on the left) was as described in Materials and methods. The off-diagonal RNA species are marked 'OD'. The 140 nt medRNA is marked with an arrow. The asterisk highlights a non-specifically hybridizing 220 nt rRNA. (**C**) Hybridization pattern of total trypanosome RNA with a 5′ mini-exon specific probe (22mer oligonucleotide indicated by the light wavy line in the schematic representation of the medRNA on the left hand side).

RNA probe (data not shown). In the 2-D gels a discrete but relatively faint, off-diagonal line of hybridization (indicated 'OD' in Figure 2B) can now be identified which must represent a population of rare structured RNA molecules, that migrate more slowly than their linear counterparts. In addition to the hybridization at the off-diagonal line, the 140 nt medRNA (indicated with arrow) and a 220 nt RNA species are also detected with this probe. The latter RNA species presumably is an abundant rRNA which migrates at that position and cross-hybridizes with the oligonucleotide probe (see RNA indicated with asterisk in Figure 2A and B).

Several features characterize the hybridization signal in the off-diagonal line. First, the hybridization is specifically observed with the 5′ donor-intron probe and not with the 5′ mini-exon probe. Its aberrant migrational behavior therefore does not result from poor denaturation of 140 nt medRNA transcripts, since these would be detected with both probes. Second, the apparent size of these RNA species varies from 180 to ~300 nt. Even though the size of these structured RNA molecules cannot be accurately determined by gel-electrophoresis, the more accurate estimates are obtained in the low percentage (4%) polyacrylamide gels. The values are however biased and represent an overestimation of the actual size of these RNA molecules. The hybridization signal with the 39mer mini-exon specific probe shows that larger RNA (>300 nt) is present on the filter but these RNA molecules all migrate at the diagonal. Larger structured RNAs (between 300 and ~800 nt) are therefore rare. In the hybridization of the 4% polyacrylamide gel (first dimension only), we can see that the 5′ donor-intron probe indeed detects a smear of hybridization between 180 and 300 nt specifically. The hybridization signal fades out in the high mol. wt range (indicated between brackets) and resumes again at ~800 nt. The larger RNA molecules (>800 nt) that hybridize with the 5′ donor-intron probe cannot be studied in this gel-electrophoresis system since they migrate only short distances in 8% polyacrylamide gels. The large RNAs (>700 nt) are therefore difficult to visualize since they do not transfer well from the 8% polyacrylamide gel to the hybridization membranes. In addition, the contribution of short branches (105 nt) is expected to affect the migrational behavior of larger RNA molecules less severely.

Debranching of the structured RNAs

We wished to determine whether the structured RNAs that hybridized with the 5′ donor-intron specific probe were

