Transcription of Kinetoplast DNA in *Trypanosoma brucei* Bloodstream and Culture Forms

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Kinetoplast DNA is the unusual mitochondrial DNA of trypanosomes. In *Trypanosoma brucei* it consists of about 10^4 minicircles (0.3 μm) and 10^2 maxicircles (6 μm) catenated into a single network. The maxicircles are probably the equivalent of mitochondrial DNA in other organisms. Here we report that a fraction of the total cellular RNA from bloodstream form and culture form *T. brucei* hybridizes with the maxicircle; we find no minicircle transcripts. Preferential hybridization with a 1.8-kb[2] maxicircle segment is shown to be due to two abundant RNA species, the 9 S and 12 S RNAs. After glyoxylation the apparent size of these putative rRNAs in gels is 1080 and 590 nucleotides, respectively; they are present in approximately equimolar amounts, lack poly(A) tails, their genes are adjacent, and they are transcribed from the same strand in the order 12 S–9 S. Six additional RNA species, varying in size from 360 to 1110 nucleotides, are transcribed from segments covering 50% of the maxicircle. These transcripts are specifically retained on oligo(dT)-cellulose and presumably represent mitochondrial messenger RNAs. Recombinant DNA plasmids containing DNA

complementary to two of these kinetoplast RNAs have been constructed and used as probes for the study of the corresponding genes. One of these complementary DNAs hybridizes with nonadjacent fragments of the maxicircle and this gene may, therefore, contain an intervening sequence(s).

Bloodstream trypanosomes, in which the synthesis of a mitochondrial respiratory chain is totally repressed, contain a 5 to 10-times lower level of maxicircle transcripts than cultured trypanosomes, in which the mitochondria are fully developed. This decrease affects all mitochondrial transcripts analyzed approximately to the same extent. Controls at the level of mitochondrial protein synthesis or membrane assembly may, therefore, contribute to the repression of mitochondrial synthesis in bloodstream-form trypanosomes.

Trypanosomes are unicellular parasitic flagellates, characterized by the unique kinetoplast DNA (kDNA) that is found in a specialized portion of the single mitochondrion of these organisms. In DNA-protein monolayers prepared for electron microscopy, kDNA appears to consist of 10^3–10^4 small circular molecules (minicircles) catenated into a complex network (reviewed by Simpson, 1972; Borst and Hoeijmakers, 1979a; Englund, 1981). Depending on the species, minicircles vary in size from 0.25 to 0.8 μm (Simpson, 1972); as shown by restriction enzyme analysis, their base sequence is usually heterogeneous (Borst and Hoeijmakers, 1979a), and very different in closely related species (Borst and Hoeij-

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[2] Abbreviations used: kDNA, kinetoplast DNA; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; rRNA, ribosomal RNA; mRNA, messenger RNA; cDNA, complementary DNA; kb, kilobase pair(s); bp, base pair(s); DBM paper, diazobenzyloxymethyl-cellulose paper; VSG, variant surface glycoprotein.

makers, 1979b). They are not transcribed in two of the three trypanosome species studied (Hoeijmakers and Borst, 1978; Borst and Hoeijmakers, 1979b; Fouts and Wolstenholme, 1979).

Enzymatic dissection of kDNA with restriction endonucleases has further uncovered the presence of 50–100 maxicircles in the network (Kleisen *et al.*, 1976a; Fairlamb *et al.*, 1978). Accumulating data strongly suggest that the maxicircle codes for gene products similar to those encoded by the mitochondrial DNA (mtDNA) of other organisms: its size (ranging from 6 μm in *T. brucei* to 12.5 μm in *Trypanosoma cruzi*) and complexity are in the same order of magnitude as mtDNA of other organisms; it has a unique, strongly conserved base sequence; in trypanosomes that have lost the ability to make a functional mitochondrion often (part of) the maxicircle is lacking; and maxicircles are transcribed (Hoeijmakers and Borst, 1978). Two major mitochondrial RNA (mtRNA) species (9 S and 12 S), transcribed from the maxicircles, have been identified in *Leishmania* (Simpson and Simpson, 1978) and the possibility has been raised that these are the mitochondrial ribosomal RNAs (rRNAs) (Borst and Hoeijmakers, 1979a).

In this paper we present our studies on kDNA transcription in *T. brucei*. This pathogen causes sleeping sickness in mammals and shares with yeast the unusual ability to repress completely mitochondrial biogenesis and live by glycolysis alone. Some of the results of this work have been summarized in a recent review (Borst *et al.*, 1980a).

MATERIALS AND METHODS

Growth of the organisms. Trypanosoma (Trypanozoon) brucei brucei, stock 427, cloned stabilates 60 and 118 were used in this study. The kDNAs of both organisms are indistinguishable by restriction enzyme analysis (see Borst *et al.* (1980b) for further details). The bloodstream form of this try-

panosome was grown in rats under standard conditions (Fairlamb *et al.*, 1978). Procyclic culture forms of the same *T. brucei* 427 stock were grown in the semidefined medium described by Brün and Schönenberger (1979). The generation time under these conditions exceeded 14 h and the maximal cell density was approximately 6 $\times$ 10^7 trypanosomes/ml. The insect trypanosome *Crithidia luciliae* was grown in Boné medium (Boné and Steinert, 1956) under conditions specified previously (Kleisen *et al.*, 1975).

Isolation of nucleic acids. kDNA and nuclear DNA were extracted from bloodstream form *T. brucei* according to published procedures (Borst and Fase-Fowler, 1979; Borst *et al.*, 1980c).

For the isolation of total cellular RNA from bloodstream and culture form trypanosomes a previously published modification of the hot phenol extraction procedure of Penman (1966) was used (Hoeijmakers *et al.*, 1980). However, the concentration of sodium EDTA in the NET-100 buffer (100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 100 mM Na-EDTA) was reduced in later experiments to 25 mM (NET-25). Poly(A)$^+$-enriched RNA was obtained by two cycles of oligo(dT)-cellulose chromatography (Hoeijmakers *et al.*, 1980).

