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In vivo labelling of intermediates in the discontinuous synthesis of mRNAs in *Trypanosoma brucei*

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Discontinuous mRNA synthesis in trypanosomes is thought to involve a 140-nucleotide precursor, called the mini-exon-derived RNA or medRNA, which contributes its 5′ 35 nucleotides to the 5′ end of nascent mRNAs. We used *in vivo* labelling of RNA to show that medRNA has a half-life of <6 min, whereas putative high mol. wt intermediates containing the 3′ part of the medRNA have an average half-life of <1 min. This eliminates priming of pre-mRNA synthesis by intact medRNA as the main mode of discontinuous mRNA synthesis. Potential intermediates of 35 and 105 nucleotides were labelled in parallel with medRNA, but their significance could not be assessed in RNA preparations containing medRNA, as they are also produced by artefactual cleavage of medRNA. We show, however, that high mol. wt RNA, free of medRNA, can release medRNA segments upon a debranching treatment. These results are consistent with a *trans* splicing mechanism involving short-lived forked intermediates, analogous to lariats in *cis* splicing systems.

Key words: discontinuous transcription/*in vivo* labelling/RNA turnover/*trans* splicing/trypanosomes

Introduction

Trypanosoma brucei is a mammalian bloodstream parasite of the protozoan order of *Kinetoplastida*. Several unique biochemical features of kinetoplastid flagellates have surfaced in the past decade (see Opperdoes, 1985; Borst, 1986; Sogin *et al.*, 1986). One of these is the discontinuous synthesis of mRNAs, i.e. the joining of products of two transcription units into one mature mRNA (Campbell *et al.*, 1984; Kooter *et al.*, 1984; Kooter and Borst, 1984; Milhausen *et al.*, 1984; Guyaux *et al.*, 1985; Boothroyd, 1985; Borst, 1986).

A key role in discontinuous mRNA synthesis is played by a 35-nucleotide sequence first found at the 5′ end of the mRNA for variant-specific surface glycoproteins (Van der Ploeg *et al.*, 1982; Boothroyd and Cross, 1982) and later at the 5′ ends of all other trypanosome mRNAs as well (De Lange *et al.*, 1984a; Nelson *et al.*, 1984; Parsons *et al.*, 1984; Cornelissen *et al.*, 1986; Walder *et al.*, 1986). This 35-nucleotide sequence is encoded by separate mini-exons present in ~200 copies in the trypanosome genome (De Lange *et al.*, 1983). Each mini-exon is part of a 1.35-kb repeat unit and most of these mini-exon repeats are tandemly linked (De Lange *et al.*, 1983,1984b; Nelson *et al.*, 1983; Dorfman and Donelson, 1984).The main transcript of mini-exon repeats is 140 nucleotides long and carries a 5′ cap (Campbell *et al.*, 1984; Kooter *et al.*, 1984; Milhausen *et al.*, 1984; Laird *et al.*, 1985; Lenardo *et al.*, 1985a). This mini-exon-derived RNA (medRNA) is thought to transfer its mini-exon sequence to the 5′ end of pre-mRNAs, but how is unknown. As shown in the schematic diagrams in Figure 1, the medRNA could prime transcription upstream of the main exons or, alternatively, the linkage could occur post-transcriptionally by RNA-RNA ligation or by *trans* splicing between the medRNA and main exon precursor transcripts. The intermediates are depicted by analogy with nuclear mRNA precursor splicing systems of other eukaryotes (see Padgett *et al.*, 1986). It is likely that a splicing event is responsible for the final joining of the two RNA segments, since the mini-exon sequence is flanked at the 3′ end by GT (De Lange *et al.*, 1983) and all main exon sequences are preceded by AG (see Borst, 1986), in compliance with the conventional splice site consensus sequences (Mount, 1982). The pyrimidine-rich stretch upstream of splice acceptor sites in other eukaryotes is less prominent in trypanosomes, while a conserved sequence TTTCPy is found almost invariably in one or more copies within 30 bp upstream of trypanosome splice acceptor sites (P.W.Laird, unpublished results). No pre-mRNA transcription unit has been fully defined yet, but, in the few cases studied, these units appear to span several protein-coding regions, which lack conventional introns (see Borst, 1986).

Studies of the mechanism of discontinuous mRNA synthesis have been hampered by the limited available methodology. We have used *in vivo* labelling of RNA to study the turnover of medRNA, to track the fate of its 5′ and 3′ segments and to identify possible intermediates.

Results

[^{3}H]Adenosine labelling of trypanosomal ATP and RNA

Initial labelling experiments using inorganic ^{32}P gave poor incorporation into RNA, possibly because of the large internal polyphosphate pools in trypanosomes (Visser, 1981). As trypanosomes are unable to synthesize purines *de novo* (Hammond and Gutteridge, 1984) and are known to have a high affinity adenosine uptake system (James and Born, 1980), we used [^{3}H]adenosine as the most effective means of labelling trypanosome RNA. The amount of ^{3}H-labelled adenosine that we added was low, compared to the adenosine concentration in the growth medium so that perturbations of the system due to the addition of label were minimal (see Materials and methods). For short time periods, the system should approximate a steady-state situation.

The labelling kinetics of trypanosomal RNA and ATP are compared in Figure 2. The ATP pool, which was representative of the other adenine nucleotides, labelled rather slowly, reaching a peak after ~1 h. We have determined that this is not due to poor uptake of adenosine but to large internal pools of purines and of purine nucleosides and nucleotides (see Materials and methods). Equilibration of these large internal pools with the external adenosine pool eventually reduces the specific activity of the adenosine in the labelling medium almost 2-fold, which in