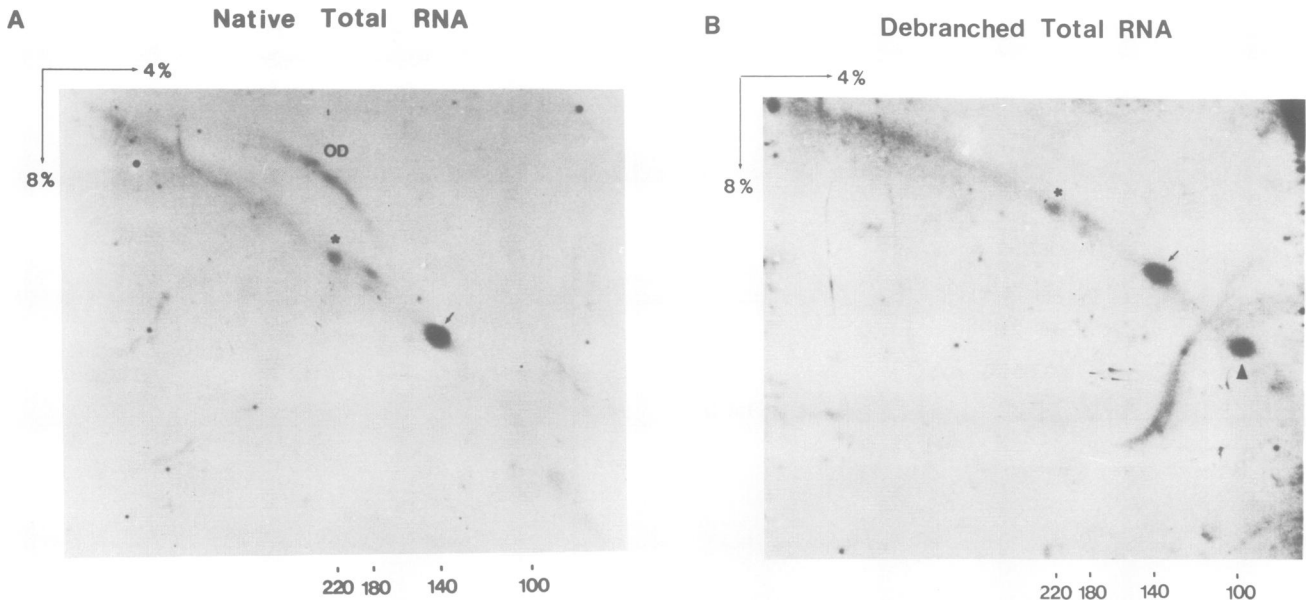

Fig. 3. Debranching of total trypanosome variant 118a RNA. (**A**) Hybridization pattern of native total RNA separated by 2-D gel-electrophoresis with a probe specific for the 5′ donor-intron. (**B**) 2-D hybridization pattern of total variant 118a RNA which has been pre-treated with a HeLa cell nuclear extract with debranching activity. The probe used was the 24mer 5′ donor-intron specific oligonucleotide. The 140 nt medRNA is indicated with an arrow. The branched 5′ donor-intron containing RNA molecules is indicated at the off-diagonal line ('OD'). The 220 nt rRNA is indicated with an asterisk, and the released 5′ donor-intron (105 nt) is indicated with an arrowhead.

sensitive to a HeLa cell nuclear extract with debranching activity. If so, then these RNAs would most likely contain the 105 nt 5′ donor-intron joined via a 2′−5′ phosphodiester bond, with other small RNAs. We separated untreated native trypanosome RNA and RNA that had been treated with a HeLa cell nuclear extract containing debranching activity using 2-D gel-electrophoresis and hybridized the Northern blots of these gels with the 5′ donor-intron probe. Figure 3 shows the control 5′ donor-intron hybridization pattern on native RNA (Figure 3A) and the hybridization pattern of RNA that has been treated with the HeLa cell extract with debranching activity (Figure 3B). Again, in the 2-D gel of native RNA an off-diagonal line of hybridization can specifically be found with the 5′ donor-intron probe. However, after debranching the hybridization at the off-diagonal line is no longer detected and a strong spot of hybridization at 105 nt (indicated with arrowhead) is now found as a result of the release of the 105 nt 5′ donor-intron. The 105 nt 5′ donor-intron has been released from larger RNAs due to debranching, thus explaining the increase in hybridization intensity at 105 nt. Since other, larger (>700 nt) RNAs that hybridize with the 5′ donor-intron specific probe exist as well (see first dimension in Figure 2B) it is not possible to prove that the 105 nt spot is derived from release of the 5′ donor-intron from the RNA that migrates at the off-diagonal line only. However, since the hybridization is now confined to the 105 and 140 nt hybridizing spots the release of the 105 nt 5′ donor-intron from RNA molecules >700 nt and the RNA molecules between 180 and 300 nt led to the increased hybridization intensity at 105 nt.