Treatment of DNA with restriction enzymes. The source and assay conditions for the endonucleases *Pst*I, *Hap*II, *Bgl*I, *Sst*I, *Hae*III, *Sau* 96-I, *Eco*RI, *Hin*dIII, and *Mbo*I are specified elsewhere (Borst and Fase-Fowler, 1979), those for *Taq*I are described by Borst *et al.* (1980b). Restriction enzymes *Mbo*II, *Bsp*I, *Msp*I, *Bam*HI, and *Hinf*I (purchased from Biolabs) were incubated for 2 h at 37°C in 10 mM Tris–HCl (pH 7.5), 8 mM MgCl$_2$, 1 mM dithiothreitol, and 0.1% gelatin. The digestion buffer for *Alu*I (Biolabs) contained 100 mM NaCl in addition. Double digestions were carried out as described (Borst and Fase-Fowler, 1979).

Gel electrophoresis of DNA. Two conditions were used for the electrophoretic separation of DNA fragments in the 0.8–1.5% agarose gels: for optimal resolution in the

low-molecular-weight range (1 kilobase pair (kb)) "high-voltage" electrophoresis was applied; larger DNA fragments were size-fractionated by the "low voltage" system (Borst *et al.*, 1980b). For photography of ethidium bromide-stained gels we refer to Borst and Fase-Fowler (1979) and Kleisen *et al.* (1976b).

Nomenclature of restriction fragments. The nomenclature of restriction fragments and sites used is a modification of the system proposed first by Sanders *et al.* (1977) and further specified by Borst and Fase-Fowler (1979) and Borst *et al.* (1980b). Short-hand abbreviations for the enzymes used are: B, *Bam*HI; G, *Bgl*II; R, *Eco*RI; D, *Hin*dIII; Ps, *Pst*I; Ss, *Sst*I; Xb, *Xba*I; E, *Hae*III (*Bsp*I); Y, *Hap*II (*Msp*I); L, *Hha*I; U, *Sau* 96-I; M, *Mbo*I; N, *Mbo*II; Q, *Taq*I; V, *Hin*fI. Any site for a given enzyme is designated using the abbreviation of the enzyme followed by the number of the site for that enzyme lying clockwise on the physical map of Fig. 2, starting from the arbitrarily chosen *Eco*RI site R1 (Borst and Fase-Fowler, 1979). Any fragment is identified by a combination of the designations of the terminal sites, the first abbreviation being the site encountered first going clockwise on the physical map of Fig. 2. The number behind each combination of sites gives the electrophoretic mobility of that fragment generated by the particular (combination of) enzyme(s). Fragments are numbered in order of increasing mobility; e.g., D3E2-4 is the fragment bordered by the third *Hin*dIII site (D3) and the second *Hae*III site (E2) mapped starting clockwise from R1 and it is the forth band in a *Hin*dIII + *Hae*III double digestion of the maxicircle. See legends to Figs. 2 and 11 for further details.

Gel electrophoresis of RNA. In most experiments the RNA was denatured by glyoxal treatment (McMaster and Carmichael, 1977) prior to electrophoresis. Untreated RNA is heated briefly (1 min) to 95–100°C to disaggregate. The RNA samples are layered in the presence of 5% Ficoll and a trace of diethylpyrocarbonate with Orange G as color marker. Electrophoresis is performed in 1.75–2.0% agarose gels at room temperature for 75–120 min at 125 mA (150 V) under ribonuclease-free conditions. The electrophoresis system used is essentially similar to the "high-voltage" DNA electrophoresis and is detailed elsewhere (Van Ommen *et al.*, 1980).

Molecular-weight determination. The molecular weights of electrophoresed DNA fragments or RNAs are calculated from their mobility in the gel relative to a set of co-electrophoresed reference molecules. The following marker sets were used:

I. Phage lambda DNA and an *Eco*RI digest of phage lambda DNA (Thomas and Davis, 1975).

II. Phage T7 DNA, phage ϕ29 DNA, and an *Eco*RI digest of phage ϕ29 DNA (Ito *et al.*, 1976).

III. Phage ϕX (replicative form) DNA digested with *Bsp*I (Sanger *et al.*, 1977).

IV. Phage ϕX (replicative form) DNA digested with *Msp*I (Sanger *et al.*, 1977).

V. Yeast RNA (1710 ± 80 and 3360 ± 80 nucleotides for the main rRNA components) (Philippsen *et al.*, 1978).

VI. *Escherichia coli* RNA (2904, 1541 nucleotides for the main rRNA components) (Brosius *et al.*, 1978, 1980).

Isolation of RNA and DNA fragments from gels. Electrophoretically separated RNA molecules were recovered from the gel by the method of Van Ommen *et al.* (1979). DNA fragments were isolated by adsorption to Sepharose-lysine (Osinga *et al.*, 1981).

Electrophoretic separation of DNA strands. DNA strands were separated using the method of Goldbach *et al.* (1978a), which is based on differential binding of poly(UG) to the two lambda strands.

Transfer of nucleic acids to nitrocellulose filters. Restriction-fragmented DNA, separated on gels was denatured with alkali and blotted onto nitrocellulose filters essentially as described by Southern (1975).

Transfer of nucleic acids to diazobenzyl-oxymethyl-cellulose (DBM) paper. Electrophoretically separated RNA or DNA fragments were treated *in situ* by alkali (50 mM NaOH, 1 h), neutralized with 0.2 M Na-phosphate buffer (pH 6.5) for 20 min at 4°C and transferred to DBM paper (Alwine *et al.*, 1977) in 25 mM Na-phosphate (pH 6.5) or 0.2 M Na-acetate (pH 4.0) under ribonuclease-free conditions according to published procedures (Alwine *et al.*, 1977; Van Ommen *et al.*, 1979).