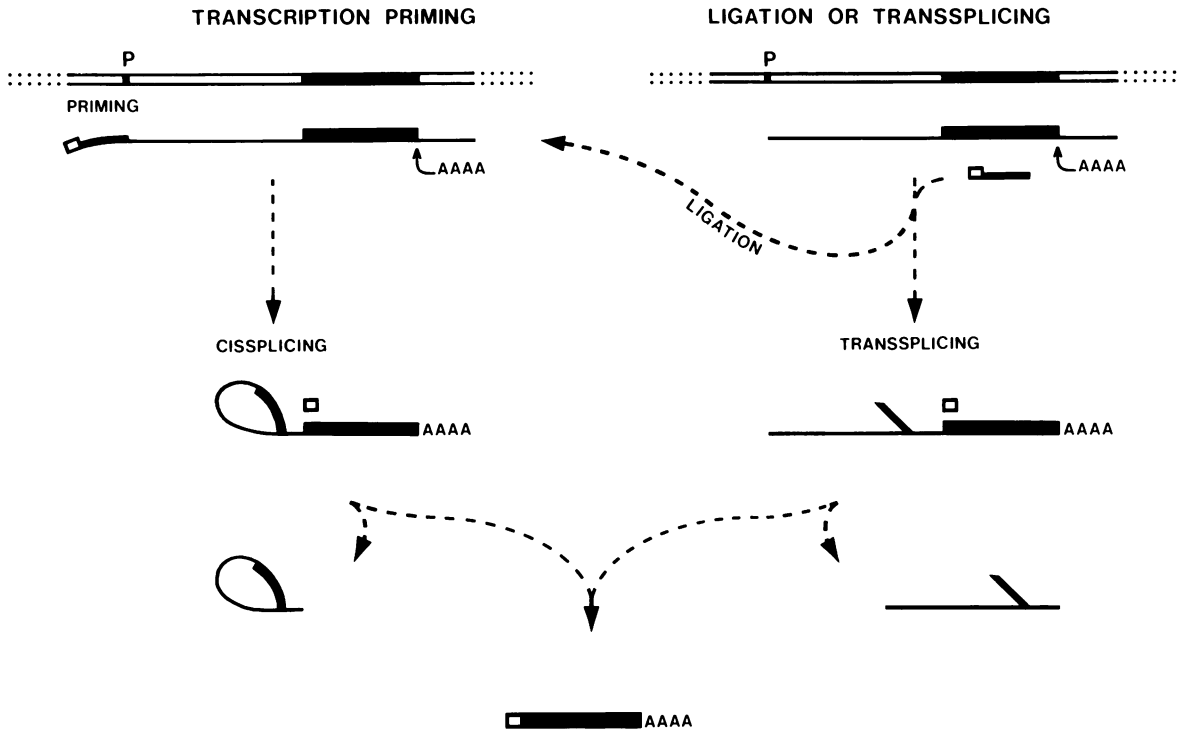

Fig. 1. Models for discontinuous mRNA synthesis in trypanosomes. The left side of the figure shows how medRNA could prime transcription upstream of a protein-coding gene, yielding a primary transcript, which could be polyadenylated and spliced in a conventional manner (see Padgett *et al.*, 1986). Alternatively, the joining could be post-transcriptional, as shown on the right side of the figure, for instance by end-to-end RNA ligation, yielding an intermediate resembling the primary transcript of the priming model or by a *trans* splicing mechanism, in which splicing initiates without prior covalent joining of medRNA to the pre-mRNA, resulting in a forked intermediate and by-product, instead of lariats. During the *cis* or *trans* splicing event, the mini-exon sequence is thought to remain associated with the main body of the RNA by protein-assisted interactions by analogy with other eukaryotic nuclear mRNA splicing systems (Brody and Abelson, 1985; Grabowski *et al.*, 1985; Frendewey and Keller, 1985). All mechanisms ultimately yield a 3′ polyadenylated mRNA with a capped mini-exon sequence at its 5′ end.

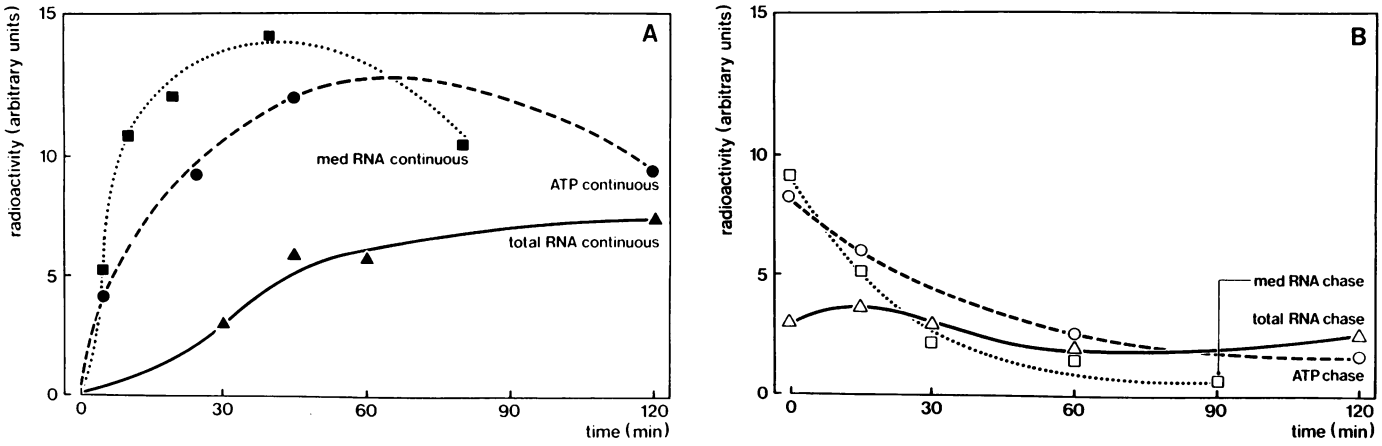

Fig. 2. [^{3}H]Adenosine labelling kinetics of ATP and RNA. The labelling of RNA and ATP pools in time was performed as described in Materials and methods in continuous labelling (**panel A**; closed symbols) and in chase (**panel B**; open symbols) experiments. The chase experiments shown in **panel B** were preceded by a 30-min pulse labelling. The arbitrary units of the vertical scale differ for each component. For ATP (circles), the vertical scale denotes the specific activity in 10^2 d.p.m./pmol. For total RNA (triangles), the incorporation into 1% of the RNA isolated from 5-ml samples is given in 10^5 d.p.m. The medRNA values (squares) are in 10^2 d.p.m./250-μl sample in **panel A** and in **panel B** in arbitrary units of absorbance in densitometric scans of short exposures of the fluorograms shown in Figure 4A and B.

turn results in the decrease in specific activity of ATP after 1 h, as can be seen in Figure 2A. It proved impractical to prevent this decrease by raising the external adenosine concentration, since this would have severely reduced the specific activity of the labelling medium or would have required inordinate amounts of ^{3}H-labelled adenosine. The slow turnover of the adenine nucleotide pool is also reflected in the partially effective chase of both cellular ATP and RNA (Figure 2B).