The off-diagonal molecules migrated in a discrete line. From the analysis of lariats in higher eukaryotes it has become clear that artificially generated branched Y-shaped RNAs that are identical in mol. wt but differ in the lengths of two of the three arms of the Y-shaped RNA molecules will migrate with identical apparent mol. wt (Zeitlin and Efstratiadis, 1984). The fact that the heterogeneous pool of structured RNA molecules in trypanosomes migrate at a discrete line in the second dimension thus indicates that all the structured RNAs that co-migrated in any particular size class are indeed of identical mol. wt. Since these RNAs most likely contain the entire 105 nt 5′ donor-intron joined in a 2′−5′ phosphodiester bond to 3′ acceptor-introns, they are expected to vary in the size of the 3′ acceptor-intron only. Roughly, the sizes of the 3′ acceptor-introns thus range from 80 (180−105) to 200 nt (300−105). It is unclear whether the position of the branchpoint in these Y-shaped RNAs is fixed with respect to the 3′ splice site.

The branched Y-shaped RNAs are not polyadenylated

The presence of branched Y-shaped splicing intermediates has been proposed by Murphy *et al.* (1986) and Sutton and Boothroyd (1986). In their studies, putative branched Y-shaped polyadenylated pre-mRNAs were treated with debranching extracts, which resulted in the release of the 105 nt 5′ donor-intron. Since the structured RNA molecules that we identified are very short (180−300 nt), they most likely do not represent polyadenylated pre-mRNA species. We therefore tested whether or not they could be the released branched Y-shaped donor- and acceptor-intron by-products of the splicing reaction. In essence this was done on the basis of their ability to be retained on oligo(dT) cellulose columns (Figure 4) and by their size reduction after treatment of oligo(dT)−poly(A) hybrids with RNase H (data not shown). Even though the poly(A) tails from control genes (VSG genes) were readily removed by RNase H, the enzyme did not affect the size of the structured RNAs in the off-diagonal line (data not shown). To obtain further proof that the RNA is not polyadenylated, we fractionated *T.brucei* RNA by oligo(dT) column chromatography. Since the medRNA is often retained in oligo(dT) selected RNA, we assumed that