Preparation of labeled probes. Double-stranded DNA was labeled *in vitro* by nick-translation (Rigby *et al.*, 1977) to a specific activity of 10^7–10^8 cpm/μg using [α-^{32}P]-dATP and [α-^{32}P]dTTP (The Radiochemical Center, Amersham; sp. act 2–3 Ci/μmol) as the labeled nucleotides. RNA was fragmented to 200–400 nucleotides with alkali and labeled by polynucleotide kinase and [γ-^{32}P]ATP as described (Goldbach *et al.*, 1978b). DNA markers labeled by polynucleotide kinase were kindly provided by Mrs. H. A. M. Hoeijmakers-Van Dommelen.

Hybridization with filter-bound nucleic acids. The hybridization conditions for DNA bound to nitrocellulose filters are specified by Hoeijmakers and Borst (1978). The same protocol was followed for hybridizations with nucleic acids covalently attached to DBM paper. In this case, however, the filters were incubated in the hybridization medium supplemented with 1% glycine for at least 3 h at 60°C prior to hybridization. In some experiments 10% (w/v) dextran sulfate (Sigma) was included in the hybridization mixture to increase the hybridization efficiency (Wahl *et al.*, 1979). To reduce the background of aspecifically bound radioactivity to acceptable levels it was necessary to wash the filters extensively after hybridization. Autoradiographic exposure of hybridized filters was for 2–20 days.

Construction of recombinant plasmids containing DNA complementary to maxicircle transcripts. The construction of double-stranded complementary DNA (cDNA)

from total poly(A)$^+$ RNA from *T. brucei* 427-118 (bloodstream form), insertion in the *Pst*I site of plasmid pBR322 by the GC tailing technique and subsequent transformation of *E. coli* strain X-1776 have been described elsewhere (Hoeijmakers *et al.*, 1980). However, size-fractionation of the cDNA was omitted. Transformants were screened by the high-density colony-filter hybridization technique (Hanahan and Meselson, 1980) using ^{32}P-labeled kDNA as probe essentially as described (Hoeijmakers *et al.*, 1980) except that poly(A) was not included in the hybridization solutions. Colonies were picked from the nitrocellulose filters and grown under conditions specified (Hoeijmakers *et al.*, 1980). For chloramphenicol amplification and purification of plasmid DNA we refer to Wilkie *et al.* (1979). The construction of the recombinant DNA clone bank was carried out under P3-EK2 containment conditions.

RESULTS

Characterization of kRNA from Bloodstream Trypanosomes

RNA from bloodstream *T. brucei* was screened for kDNA transcripts by two types of hybridization experiments:

1. *In vitro* labeled total cellular RNA was hybridized with Southern filters containing electrophoretically separated restriction enzyme fragments of trypanosomal DNAs. RNA hybridized to the filter-bound DNA was visualized by autoradiography. Figure 1 presents the result of one such experiment. The photograph of the ethidium-stained gel with restriction digests of *T. brucei* kDNA shows maxicircle fragments in the upper part of the gel and more prominent minicircle bands in the lower regions (panel I). The corresponding autoradiogram (panel II) shows only maxicircle fragments hybridizing, a picture similar to what we have seen in the insect trypanosome *C. luciliae* (Hoeijmakers and Borst, 1978). This hybridization is specific and not attributable to nuclear
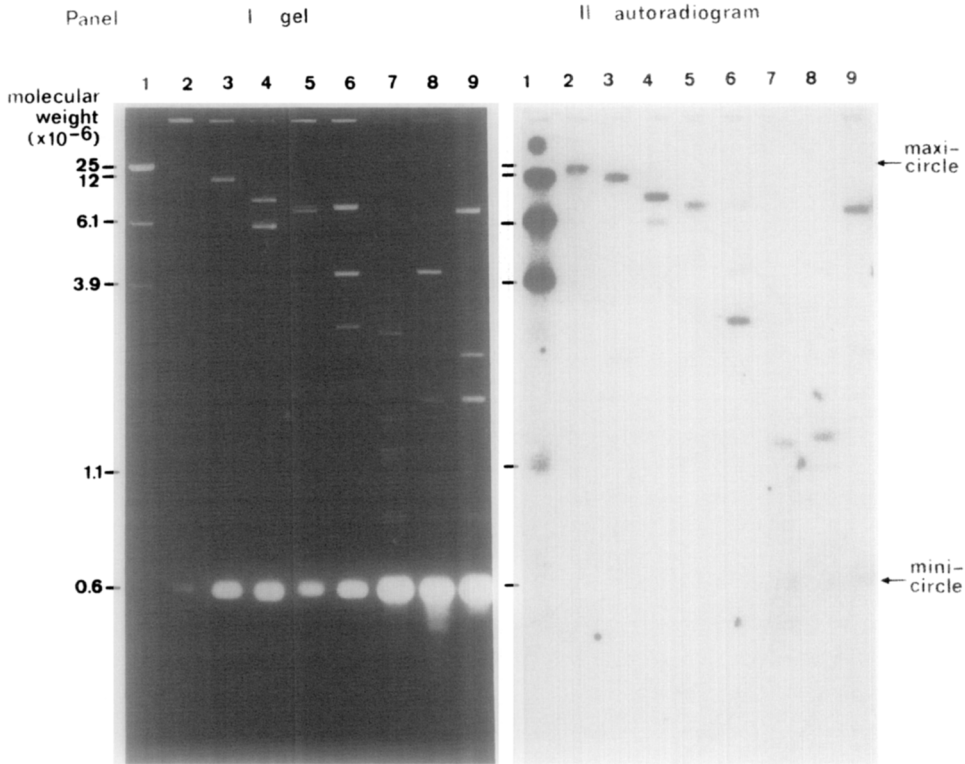
FIG. 1. Hybridization of total cellular RNA from bloodstream *T. brucei* with restriction fragments of kDNA. kDNA (0.25 μg, panel I) of *T. brucei* was electrophoresed in 0.8% agarose after digestion with the following restriction endonucleases. Lanes: 2, *Sst*I; 3, *Hae*III; 4, *Hap*II; 5, *Bgl*I; 6, *Eco*RI; 7, *Alu*I; 8, *Mbo*I; 9, *Sau* 96-I. The *Alu*I digestion is not complete. The DNA was denatured *in situ*, transferred to a nitrocellulose filter by the Southern technique, and hybridized with *in vitro* labeled total cellular RNA from *T. brucei* bloodstream form. Panel II shows the autoradiogram of the gel of panel I (after 2 weeks exposure). Lanes 1 contains molecular weight marker set II.