The size distribution of the ^{3}H-labelled RNA was analysed by agarose and polyacrylamide gel electrophoresis followed by fluorography, as shown in Figure 3. After a 30-min incubation, the label is found mainly in very high mol. wt RNA. During the chase, this RNA is processed to smaller molecules, of which the most prominent species are rRNAs. In *T. brucei*, the 28S

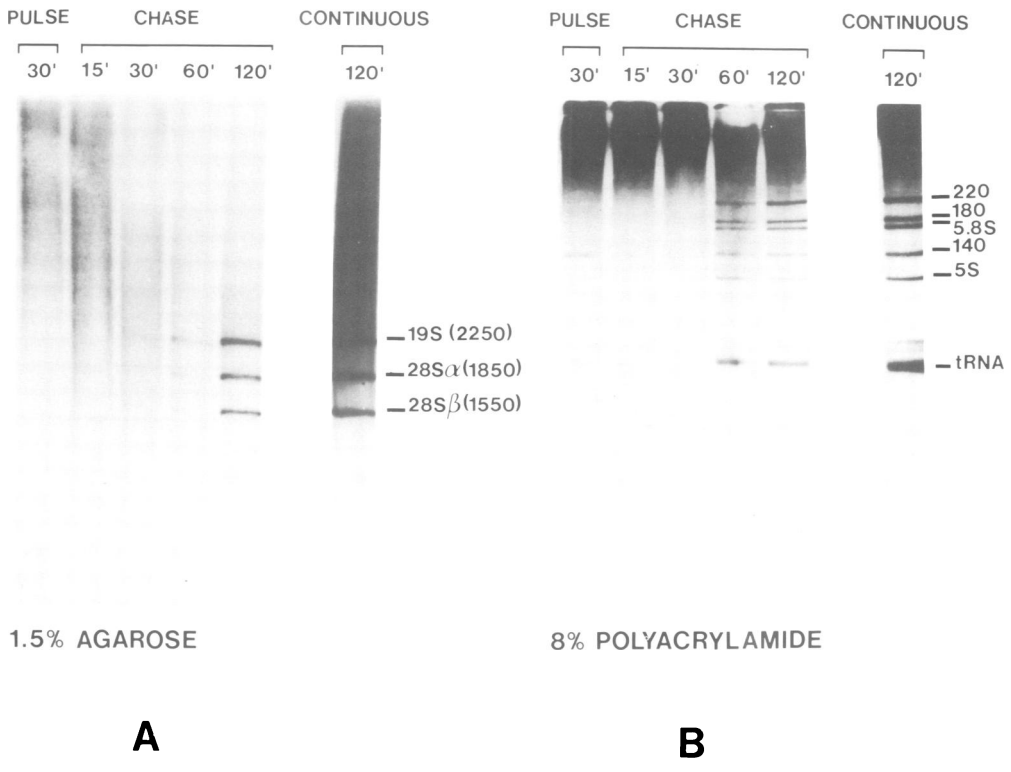

Fig. 3. Gel analysis of ^{3}H-labelled total RNA. ^{3}H-Labelled RNA was isolated from 5-ml time point samples and 1% of the yield was loaded on either a 1.5% agarose gel (**panel A**) or on a 7 M urea, 8% polyacrylamide gel (**panel B**). The ^{3}H-labelled RNA was visualized by fluorography as described in Materials and methods. The time points are indicated at the top of the lanes. The prominent bands indicated represent rRNAs and tRNA (see text).

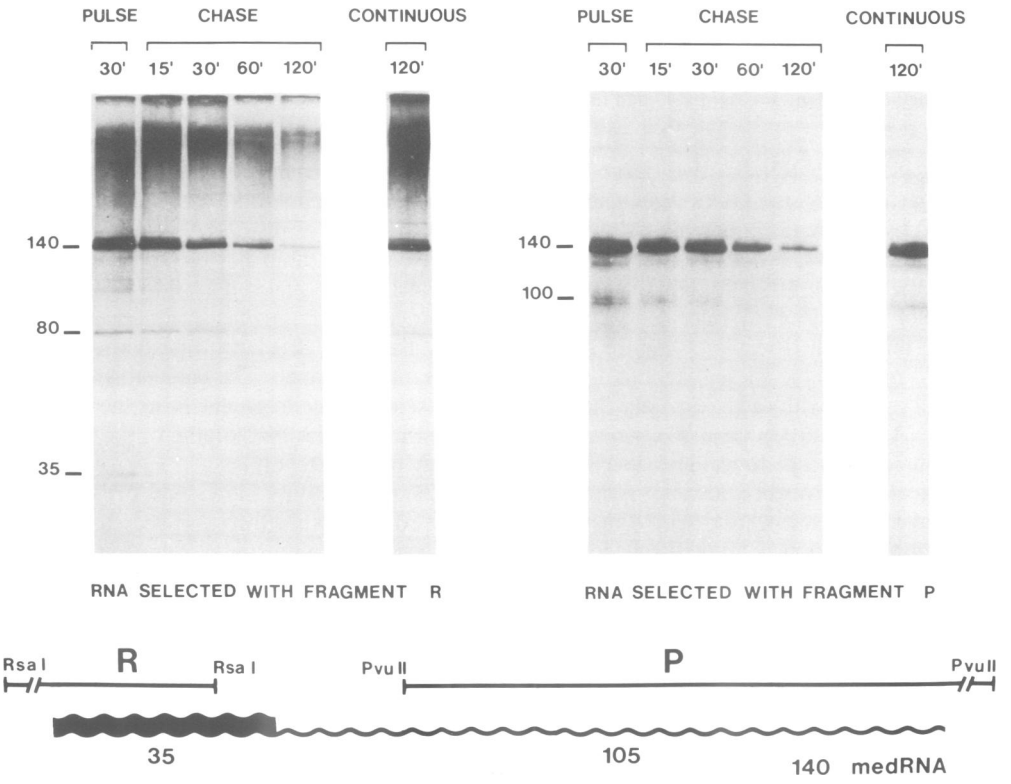

Fig. 4. Hybridization selection of ^{3}H-labelled RNA for medRNA sequences. ^{3}H-Labelled RNA was isolated from 5-ml time point samples and 12.5% of the RNA was hybrid selected with either fragment R covering part of the mini-exon (left six lanes) or fragment P covering part of the 3′ segment of medRNA (right six lanes) as described in Materials and methods. The selected RNA was loaded onto a 7 M urea, 8% polyacrylamide gel and visualized by fluorography. The time points are indicated at the top of the lanes. The fragments used for hybrid selection are shown at the bottom of the figure.