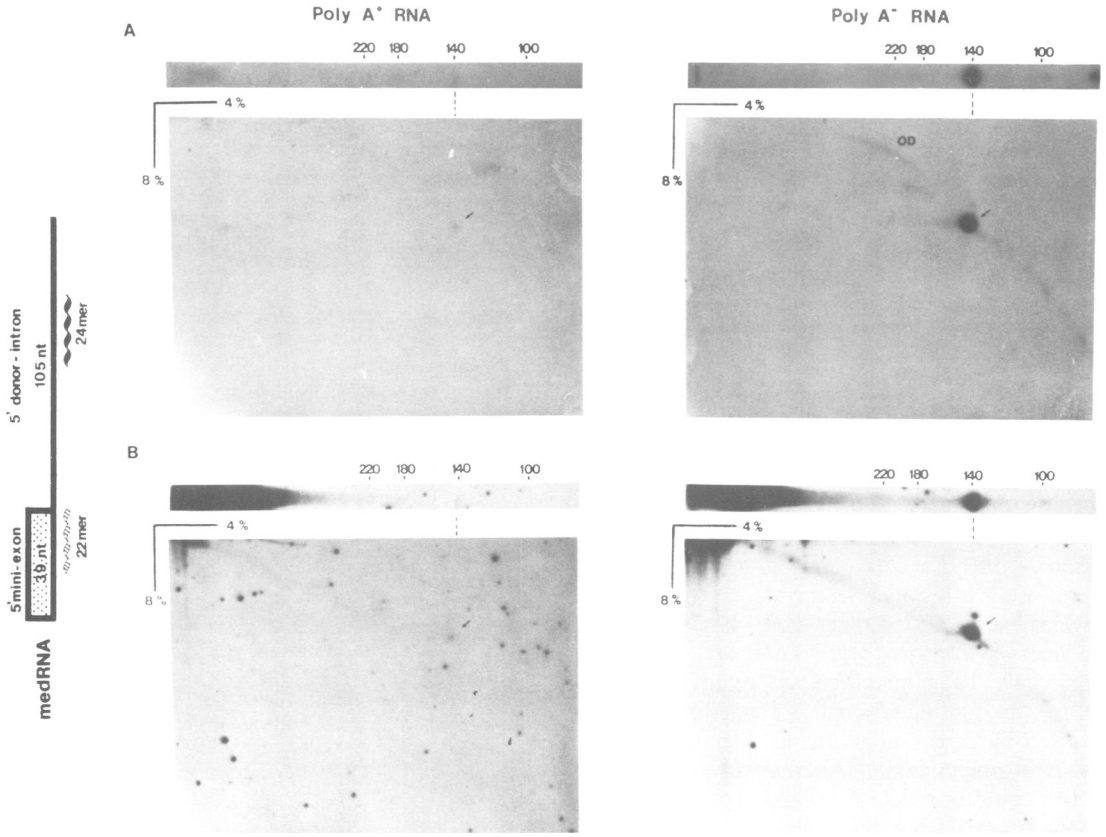

Fig. 4. 2-D separation patterns in poly(A)$^+$ and poly(A)$^-$ RNA. Total RNA (variant 118a) was fractionated in an oligo (dT) cellulose column as described in Materials and methods. (**A**) Left and right hand panels show the hybridization patterns of poly(A)$^+$ and poly(A)$^-$ RNA respectively, in the 4% polyacrylamide first dimension (top panel) and 2D gels (bottom panels). The probe used was a 5′ donor-intron specific 24mer, represented by a black wavy line in the schematic indicated on the left hand side. (**B**) The same filters bearing poly(A)$^+$ and poly(A)$^-$ RNA were rehybridized with a mini-exon specific probe (indicated by the light wavy line in the schematic representation of the medRNA on the left hand side of the figure).

this was due to its association or trapping in poly(A) fractionated RNA. To circumvent this non-specific selection, we first denatured RNA in formamide preceding its column fractionation. The poly(A)$^+$ and (A)$^-$ RNA fractions were next separated by 1-D and 2-D PAGE. In Figure 4B, the hybridization with the 39 nt 5′ mini-exon specific probe is shown. As expected only in the poly(A)$^-$ fraction can we detect a signal of the non-polyadenylated 140 nt medRNA (indicated with arrow). The parallel filters of the 4% polyacrylamide first dimensions are shown as controls, aligned on top of the 2-D gels. In these gels the larger (abundant) polyadenylated 105 nt 5′ donor-intron containing RNAs are only visible in the first dimension of the 2-D gel-electrophoresis (Figure 4B). A faint signal of hybridization of residual 140 nt medRNA can still be detected in the poly(A)$^+$ RNA (indicated with dotted line and arrow). Note that since the high mol. wt RNA does not migrate into 8% polyacrylamide its hybridization intensity is drastically reduced in the 2-D gels.

These same filters were next hybridized with a probe that is specific for the 5′ donor-intron. Only in the (A)$^-$ fraction could the structured 180−300 nt RNA molecules be detected (Figure 4A right hand panel, as indicated by the off-diagonal line). An obvious difference in the intensity of hybridization of the 5′ donor-intron containing larger RNA (>700 nt) in the first dimensions of Figure 2B and Figure 4A (top left hand panel) results from the use of different RNA preparations. We have found that different batches of RNA vary in the actual amount of 5′ donor-intron containing large (>700 nt) poly(A)$^+$ RNA.

By using 2-D gel-electrophoresis we thus identified a class of small (180−300 nt) non-abundant structured non-polyadenylated RNAs. These RNAs contain the 105 nt 5′ donor-intron joined via a 2′−5′ phosphodiester bond to the putative 3′ acceptor-introns of protein coding genes. The branched RNAs therefore most likely represent the free donor- and acceptor-intron sequences, released as side-products of the splicing reaction.