DNA contamination in the kDNA, because nuclear DNA digested with the same set of enzymes gives entirely different hybridization patterns (not shown). The most prominently hybridizing maxicircle fragment (Fig. 1, lanes 7–9) is localized on the *T. brucei* maxicircle map between sites D3 (*Hin*dIII) and E2 (*Hae*III) (see Fig. 2), covering a region of 1.8–2.0 kb (see also Borst and Hoeijmakers, 1979b). An analogous predominantly transcribed maxicircle segment of only 1.5 kb is found in *C. luciliae* and *C. fasciculata* (unpublished observations).

2. To obtain more information on the RNA(s) that hybridize to kDNA fragments, we have carried out "Northern" or "RNA blot" hybridization experiments using elec-

trophoretically fractionated, filter-bound RNA and *in vitro* labeled kDNA probes. Upon electrophoresis in agarose gels, total cellular RNA of *T. brucei* shows three high- and at least five low-molecular-weight components (Fig. 3, panel I, lane 3). This unusual profile resembles that of other trypanosomatids like *Crithidia* (panel I, lane 2) in which case it has been demonstrated that the rRNA from the large subunit of cell-sap ribosomes contains *in vivo* at least one—possibly more—hidden breaks (Spencer and Cross, 1976; Garvin *et al.*, 1978; Morales and Roberts, 1978; Gray, 1979). Similarly, the large rRNA of *T. brucei* cell-sap ribosomes disappears upon heat denaturation and generates at least two lower-molecular-weight RNA components, indi-
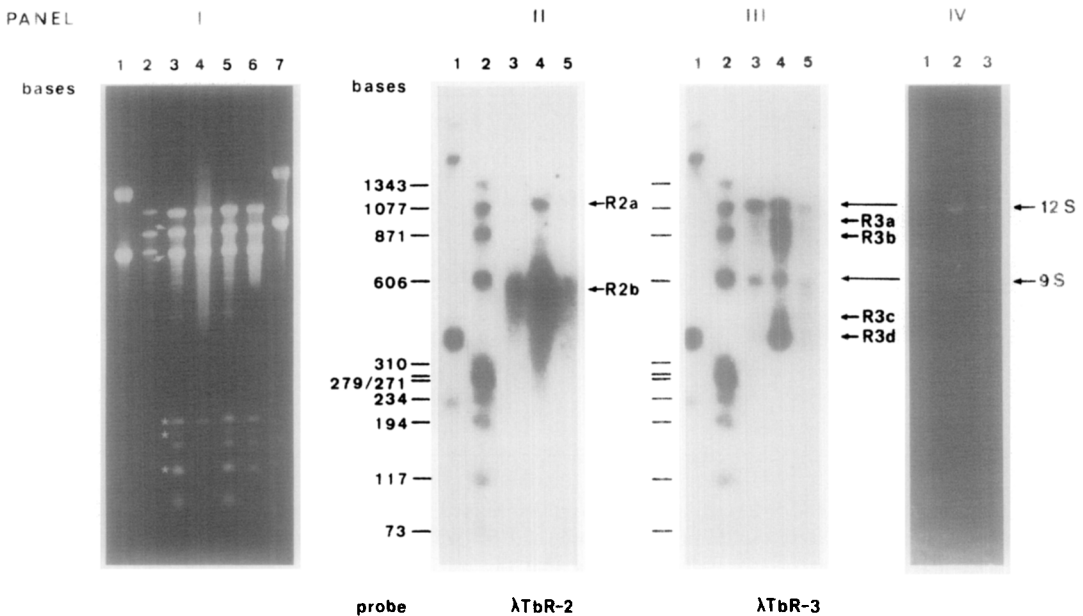
FIG. 2. Partial transcription map of the maxicircle of *T. brucei* 427. The location of the recognition sites for most of the enzymes is from Borst and Fase-Fowler (1979). The position of (some of) the *Taq*I and *Mbo*I sites is deduced in part from experiments presented here or elsewhere (Borst *et al.*, 1980b) and in part from unpublished experiments. Interrupted regions indicate maxicircle segments with many sites (for the enzyme bordering the region) of which the exact position is not known. The genes for the 9 S and 12 S RNA species and Tck-1, -2 and Tck-3 are indicated by the heavy bars. The location of the genes for the other RNA species (indicated with °) is not further determined within RR-2 or RR-3 and they are, therefore, placed arbitrarily. The direction of transcription of the 9 S and 12 S RNA genes, deduced from Figs. 7 and 8, is indicated by an arrow. For a detailed map of this region including also sites for the enzymes *Hinf*I and *Mbo*II, we refer to Fig. 11. Polymorphic sites (Borst *et al.*, 1980b) are connected to the circle by an interrupted line. (*) Indicates the polymorphic site absent in *T. brucei* 427. The size of the RR-3 fragment is reduced to 4 kb (instead of the 4.7 kb of Fig. 11) to fit earlier size determinations; all fragments within RR-3 are reduced by the same factor. Details on the "variable region" can be found in Borst *et al.* (1980b). B, *Bam*HI; D, *Hind*III; E, *Hae*III; G, *Bgl*II; L, *Hha*I; M, *Mbo*I; Ps, *Pst*I; Q, *Taq*I; R, *Eco*RI; Ss, *Sst*I; U, *Sau* 96I; Xb, *Xba*I; Y, *Hap*II (from Borst *et al.*, 1980a).