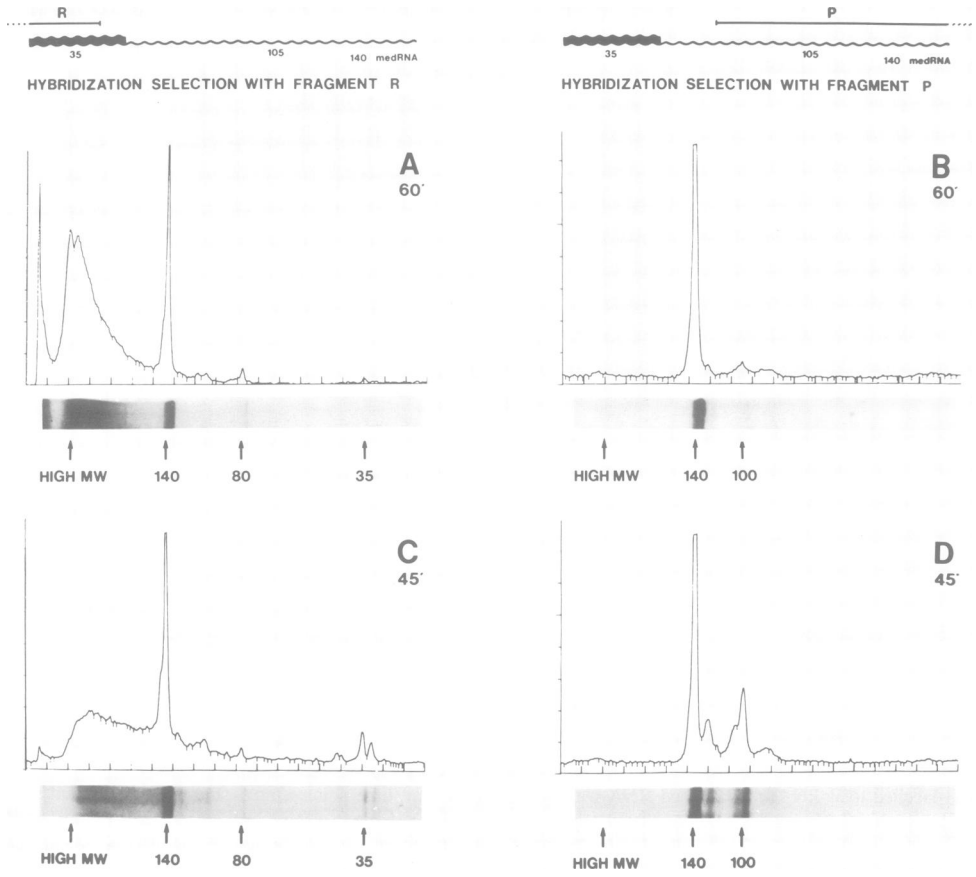

Fig. 5. Densitometric analysis of hybrid-selected ^{3}H-labelled RNA samples. ^{3}H-Labelled RNA from two time point samples was hybrid selected and size-fractionated as described in Figure 4. The fluorograms and their corresponding densitometer traces are shown. The regions of medRNA, covered by fragments R and P, are shown at the top of the figure. (**A**) RNA from a 60-min time point sample hybrid selected with fragment R. (**B**) RNA from a 60-min time point sample hybrid selected with fragment P. (**C**) RNA from a 45-min time point sample hybrid selected with fragment R. (**D**) RNA from a 45-min time point sample hybrid selected with fragment P.

rRNA is split into six separate RNAs: two large RNAs designated 28Sα and 28Sβ, and four small ones designated 220, 180, 140 and 70 (masked by tRNA in Figure 3B) (Cordingley and Turner, 1980; Hasan *et al.*, 1984; White *et al.*, 1986). These RNAs, along with the 5.8S and the 19S rRNAs are processed from one large precursor transcript (White *et al.*, 1986; P.J.Johnson and P.Borst, unpublished results). Mature rRNAs are first detectable after 30 min into the chase, which is 1 h after addition of label. They all appear with approximately the same rate, except for the 5S rRNA and a 140-nucleotide species which are both prominent within 30 min labelling. The 5S rRNA is encoded in a separate repeat and probably does not require extensive post-transcriptional processing, since it has a 5′ triphosphate end and its coding region is immediately followed by a standard RNA PolIII termination signal (Lenardo *et al.*, 1985b; Cordingley, 1985; Laird *et al.*, 1985). This explains the clear difference in incorporation between 5S rRNA and other small rRNAs early in the labelling, whereas rough stoichiometry is reached after 2 h of continuous labelling or of chase. The other prominent band visible in the early time point samples in Figure 3 migrates at the position of the 140 rRNA. This is due to the co-migration of this rRNA with medRNA, which labels very rapidly (see below).

Turnover of medRNA

We have used hybridization selection to study further individual RNA species. Figure 4 shows the two fragments used to select medRNA: fragment R, which covers part of the mini-exon sequence and fragment P, which covers part of the 3′ 105-nucleotide sequence of medRNA, not found in mature mRNAs. The selected RNA samples were run on polyacrylamide gels and the ^{3}H label was visualized by fluorography, as shown in Figure 4. Whereas both fragments select for the 140-nucleotide medRNA, only fragment R selects for significant amounts of RNA in the high mol. wt range. The fact that large amounts of labelled RNA are selected by fragment R in the high mol. wt range within 30 min indicates that joining of the mini-exon sequence to large RNA occurs rapidly.

The radioactivity in medRNA reaches a maximum within the first 30 min of labelling and decreases rapidly during the chase, indicating a high turnover rate. Figure 2A shows that the labelling of medRNA is even more rapid than that of the total ATP pool. For technical reasons, the RNA and h.p.l.c. analyses were done on separate labelling experiments, which excludes an unequivocal comparison. However, the extremely rapid labelling of medRNA was reproduced several times in duplicate. The chase kinetics shown in Figure 2B confirm that medRNA turnover is more rapid than the turnover of the total ATP pool. This remarkable result can be explained if one assumes that large ATP pools in organelles exchange poorly with the cytoplasmic and nuclear pools, so that total cellular ATP turnover is slower than that of the ATP pool used for RNA synthesis in the nucleus. The approach to equilibrium labelling of medRNA shown in Figure 2A gives a 50% labelling level within 6 min, which, in a steady-