Discussion

mRNAs in trypanosomes are derived from the discontinuous transcription of two separate genes. The medRNA gene encodes the capped common 5′ mini-exon, found on all mRNAs in trypanosomes. Many of the pre-mRNAs of the protein coding genes may be polycistronic but only one transcription initiation site of a polycistronic transcript has so far been identified (Shea *et al.*, 1987). To understand the joining of the mini-exon and the protein coding-exon, several of the splicing intermediates have been characterized (Figure 1). Here we present evidence for the existence of non-polyadenylated branched Y-shaped free intron-like side products of the splicing reaction, of relatively small size (≤180−300 nt). The identification of these Y-shaped RNA molecules provides strong evidence supporting *trans*-splicing, since the Y-shaped RNAs are the predicted side products of the reac-

tion (see Figure 1). All attempts to use 3′ acceptor-intron specific probes of protein coding genes to identify the released Y-shaped intron side products of splicing (hsp70, tubulin, VSG 118 clone 1) and to locate their branch points have failed thus far, presumably due to their extremely low abundance. Their low abundance has also made size fractionation and isolation of these RNAs for further analysis difficult.

Discontinuous transcription results in the generation of distinct RNA precursors that are joined post-transcriptionally. All intermediates of the splicing reaction that have been identified in trypanosomes so far, resemble those of the *cis*-splicing process in higher eukaryotes. A major difference is that the precursor RNAs with the 5′ and 3′ exons are discontinuously transcribed and are apparently never joined to form a single co-linear precursor (since the resulting intermediates are Y-shaped). It will be of interest to determine the mechanism by which the two different RNA precursors are brought together in the splicing complex.

Transcription of mRNAs in trypanosomes, unlike other eukaryotes, can occur in a polycistronic manner (Gonzalez *et al.*, 1985; Shea *et al.*, 1987; Kooter *et al.*, 1987; Johnson *et al.*, 1987). Since the intergenic regions of the polycistronic transcripts that have been identified are relatively short (~200–400 nt), the finding of small Y-shaped structured RNAs may reflect the preferential organization of trypanosome genes into polycistrons. Alternatively, the free branched RNAs could originate from splicing at the very 5′ ends of polycistronic RNA molecules.

It is not clear how the processing of polycistronic transcripts which results in the release of the donor- and acceptor-introns can occur, but two mechanisms can be considered: the first mechanism predicts that, if polycistronic transcripts are efficiently processed, utilizing each gene of the transcript, it may be required that poly(A) addition and splicing are coupled events. Coupling these processes will prevent a situation in which cleavage of a polycistronic RNA, to allow poly(A) addition, results in the accumulation of uncapped pre-mRNAs, with a limited half-life. The second mechanism predicts that if splicing can precede poly(A) addition splicing intermediates may be generated that carry the branched 105 nt 5′ donor-intron at the 3′ end of the pre-mRNA of the preceding gene. The fact that we cannot identify a population of larger (>700 nt) poly(A)$^-$ RNA molecules (see 4% first dimension in Figure 4A) that hybridizes with the 5′ donor-intron specific probe indicates that such RNAs are either short lived or that they do not exist at all.

The free branched Y-shaped by-products of the splicing reaction may thus have been released from polycistronic precursor RNA molecules as a result of two events: cleavage for 3′ end maturation and poly(A) addition followed by cleavage at the AG dinucleotide of the 3′ splice site and branch formation.

Materials and methods

Propagation of trypanosomes

Bloodstream form trypanosomes were propagated in Wistar rats (250 g). The rats were pre-exposed to 600 rads gamma irradiation to compromise their immune system. All trypanosomes used in this study were either variant 118 clone 1 (Lee and Van der Ploeg, 1987) or variant 118a (Cross, 1975; Hoeijmakers *et al.*, 1980; Michels *et al.*, 1983). All trypanosomes were collected by heart puncture of infected animals. The blood was supplemented with 0.37% sodium citrate to prevent clotting. Erythrocytes and trypanosomes were separated by centrifugation after which the buffy coat containing trypanosomes was collected.