FIG. 3. Hybridization of size-fractionated RNA of *T. brucei* (bloodstream form) with cloned maxi-circle fragments. 3 µg of total cellular RNA, RNA enriched in poly(A)$^+$ RNA by two cycles of oligo(dT)-cellulose chromatography (poly(A)$^+$ RNA) and the unbound fraction (poly(A)$^-$ RNA) from *T. brucei* bloodstream form was denatured by glyoxal treatment (except for panel I), electrophoresed in 1.8% agarose, and transferred to activated DBM paper. The filter-bound RNA was hybridized with ^{32}P-labeled lambda·TbR-2 and lambda·TbR-3 maxicircle probes. Panel I: the ethidium-stained gel containing total cellular RNA from *C. luciliae* (lane 2); *T. brucei* bloodstream form (3); the corresponding poly(A)$^+$ (4) and poly(A)$^-$ RNA (5); RNA from purified *T. brucei* cell-sap ribosomes (6); as molecular weight markers *E. coli* rRNA (1 and panel IV, lane 1) and yeast rRNA (7). To visualize the RNA species better, these samples were not treated with glyoxal prior to layering. The arrowheads in lane 3 indicate RNA fragments that are generated from the large subunit rRNA upon heat denaturation of the RNA. Asterisks point to RNA species found to be associated with rRNAs upon size fractionation on a Sephacryl S300 column of total cellular RNA (unpublished). Panels II and III: the autoradiograms of filter-bound RNA after hybridization with lambda·TbR-2 (panel II) and lambda·TbR-3 (panel III). The RNAs were: lanes 3, total cellular RNA; 4, poly(A)$^+$ RNA; 5, poly(A)$^-$ RNA (in lane 5 of panel III less RNA was layered). Prior to electrophoresis the RNA samples were denatured by treatment with glyoxal. After ethidium staining they show a pattern as depicted in lanes 2 and 3 of panel IV. The arrows indicate the position of the bands of the presumptive 9 S and 12 S mtRNAs. The ^{32}P-labeled molecular-weight markers are: lane 1, phage φX174 DNA (RF) digested with BspI; 2, digested with MspI. The gel of panel IV is from a separate run.

cated in lane 3 by arrowheads (experiments not shown). In fact, the results with RNA extracted from purified ribosomes indicate that all prominent RNAs (except for the 4 S RNA) are of ribosomal origin (lane 6, see also Cordingley and Turner (1980) and Simpson and Simpson (1978)). Total cellular RNA, enriched in poly(A)$^+$ RNA by two cycles of oligo(dT) chromatography is electrophoresed in lane 4, the corresponding, unbound fraction (poly(A)$^-$) in lane 5 of Fig. 3.

Glyoxal-denatured RNA (McMaster and Carmichael, 1977) with the profile shown in panel IV (lanes 2 and 3) was transferred to activated DBM paper. To detect maxicircle transcripts, the filter-bound RNA was hybridized with two lambda·gt·*WES*·kDNA recombinants, containing the second and third *Eco*RI maxicircle fragment (R1R2-2 and R3R1-3 in Fig. 2) (Brunel *et al.*, 1980), further designated as lambda-TbR-2 and lambda-TbR-3, respectively. The cloned fragments together cover slightly more than

TABLE 1

Properties of Maxicircle Transcripts

Designation RNA species	Apparent size[a]	Binding to oligo(dT)	Hybridization with	cDNA clone	Size (bp)
12 S[b]	1080	−	RR-3		
9 S[b]	590	−	RR-3		
R-2a	1110	+	RR-2		
R-2b	570	+	RR-2	Tck-1,-2	540,540
R-3a	920	+	RR-3		
R-3b	780	+	RR-3		
R-3c	440	+	RR-3		
R-3d	360	+	RR-3		
R-3e	?	?	RR-3	Tck-3	470

[a] In nucleotides.

[b] The size of 9 S and 12 S RNAs in *Leishmania* determined on methylmercurihydroxide gels is 522 and 1022 nucleotides, respectively (Simpson and Simpson, 1978). We attribute the difference with our *T. brucei* values to differences in gel systems. It is possible that the true size of these RNAs is larger, as we have recently observed that the DNA fragments protected against S_1 nuclease by hybridization with 9 S and 12 S RNAs are about 640 and 1230 bp, respectively.

50% of the maxicircle. The smallest *Eco*RI fragment (RR-3; see Fig. 2), which shows the strongest hybridization in Fig. 1 (lane 6), hybridizes with two major and at least four minor RNA species (Fig. 3, panel III, lanes 3 and 4). RR-2 hybridizes only with two minor RNAs (Fig. 3, panel II).

Table 1 compiles some of the properties of these RNA molecules. We presume that the two major RNAs are identical to the 9 S and 12 S RNAs described by Simpson and Simpson (1978) in *Leishmania* mitochondria for the following reasons:

1. They are similar in size (see Table 1).

2. They are the most abundant (mitochondrial) RNAs (Fig. 3).

3. They are not specifically retained on oligo(dT)-cellulose (in contrast to all minor RNA species; Fig. 3).

4. After glyoxal denaturation, which blocks GC base pair formation, the 9 and 12 S RNAs stain relatively stronger with ethidium bromide than the other RNAs (compare panels I and IV of Fig. 3). This suggests that they have a high A + U content (as reported for the 9 S and 12 S RNA (Simpson and Simpson, 1978)) that stabilizes their secondary structure.

Comparison of Bloodstream and Culture form kRNA

In the mammalian bloodstream, *T. brucei* relies for its energy supply on glycolysis alone and CN^--sensitive mitochondrial respiration is completely absent. Mitochondrial respiration and oxidative phosphorylation are fully developed in the insect as well as in culture (see Borst and Hoeijmakers, 1979a). To see whether a correlation exists between mitochondrial activity and the transcription level of kDNA we compared in Fig. 4 the hybridization of total cellular RNA from bloodstream-form and culture-form trypanosomes to filter-bound DNAs. For proper comparison of both kRNA hybridizations (lanes 2–4), the autoradiograms of panel II (hybridization with culture form RNA) and panel III (hybridization with bloodstream form RNA) were exposed to give the same hybridization intensity with the nuclear DNA (lanes 5–7). Clearly, the overall level of kDNA transcripts is considerably increased in culture-form RNA and again only maxicircle transcripts are found.