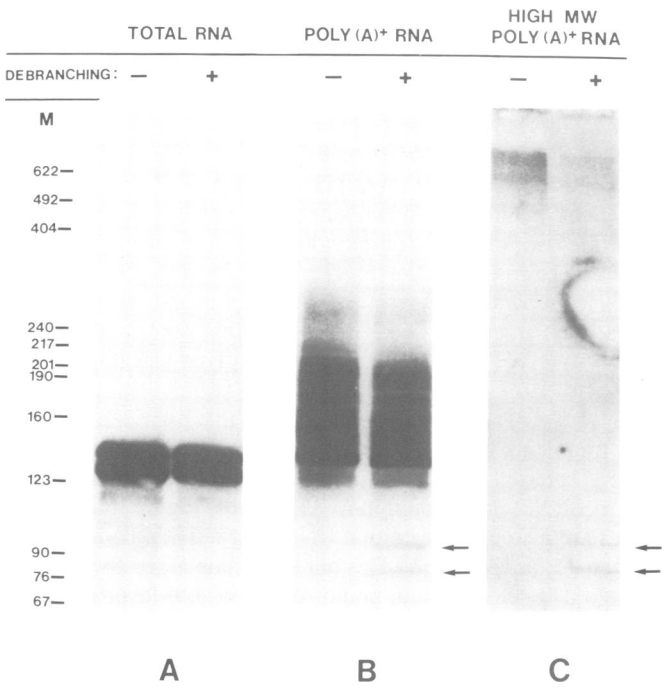

Fig. 6. Debranching treatment of total and poly(A)$^+$ RNA. RNA was treated with a HeLa cell extract with debranching activity or mock treated and then size-fractionated on a 7 M urea, 8% polyacrylamide gel, electroblotted and hybridized with nick-translated fragment P (see Figure 4) as described in Materials and methods. (**A**) 8 μg total RNA, with either a mock treatment (−) or with a debranching treatment (+). (**B**) 8 μg poly(A)-enriched RNA with either a mock treatment (−) or with a debranching treatment (+). (**C**) 8 μg poly(A)-enriched RNA, depleted of low mol. wt RNA by a size-fractionation on an S400 Sephacryl column as described in Materials and methods, with either a mock treatment (−) or with a debranching treatment (+). Arrows to the right of **panels B** and **C** indicate 84- and 96-nucleotide RNA species. The migration of an *Msp*I digest of the plasmid pAT153 used as a marker (M) is indicated to the left of **panel A**.

state situation, implies a half-life of 6 min as well. The actual half-life of medRNA may be much shorter, but an accurate determination would require a faster labelling of the nucleotide pool.

The high mol. wt. RNA selected with the mini-exon specific fragment R in Figure 4 consists of mRNAs and possibly also of intermediates in processing. Densitometry of the fluorograms shows that the signal in high mol. wt RNA at 30 min labelling decreases to about half after 60 min chase. Since this signal must be biased towards short-lived mRNAs, we conclude that the mRNA pool has an average half-life of at least 1 h. This is in agreement with the average half-life of >1 h recently found for poly(A)$^+$ RNA by B.Ehlers, J.Czichos and P.Overath (Tübingen, FRG, personal communication). Hybridization of blots of total RNA with a mini-exon-specific probe shows that the steady-state concentration of mRNA exceeds that of medRNA by at least a factor of 10 (Campbell *et al.*, 1984; Kooter *et al.*, 1984; Milhausen *et al.*, 1984; Laird *et al.*, 1985). This corresponds well with at least a 10-fold longer half-life for mRNAs, determined in our experiments. This indicates that the mini-exon-derived RNA has turnover kinetics compatible with those of a true intermediate in mRNA synthesis.

medRNA is unstable

Hybridization selection for medRNA sequences reveals a number of smaller RNA species aside from the medRNA, which could be intermediates in the processing of mRNAs. Selection with fragment R yields several species ~100 nucleotides, a prominent species of 80 nucleotides, but most strikingly two species of ~35 nucleotides. Selection with fragment P yields several prominent bands of ~100 nucleotides.

Although the 35-nucleotide RNA is an expected intermediate and the 105-nucleotide RNA a possible by-product in some processing pathways for medRNA (see Figure 1), they do not behave as true kinetic intermediates in our pulse−chase experiments. We also noted that the level of these RNAs varied in different samples: in some they were hardly detectable, but in one, shown in Figure 5C and D, the level was substantial after 45 min of continuous labelling, but not after 60 min (Figure 5A and B) or 30 min (not shown). A comparison of the distribution of high mol. wt RNA in Figure 5A and C shows that the higher levels of the 35- and 100-nucleotide species coincide with an increase in the degradation of the RNA. Apparently, medRNA is readily cleaved in the vicinity of the splice site if limited degradation of the RNA occurs.

Structure of high mol. wt RNA containing the 3′ part of medRNA

We have previously tried to detect high mol. wt RNA containing a covalently bound 3′ part of the medRNA by RNA blot hybridizations, but without success (Laird *et al.*, 1985). In the experiment shown in Figure 5B, low levels of high mol. wt RNA selected with fragment P can be detected. After correction for background, the ratio of radioactivity in medRNA and high mol. wt RNA is 9.7, calculated from integration of the relevant areas of the scans in Figure 5B. The higher ratio of high mol. wt RNA versus medRNA in this experiment is probably due to the presence of ^{3}H label throughout these molecules as well as in the 3′ medRNA part and due to the fact that the medRNA band is overexposed. It is obvious that intermediates in mRNA synthesis that contain the 3′ part of the medRNA sequence must be present at very low concentration. If the majority of the medRNA population is used to generate mRNAs through such intermediates, then the synthesis rates of medRNA and of these intermediates should be about equal in a steady-state situation. Any difference in steady-state RNA levels is then attributable to a difference in half-lives. A >10-fold lower steady-state level of such high mol. wt intermediates than of medRNA thus implies at least a 10-fold shorter half-life.

Of the mechanisms depicted in Figure 1, the *trans* splicing model best accommodates a very short half-life of high mol. wt intermediates containing the 3′ part of medRNA. The *trans* splicing model predicts that these short-lived intermediates contain the 3′ part of medRNA as a branch of a forked molecule, covalently attached by a 2′,5′-phosphodiester bond by analogy to nuclear pre-mRNA splicing of other eukaryotes (Konarska *et al.*, 1985a). We use the specific debranching activity of HeLa cell extracts (Ruskin and Green, 1985) to explore the possible existence of such molecules in steady-state RNA. Figure 6A and B shows electroblots of total RNA and of poly(A)$^+$ RNA with and without a debranching treatment, hybridized with a 3′ medRNA probe. The debranching activity had no significant effect on total RNA but resulted in the appearance of two additional weak bands, at about 96 and 84 nucleotides with poly(A)$^+$ RNA. To test whether these bands were derived from large RNA or from the medRNA species abundantly present in the poly(A)$^+$RNA, we removed all low mol. wt RNA from the poly(A)$^+$ RNA by size fractionation on a Sephacryl S400 column. The two lanes in Figure 6C show that virtually all low mol. wt RNA can be removed by this fractionation procedure. Nevertheless, the 84- and 96-nucleotide species still appear upon debranching of this RNA, proving that these species are indeed derived from high mol. wt RNA. These results concur with those

recently obtained by Murphy *et al.* (1986) and Sutton and Boothroyd (1986).