2-D gel-electrophoresis

RNA was harvested from trypanosomes by resuspending the trypanosome pellets in 10 volumes of 6 M urea, 3 M LiCl followed by homogenization with a polytron for 90 s at 0°C. After a 12 h incubation at 0°C, the RNA was collected by centrifugation at 20 000 r.p.m. in an SW28 ultracentrifuge rotor. The RNA was prepared for electrophoresis by resuspending it in 10 μl of deionized formamide followed by heating to 55°C for 5 min. Tracking dyes were added to the RNA and it was electrophoresed in a denaturing polyacrylamide gel (8.5 M urea) buffered with 80 mM Mops (pH 7.0), 1.0 mM EDTA, 10 mM sodium acetate. For the second dimension, the lane containing RNA was cut from the 4% gel, rotated 90° and an 8% gel was cast around the 4% lane. After electrophoresis the RNA was electroblotted to Genescreen Plus (NEN).

Hybridization probes

Oligonucleotides with sequences complementary to either the 5′ mini-exon (22mer, position nt 17–39) or to the 5′ donor-intron (24mer, position nt 90–114) were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). The kinased ^{32}P-labeled oligonucleotides were hybridized to the Genescreen blots of trypanosome RNA in 5% dextran sulfate, 5 × Denhardt's, 0.1% Na pyrophosphate, 0.75 M NaCl, 0.15 M Tris–HCl (pH 7.0), 0.1 M Na phosphate, 0.2% SDS. Hybridizations were carried out at 40°C for 24 h. The blots were washed in 3 × SSC, 1% SDS at 40°C for ~2 h and put up for autoradiography.

Oligo(dT) column fractionation

RNA pellets were dissolved in 100% formamide, incubated at 55°C for 10 min prior to being loaded on the oligo(dT) cellulose column. Fractionation of poly(A)$^+$ RNA was done essentially as described by Maniatis *et al.* (1982).

Debranching of RNA

HeLa cell nuclear extracts with *in vitro* splicing activity were a gift from Dr A.Efstratiadis. Total trypanosome RNA (20 μg) was ethanol precipitated and resuspended in 16 μl H_2O. Next, 1 μl 100 mM DTT, 1.5 μl 500 mM EDTA and 30 μl of HeLa nuclear cell extract (Dignam *et al.*, 1983; Zeitlin *et al.*, 1987) supplemented with 60 U RNasin. These HeLa cell nuclear extracts do not splice eukaryotic pre-mRNAs in the absence of ATP and Mg^{2+}. These conditions do however allow hydrolysis of 2′–5′ phosphodiester bonds of RNAs that are branched by-products of RNA splicing reactions (Ruskin and Green, 1985).

Acknowledgements

We thank all colleagues from the laboratory for critical reading of the manuscript and Argiris Efstratiadis for gifts of HeLa cell nuclear extracts. We also thank Scott Zeitlin, John Welsh, Bob Wilson, Pierre Jelenc and Annette Parent for discussion. This work was supported by a grant from the John D. and Catherine T.MacArthur Foundation and by NIH grant AI21784.

References

Boothroyd,J.C. and Cross,G.A.M. (1982) *Gene*, **20**, 281–289.
Borst,P. (1986) *Annu. Rev. Biochem.*, **55**, 701–732.
Campbell,D.A., Thornton,D.A. and Boothroyd,J. (1984) *Nature*, **311**, 350–355.
Cross,G.A.M. (1975) *Parasitology*, **71**, 393–417.
Krause,M. and Hirsch,D. (1987) *Cell*, **49**, 753–761.
De Lange,T., Liu,A.Y.C., Van der Ploeg,L.H.T., Tromp,M.C. and Van Boom,J.H. (1983) *Cell*, **34**, 891–900.
Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
Freistadt,M.S., Cross,G.A.M., Branch,A.D. and Robertson,H.D. (1987) *Nucleic Acids Res.*, **15**, 9861–9879.
Glass,D.J., Polvere,R.I. and Van der Ploeg,L.H.T. (1986) *Mol. Cell. Biol.*, **6**, 4657–4666.
Gonzalez,A., Lerner,T.J., Huecas,M., Sosa-Pineda,B., Nogueira,N. and Lizardi,P. (1985) *Nucleic Acids Res.*, **13**, 5789–5804.
Grabowski,P.J., Padgett,R.A. and Sharp,P.A. (1984) *Cell*, **37**, 415–427.
Guyaux,M., Cornelissen,A.W.C.A., Pays,E., Steinert,M. and Borst,P. (1985) *EMBO J.*, **4**, 995–998.