Comparison of panels II and IV (which is a longer exposure of the kDNA part of panel III) suggests that the concentration of some

kRNAs is affected more than of others (arrowheads). The significance of this is doubtful, however, since the relative hybridization intensities to different maxicircle parts varied sometimes also between different bloodstream-form RNA preparations (compare Fig. 1, lane 8, and Fig. 4, lane 3 of panel IV). The lack of specific differences between bloodstream- and culture-form RNA is confirmed by the "Northern" hybridization experiment presented in Fig. 5 (panels I and II). This shows that the concentration of all RNAs is affected to about the same extent at least with the two *Eco*RI maxicircle fragments used as probes (for the hybridization with lambda·TbR-2 compare also Fig. 3, panel II).

Characterization of Recombinant Plasmids Containing Copy DNA Complementary to Maxicircle Transcription Products

To study the precise genetic location, synthesis, and possible function of some of the transcription products identified above we have screened an *Escherichia coli* clone bank, constructed for the isolation of plasmids containing DNA complementary to variant surface glycoprotein (VSG) messenger RNAs (mRNAs) (Hoeijmakers *et al.*, 1980) for the presence of recombinants with kDNA transcript sequences. This cDNA bank was made as described under Materials and Methods starting from total poly(A)$^+$ RNA of bloodstream-form *T. brucei* which (as shown in Fig. 3, panels II and III, lanes 4) contains also maxicircle transcripts. Among the approximately 4×10^3 transformants screened in this way we have found 11 (weakly) hybridizing colonies (i.e., 0.3%). Plasmid DNA from three of these recombinants (Tck-1, -2, and -3) was prepared for further analysis. Since Tck-1 and Tck-2 were found to be cDNA clones derived from the same RNA, we present only the results for Tck-1 and Tck-3. The partially purified hybrid plasmids were characterized by re-
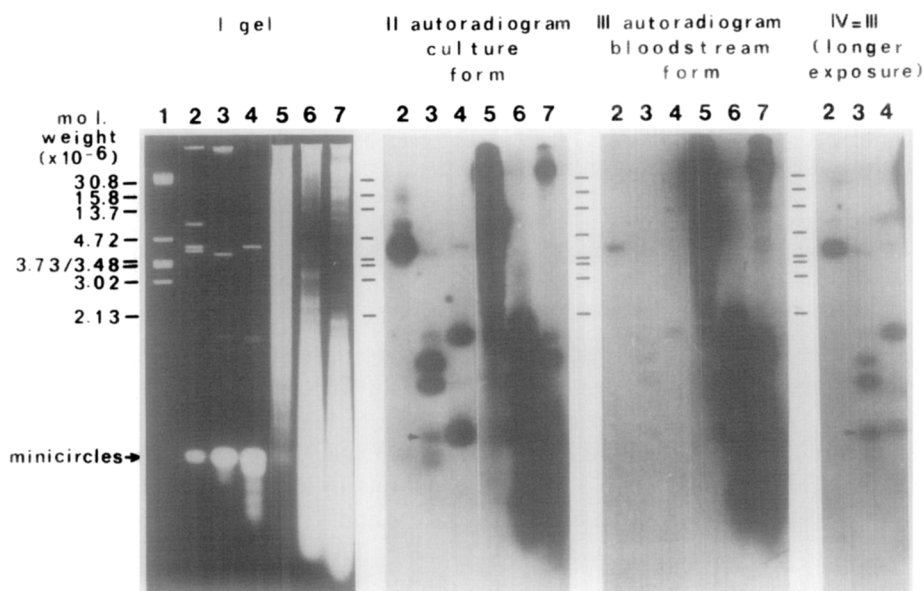