Discussion

Our experiments have yielded three results that bear on the mechanism of discontinuous mRNA synthesis.

(i) The turnover rate of medRNA is high. This is most convincingly demonstrated by the approach to equilibrium labelling experiments, in which half-maximal labelling was reached after ~6 min. This must still be an underestimate of the true turnover, since there is always a lag in the labelling of a nuclear ATP pool following the addition of label. The average half-life and the steady-state concentration of mRNA are at least 10-fold higher than those of medRNA. Our results therefore show that the turnover of medRNA is compatible with its postulated role as an obligatory intermediate in discontinuous mRNA synthesis.

(ii) The concentration of high mol. wt RNAs containing the 3′ part of medRNA is very low, at least 10-fold lower than that of medRNA. This conclusion follows from labelling experiments as in Figure 5B and from the hybridization analysis in Figure 6 and extends earlier hybridization experiments (Campbell *et al.*, 1984; Kooter *et al.*, 1984; Laird *et al.*, 1985). We conclude that the half-life of these putative intermediates in mRNA synthesis must be less than one-tenth of that of medRNA, i.e. far below 1 min. Such a short half-life virtually rules out a priming mechanism for discontinuous mRNA synthesis, as depicted in the left-hand part of Figure 1. Every protein-coding gene we have studied with run-on transcription and inactivation by u.v. light appears to be part of a single multi-cistronic transcription unit of at least 10 kb (Borst, 1986; J.M.Kooter, B.W.Swinkels and P.J.Johnson, unpublished results). The distribution of ^{3}H label in the very high mol. wt range early in labelling in Figure 3A is consistent with a large average primary transcript length in trypanosomes. In other eukaryotes, transcription of a 10-kb unit and the subsquent removal of intron sequences requires at least 5 min (e.g. Darnell, 1982). We conclude that priming of pre-mRNA synthesis by a complete medRNA molecule cannot be the main mechanism for discontinuous mRNA synthesis; priming by a 5′ segment of medRNA (cf. Borst, 1986) cannot be ruled out on the basis of these results, however.

(iii) High mol. wt RNA, enriched on oligo(dT)-cellulose and free of medRNA, yields two unique truncated medRNA species during an incubation in a HeLa cell extract with debranching activity. Presumably these medRNA segments are derived from branched molecules, analogous to the one depicted in the right-hand half of Figure 1. Why debranching yields two different RNAs instead of one, and why even the largest RNA seems smaller than the 105-nucleotide expected, is unclear. If the branch is indeed either 96 or 84 nucleotides *in vivo*, then all 100–105-nucleotide species in a steady-state RNA may be the result of artefactual degradation of medRNA. The existence of forked molecules is compatible with *trans* splicing, but it does not prove that this is the main route for trypanosome mRNA synthesis, since occasional aberrant *trans* splicing in a normally *cis* splicing system may occur, as has been found *in vitro* in other systems (Konarska *et al.*, 1985b; Solnick, 1985). Particularly in the case of trypanosomes, intermediates in such an aberrant process would be more readily detected, since the medRNA is possibly the only available splice donor. Definitive proof that *trans* splicing is the main route for mRNA synthesis in trypanosomes would be provided by a quantitative tracing of the majority of the labelled medRNA molecules through these intermediates in pulse–chase experiments *in vivo*. The short half-lives and low concentrations of the forked intermediates makes this a daunting task.

Even through trypanosomes are unable to synthesize purines *de novo*, the labelling of cellular ATP with exogenous [^{3}H]-adenosine is not as effective as we had hoped. The problems are vividly illustrated by the sluggish chase of the ATP pool in Figure 2B, which contains at least two kinetic components. It seems likely that the poor chase is not only due to a large purine pool, but also to compartmentalization of adenine nucleotides. Obvious candidates for separate compartments are the mitochondrion and the glycosomes, microbody-like organelles that contain the ATP-utilizing and ATP-producing reactions of glycolysis (Opperdoes and Borst, 1977; Opperdoes, 1985).

We have observed several small RNAs that hybridize to medRNA fragments in addition to medRNA itself. There is for instance a heterogeneous population of molecules slightly larger than medRNA in the poly(A)$^+$ RNA of Figure 6B. These species are also present in total RNA, but not visible in the exposure used for Figure 6A. This minor fraction is greatly enriched by oligo(dT) selection and could represent low levels of aberrant polyadenylation of medRNA. Another possibility stems from the presence of a (T)-stretch downstream of the medRNA transcription unit (De Lange *et al.*, 1984b). Occasional read-through of medRNA transcription would yield RNAs with a highly (U)-rich 3′ tail which could be retained on oligo(dT)-cellulose by sandwich hybridization to poly(A) tails of mRNAs. The nature of these molecules is under investigation. Another RNA hybridizing with medRNA probes migrates at ~130 nucleotides and is especially prominent in Figure 6A. This RNA is probably identical with a minor truncated medRNA mapped previously (Kooter *et al.*, 1984; Laird *et al.*, 1985). The amount of this RNA is variable and it is presumably a degradation artefact. The 80-nucleotide RNA, selected with a mini-exon probe (see Figures 4 and 5), is not enriched in partially degraded RNA. This RNA may result from premature termination of transcription or from medRNA processing.

Our finding that medRNA artefactually breaks into pieces of ~105 and 35 nucleotides is clearly a major complication in assessing the role of free 35- and 105-nucleotide species in discontinuous mRNA synthesis. We do not think that the facile cleavage of the medRNA near the splice site is a coincidence. The medRNA is used to generate all mRNAs and it is conceivable that its structure and sequence have evolved to facilitate cleavage at the splice site. This could be effected by an easy accessibility to enzymes or even by a structurally induced strain in the phosphodiester bonds near this site. Secondary structure models of medRNA indicate that the splice donor region in medRNA could be rather exposed to nucleases. It is clear from our results that any medRNA fragment present or produced in RNA preparations containing medRNA must be viewed with suspicion. Such fragments must be considered artefacts, unless rigorous proof for a physiological origin is provided.