Hoeijmakers,J.H.J., Borst,P., Van der Burg,J., Weissmann,C. and Cross, G.A.M. (1980) *Gene*, **8**, 391–417.
Johnson,P.J., Kooter,J.M. and Borst,P. (1987) *Cell*, **51**, 273–281.
Knapp,G., Ogden,R.C., Peebles,C.C. and Abelson,J. (1979) *Cell*, **18**, 37–45.
Kooter,J.M. and Borst,P. (1984) *Nucleic Acids Res.*, **12**, 9457–9472.
Kooter,J.M., De Lange,T. and Borst,P. (1984) *EMBO J.*, **3**, 2387–2392.
Kooter,J.M., Van der Spek,H.J., Wagter,R., d'Oliveira,C.E., Van der Hoeven,F., Johnson,P.J. and Borst,P. (1987) *Cell*, **51**, 261–272.
Laird,P.W., Zomerdijk,J.C.B.M., de Korte,D. and Borst,P. (1987) *EMBO J.*, **6**, 1055–1062.
Lee,M.G.-S. and Van der Ploeg,L.H.T. (1987) *Mol. Cell. Biol.*, **7**, 357–366.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Michels,P.A.M., Liu,A.Y.C., Bernards,A., Sloof,P., Van der Bijl, M.M.W., Schinkel,A., Menke,H.H., Borst,P., Veeneman,G.H., Tromp, M.C. and Van Boom,J.H. (1983) *J. Mol. Biol.*, **166**, 537–556.
Mount,S. (1982) *Nucleic Acids Res.*, **10**, 459–472.
Murphy,W.J., Watkins,K.P. and Agabian,N. (1986) *Cell*, **47**, 517–525.
Nelson,R.G., Parsons,M., Barr,P.J., Stuart,K., Selkirk,M. and Agabian,N. (1983) *Cell*, **34**, 901–909.
Parsons,M., Nelson,R.G., Watkins,K.P. and Agabian,N. (1984) *Cell*, **38**, 309–316.
Ruskin,B. and Green,M.R. (1985) *Science*, **229**, 140–145.
Ruskin,B., Krainer,A.R., Maniatis,T. and Green,M.R. (1984) *Cell*, **38**, 317–331.
Shea,C., Lee,M.G.-S. and Van der Ploeg,L.H.T. (1987) *Cell*, **50**, 603–612.
Sutton,R.E. and Boothroyd,J.C. (1986) *Cell*, **47**, 527–535.
Van der Ploeg,L.H.T. (1986) *Cell*, **47**, 479–480.
Van der Ploeg,L.H.T., Liu,A.Y.C., Michels,P.A.M., De Lange,T., Borst,P., Majumber,H.K., Weber,H., Veeneman,G.H. and Van Boom, J.H. (1982) *Nucleic Acids Res.*, **10**, 3591–3604.
Van der Ploeg,L.H.T., Cornelissen,A.W.C.A., Michels,P.A.M. and Borst,P (1984) *Cell*, **39**, 213–221.
Zeitlin,S. and Efstratiadis,A. (1984) *Cell*, **39**, 589–602.
Zeitlin,S., Parent,A., Silverstein,S. and Efstratiadis,A. (1987) *Mol. Cell. Biol.*, **7**, 111–120.

Received on April 6, 1988; revised on April 29, 1988