FIG. 4. Comparison of the hybridization of *T. brucei* culture-form and bloodstream-form RNAs with restriction fragments of kDNA and nuclear DNA. kDNA (0.25 μg) and nuclear DNA (1 μg) from *T. brucei* was digested with: lanes 2 and 5, *Hin*dIII; 3 and 6, *Mbo*I; 4 and 7, *Taq*I; and electrophoresed in 0.8% agarose together with molecular-weight marker set I. The *in situ* denatured DNA was blotted onto nitrocellulose filters and hybridized with ^{32}P-labeled total cellular RNA from *T. brucei* culture form (panel II) and bloodstream form (panels III and IV). The autoradiogram of panel IV is exposed about 3.5 times longer than that of panel III. The arrowheads indicate bands with a relative hybridization intensity that differs in bloodstream-form and culture-form RNAs.
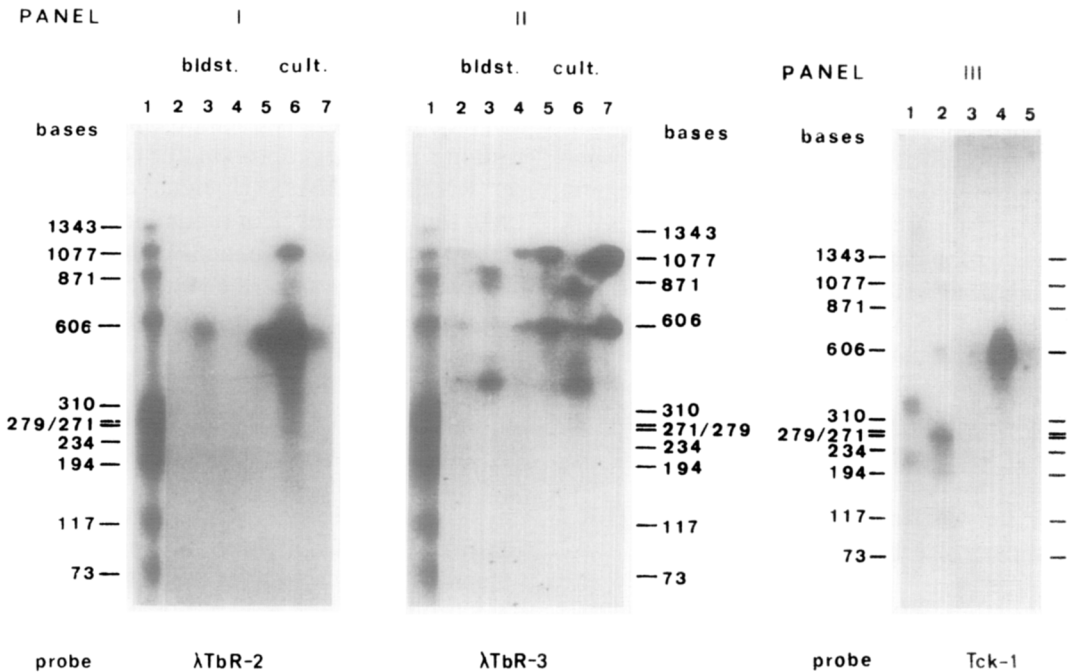
FIG. 5. Comparison of the hybridization of size-fractionated RNA from *T. brucei* bloodstream-form and culture-form with cloned maxicircle fragments and cDNA clone Tck-1. Panels I and II: lanes 2 and 5, glyoxal-denatured total cellular RNA; 3 and 6, poly(A)+ RNA; 4 and 7, poly(A)− RNA of bloodstream-form (2–4) and culture-form (5–7) *T. brucei*. (The oligo(dT)-cellulose chromatography in this experiment has been more efficient in removal of the 9 and 12 S RNAs from the poly(A)+ RNA fraction than in the poly(A)+ RNA of Fig. 3.) The RNAs were electrophoresed in 2.0% agarose, transferred to DBM paper, and hybridized with ^{32}P-labeled lambda·TbR-2 and lambda·TbR-3 maxicircle probes (panels I and II, respectively). In each lane 3.0 μg of RNA is layered except for lanes 5 and 6 where approximately 1 μg of RNA was used. Panel I shows the autoradiogram after hybridization with the RR-2 cloned maxicircle fragment (exposure time about 2 weeks); panel II after hybridization with RR-3 (exposure time 5 days). Lane 1, marker set III, labeled by polynucleotide kinase and [α-^{32}P]ATP. Panel III, RNAs corresponding with lanes 5–7 of panels I and II were electrophoresed and blotted as above. Hybridization of the filters was with labeled Tck-1. Lanes 1 and 2, marker sets IV and III, respectively, labeled as above.

striction enzyme analysis. Digestion with *Pst*I, which cleaves at the borders of the inserts (Bolivar *et al.*, 1977), generates two main bands: the linearized plasmid and the insert band (Fig. 6, panel I, lanes 3 and 9). The sizes of the inserts are given in Table 1.

By digesting the plasmids with endonucleases *Taq*I, *Mbo*I (only with Tck-3), and *Mbo*II (only with Tck-1) either alone or in combination with *Pst*I we have constructed the physical maps shown in the lower part of Fig. 11 (experiment presented in part in Fig. 6, in part not shown). The electrophoresed plasmid DNA fragments shown in Fig. 6 were denatured *in situ*, blotted onto nitro-

cellulose filters, and hybridized with labeled lambda·TbR-2 and -3 maxicircle probes. Figure 6, lanes 8 and 13 (corresponding to lanes 3 and 9, respectively) show that Tck-1 contains in insert homologous to the RR-2 probe and Tck-3 to the RR-3 probe, respectively. The RNAs from which the cDNA clones were derived were identified by Northern hybridization experiments. The autoradiogram of Fig. 5 (panel III) shows that Tck-1 is complementary to kinetoplast RNA R-2b, the main RNA component specified by RR-2 (compare lane 6 of panel I). The same was found for Tck-2 (not shown). From Table I we conclude that both Tck-1

and Tck-2 cover more than 90% of the corresponding RNA, disregarding the contribution of the GC tails which usually do not exceed 30 base pairs (bp). The fact that Tck-1 and Tck-2 do not show hybridization with kinetoplast RNA R-2a suggests that this RNA is not a precursor of R-2b.

All attempts to identify in the same way the RNA from which Tck-3 was derived have failed. The most likely explanation for this is that the steady-state level of this transcript in the cell is too low for it to be detected by the Northern technique. This indicates at least that it is not one of the RNAs R3a–d, because these can easily be detected in similar hybridization experiments using lambda·TbR-3 as probe (Fig. 3, panel III). More sensitive techniques (like protection against degradation by S_1 nuclease (Berk and Sharp, 1977)) are required to confirm this.

Determination of the Direction of Transcription of the 9 S and 12 S RNA on the Maxicircle