After this work was completed, evidence for branched RNA molecules as possible intermediates in discontinuous mRNA synthesis was also reported by Murphy *et al.* (1986) and Sutton and Boothroyd (1986).

Materials and methods

Trypanosomes

The trypanosomes used in this study belong to strain 427 of *T. brucei brucei* (Cross, 1975). The procyclic form trypanosomes were cultivated at 28°C in

SDM-79 medium (Brun and Schönenberger, 1979), supplemented with 10% v/v fetal calf serum and 2 ml/l sterile hemin solution (2.5 mg/ml in 50 mM NaOH).

RNA isolation and further RNA manipulation

Trypanosomes were harvested by centrifugation for 5 min at 5000 r.p.m. After resuspending the pellet in 1/2 culture volume of 50 mM glucose, 10 mM EDTA, 100 mM NaCl, 50 mM Tris−HCl, pH 8.0, an equal volume of 2% SDS, 1 mg proteinase K/ml was added and the lysate was incubated for 30 min at 50°C. The lysate was first extracted with phenol/chloroform/isoamyl alcohol (25:24:1) until no interphase appeared and then extracted twice with chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated overnight with 1/10 volume 3 M sodium acetate and 2.5 vol. 96% ethanol at −20°C and collected by centrifugation for 20 min at 10 000 r.p.m. The pellet was washed with 70% ethanol, dried and dissolved in 0.1 mM EDTA, 10 mM Tris−HCl, pH 7.5. This solution was then brought to 2 mM $CaCl_2$, 5 mM $MgCl_2$; 10 μg proteinase K-treated DNase I/ml was added (Tullis and Rubin, 1980) and the mixture was incubated for 15 min at 37°C. The RNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1) and then ethanol precipitated.

RNA was glyoxylated according to the procedure of Thomas (1980). Electrophoresis of glyoxylated RNA through agarose slab gels was done in a continuously circulating, low ionic strength buffer (10 mM sodium phosphate buffer, pH 7.0) for 16 h at 3 V/cm (50 mA).

Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography according to Hoeijmakers *et al.* (1980).

Poly(A)-enriched RNA was size fractionated on a Sephacryl S400 Superfine (Pharmacia) column with a cross-sectional area of 0.79 cm^2 and a packed bed volume of 12 ml. Fractions were collected and analysed by agarose gel electrophoresis. Fractions without detectable contamination of low mol. wt RNA were pooled, and stored as ethanol precipitates.

In vivo labelling of trypanosomes with ^{3}H-labelled adenosine

Mid- to late-logarithmic phase trypanosomes (2 × 10^7cells/ml) (the adenosine concentration was determined to then be 10 μM) were incubated in the presence of 100 μCi/ml [2,5′,8-^{3}H]adenosine (42−50 Ci/mmol, Amersham International) at 28°C in SDM-79 medium (Brun and Schönenberger, 1979). Samples were taken by centrifuging 5 ml (RNA isolation) or 1 ml (h.p.l.c. analysis) at 2500 r.p.m. for 5 min followed by lysis with SDS and proteinase K for RNA isolation or by perchloric acid for h.p.l.c. analysis (see these sections for details). The labelling was chased by collecting the trypanosomes by centrifugation for 5 min at 2500 r.p.m. and resuspending the pellet in the same initial volume of conditioned SDM-79 medium (i.e. filter sterilized medium from a parallel culture) to which an extra 50 μM adenosine was added. Time point samples were taken as described above for continuous labelling. More rapid time point samples for the approach to equilibrium labelling of medRNA, shown in Figure 2A, were taken by disrupting the trypanosomes (250 μl; 0.5 × 10^7 cells) in medium without prior centrifugation. This was accomplished by adding the 250-μl sample to a tube containing 25 μl 10% SDS; 5 μl of a solution with 20 mg proteinase K/ml and 5 μl of 500 mM EDTA, pH 8.0. The RNA isolation was then continued as described above.

The initial rate of uptake of [^{3}H]adenosine in whole cells was determined to be ~10 pmol/min/10^6 trypanosomes. This compares well with the 6−20 pmol/min/10^6 trypanosomes determined by James and Born (1980). However, this is ~10 times as much as the amount calculated to be necessary for net population expansion and at this high a rate the adenosine in the medium would be depleted within several hours. We have determined that this is not the case, and we conclude that most of the adenosine taken up is excreted again as adenosine or as adenine. This is in agreement with the composition of post-incubation medium after labelling with [^{14}C]adenosine as determined by Fish *et al.* (1982).

The size of the total internal adenine and adenine nucleoside and nucleotide pool was calculated from the percentage of initial ^{3}H label, which had been taken up by a given number of cells at a known specific activity of the medium, after pool equilibrium had been reached (1 h). This gives a value of 200 pmol/10^6 trypanosomes, which corresponds well with the values we obtained with the h.p.l.c. analyses (e.g. 60 pmol/10^6 cells for ATP) and those found by Fish *et al.* (1982).

H.p.l.c. analysis of nucleotide pools

Trypanosomes (2 × 10^7 cells/ml) were cultured and labelled with [^{3}H]adenosine as described above. Time point samples (1 ml) were taken by centrifugation. The supernatant was discarded and the nucleotides were extracted by resuspension of the cell pellet in 200 μl of ice-cold 0.4 M perchloric acid. The extract was left at 0°C for 15 min, mixed, and then centrifuged for 2 min in an Eppendorf centrifuge. The supernatant was neutralized with 5 μl of 5.0 M potassium carbonate and stored at −70°C until h.p.l.c. analysis. The nucleotides in the extracts were separated with an anion-exchange h.p.l.c. method as described elsewhere (De Korte *et al.*, 1985). Columns were pre-packed Partisil-10 SAX cartridges (10 × 0.8 cm) radially compressed in an RCM-Z module (Waters Associates, Inc., USA). The separations were performed at a flow rate of 2 ml/min. The radioactivity incorporated in the various compounds was monitored by an on-line heterogeneous system, consisting of the Ramona D (Isomess, FRG) with a siliconized yttrium flow-cell (volume 600 μl), in combination with a variable-wavelength spectrophotometer set at 254 nm (Perkin−Elmer model LC75) and an Apple IIe computer for quantitation of the digital signal with dual-trace Radio-Chromato-Graphic System Software (IM 2006; Isomess). The nucleotide peaks were identified by comparison of retention times with those of standard nucleotides. The specific activity of nucleotide pools was calculated from the determined radioactivity and A_{254}, calibrated with standard nucleotide solutions.

Plasmids and restriction fragments

Convenient fragments for hybridization selection were obtained from clones of a *Bal*31 deletion series. These clones were constructed as follows: the mini-exon repeat clone pCL102 (Kooter *et al.*, 1984) was digested with *Nar*I; the 1310-bp fragment containing the mini-exon was isolated and digested with *Bal*31. Fragments of suitable length were treated with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of deoxynucleotides. A second digestion with *Sau*3AI followed and fragments of the right length were isolated and cloned in pSP65 (Promega Biotec), which had been digested with *Sma*I and *Bam*HI. The resulting clones had inserts running from variable positions upstream of or within the 140-bp medRNA coding region to a common *Sau*3AI site 40 bp downstream of the 3′ end of the region encoding the medRNA. Clone pSPMEB5 contains an insert starting ~15 bp upstream of the 5′ end of the mini-exon and running to the common *Sau*3AI site, and therefore contains the complete medRNA coding region. Clone pSPMEB9 contains an insert starting at ~60 bp downstream of the 5′ end of the mini-exon and running to the common *Sau*3AI site, and therefore lacks any mini-exon sequences. Fragment R is a 144-bp *Rsa*I restiction fragment of pSPMEB5, running from an *Rsa*I site in the vector through the 5′ end of the mini-exon sequence to the *Rsa*I site within the mini-exon. Fragment P is a 328-bp *Pvu*II restriction fragment of pSPMEB5, running from the *Pvu*II site, 58 bp downstream of the 5′ end of the mini-exon through the 3′ end of the medRNA coding region to a *Pvu*II site in the vector. Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (1979), and purified on cesium chloride−ethidium bromide equilibrium gradients. Restriction fragments were isolated from low melting point agarose gels.

Hybridization selection

Plasmid DNA or restriction fragments were bound to nitrocellulose filters essentially according to Kafatos *et al.* (1979). The DNA (<0.5 μg/ml) was denatured, by boiling for 10 min, chilled in an ice−water bath, and incubated at room temperature for 20 min after addition of NaOH to 0.4 M. An equal volume of ice-cold 2 M ammonium acetate was added and the DNA was then spotted onto 0.2 cm^2 nitrocellulose filters (<50 μg/cm^2) and allowed to adsorb. The filters were first rinsed with 1 M ammonium acetate and then with 6 × SSC. The filters were dried at room temperature and baked at 80°C for 2 h. Before pre-hybridization, the filters were first boiled for 3 min in 1 mM EDTA, pH 8.0, washed twice in 1 mM EDTA, pH 8.0 at room temperature to remove loosely bound DNA and then allowed to dry. The filters were pre-hybridized for 2 h at the hybridization temperature (30°C for fragment R and 65°C for fragment P) in 300 μl 3 × SSC, 0.1% Ficoll 400, 0.1% bovine serum albumin, 0.1% polyvinyl-pyrollidone, 0.1% SDS; 50 μg denatured salmon sperm DNA/ml, 2 mM sodium pyrophosphate, 100 μg *E. coli* tRNA/ml in siliconized tubes. In each hybridization selection, single-stranded DNA of the other fragment than used to select with was added to a concentration of 1 μg/ml as competitor. The ^{3}H-labelled RNA was dissolved in 10 μl of 0.1 mM EDTA, 10 mM Tris−HCl, pH 7.5, boiled for 5 min, quenched on ice, added to the pre-hybridization and hybridized for 16 h at the temperatures specified above. Filters containing fragment R were washed to a stringency of 1 × SSC, 30°C. Those containing fragment P were washed to a stringency of 1 × SSC, 65°C. The RNA was eluted by boiling the filters for 4 min in 300 μl of 1 M EDTA, pH 8.0, 10 μg *E. coli* tRNA/ml. The filters were removed and the RNA was precipitated overnight at −20°C with 1/10 volume 3 M sodium acetate and 2.5 volumes of ethanol.

For the approach to equilibrium experiment of medRNA, shown in Figure 2A, the ^{3}H-labelled RNA was hybridized to 10 μg of pSPMEB9 plasmid DNA immobilized on nitrocellulose filters as described above, and to filters containing pAT153 plasmid DNA, to correct for aspecific hybridization. However, instead of eluting the RNA from the filters after the washes, the filters were dried and transferred to scintillation vials containing 10 ml of Insta-gel (United Technologies Packard, USA). Samples were counted in a liquid scintillation counter (Searle Analytic Inc., type Mark III, USA) for 5 min periods. The results were corrected for quenching and for aspecific hybridizations to pAT153.

Fluorography and densitometric scanning

Polyacrylamide or agarose gels containing ^{3}H-labelled RNA were fixed for 45 min in 10% acetic acid and then rinsed in redistilled water for 30 min with two changes of water. The fixed gels were then impregnated for 30 min with the scintillator Amplify (Amersham, UK). The gels were then transferred to two layers of pre-wetted 3 MM Whatman paper, overlayed with plastic wrap and

dried under vacuum for ~1 h at 60°C. Dried gels were exposed to Kodak XAR-5 films at −70°C with a tungsten intensifying screen.

Densitometric measurements of fluorographs/autoradiograms were carried out with a Beckman DU-8 gel scanning system and were corrected for background.

Debranching treatment

A post-nuclear S100 extract of HeLa cells was used to break 2′−5′phosphodiester bonds in RNA (Ruskin and Green, 1985). The extract was first cleared of precipitates by a 15-s centrifugation in an Eppendorf centrifuge. A standard reaction consisted of 30 μl extract, 6 μl 50 mM EDTA, pH 8.0 and variable amounts of RNA in 0.1 mM EDTA, 10 mM Tris−HCl, pH 7.5 in a total volume of 60 μl. As a mock treatment, 30 μl HeLa extract storage buffer (20 mM Tris−HCl, pH 7.5, 20% glycerol, 100 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$ and 0.5 mM DTT) was used, instead of the extract. The reaction mixtures were incubated for 1 h at 30°C. After a proteinase K (1 mg/reaction) digestion for 30 min at 30°C in 1% SDS the mixture was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. The RNA samples were size-fractionated on a 7 M urea, 8% polyacrylamide gel and and transferred to GeneScreenPlus membrane (NEN−Dupont) and hybridized according to Church and Gilbert (1984). The extract was kindly provided by Dr M.Aebi and Dr C.Weissmann, University of Zürich, Switzerland.

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