The direction of transcription of the 9 S and 12 S RNA genes on the maxicircle was determined as follows: 9 S and 12 S RNA were purified by preparative agarose electrophoresis of glyoxal-denatured culture form RNA, followed by excision of the ethidium-stained RNAs (cf. Fig. 3, panel IV). Light and heavy chains of lambda-TbR-3 were separated by electrophoresis in the presence of poly(UG) (Goldbach *et al.*, 1978a) and the separated strands hybridized with *in vitro* labeled 9 S and 12 S RNA. Both RNAs hybridize with the RR-3 strand that is inserted in the strand of lambda·gt·*WES* with the highest mobility in gels (Fig. 7). The faster migrating strand corresponds to the light lambda chain (i.e., the strand with the
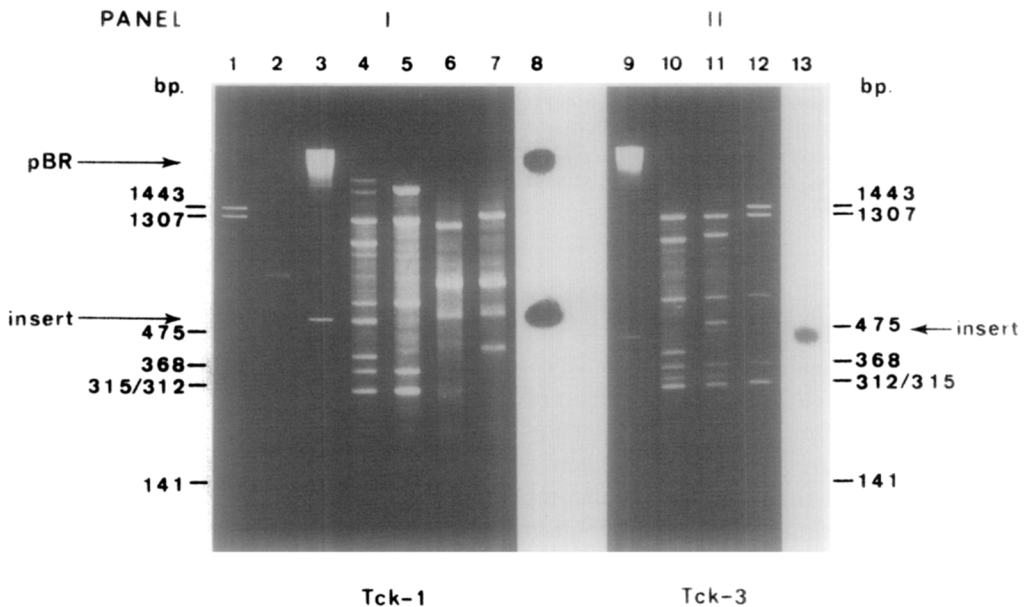

FIG. 6. Characterization of recombinant plasmids containing cDNA from maxicircle transcripts by restriction enzyme analysis and hybridization. 0.2–0.4 µg of the (partly pure recombinant) plasmid DNAs Tck-1 (3–7), Tck-3 (10, 11), and pBR322 (1, 2, 12) were digested with *Pst*I (3, 9), *Taq*I (1, 5, 11, 12), and *Taq*I + *Pst*I (4, 10), *Mbo*II (2, 7), and *Mbo*II + *Pst*I (6), and electrophoresed in 1.75% agarose. After denaturation the DNA fragments were blotted onto nitrocellulose filters and hybridized with lambda·TbR-2 (8) and lambda·TbR-3 (13); only the autoradiograms of lanes 3 and 9 are shown (lanes 8 and 13, respectively). The *Pst*I digestion in lane 3 is incomplete, as shown by the hybridization of the pBR band. For unknown reasons our *Mbo*II enzyme did not cleave the pBR322 DNA when a TC dinucleotide followed the GAAGA recognition sequence.
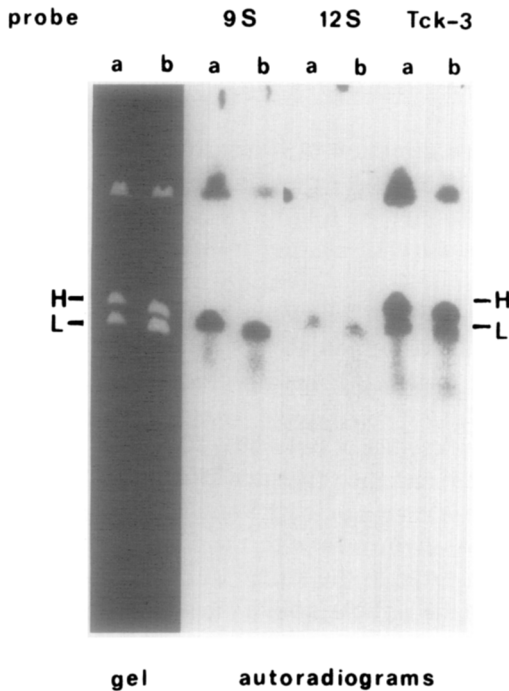
probe   9 S   12 S   Tck-3

   a   b   a   b   a   b   a   b

H—
L—
                        —H
                        —L

gel   autoradiograms

FIG. 7. Determination of the codogenic strand for 9 S and 12 S RNA in lambda·TbR-3. Lambda·TbR-3 (0.4 μg) was briefly heated in the presence of poly(UG) (6 μg for lanes a and 3 μg for lanes b) and then electrophoresed in 0.3% agarose to separate the strands. The DNA was transferred to nitrocellulose filters and hybridized with labeled 9 S RNA, 12 S RNA, or lambda·TbR-3 DNA as indicated. H, heavy strand; L, light strand.

5'-terminus at the left lambda arm (see Fig. 8 and Hradecna and Szybalski, 1967), assuming that the light and heavy lambda DNA strands are not reversed by the deletion of the middle lambda portion and the insertion of the RR-3 maxicircle fragment. (Sequence analysis of more than 50% of RR-3 indicates that there is no unequal T + G distribution over both strands (I. C. Eperon, personal communication).) The control experiment with double-stranded cDNA clone Tck-3 shows that both strands are able to hybridize (Fig. 7); the hybridization with the high-molecular-weight band is due to the presence of native lambda·TbR-3.

In principle the RR-3 fragment can be inserted in the lambda·gt·WES vector in two orientations, depicted in Fig. 8 as alterna-

tives a and b. Because of the asymmetrical location of the single HindIII site in RR-3 (see Fig. 2, and Borst and Fase-Fowler, 1979) we expect upon cleavage with this enzyme a hybrid fragment of about 8.6 kb in case a and of 6.6 kb in case b (see Fig. 8). The position of the HindIII sites in lambda DNA is deduced from Daniels et al. (1980) and Leder et al. (1977). The HindIII digest in Fig. 8, lane c-1, shows the presence of the 6.6-kb band as predicted from orientation b, whereas an 8.6-kb fragment is absent. Hybridization with Tck-3 (lane c-2) confirms that the 6.6-kb band is indeed one of the expected hybrid fragments, ruling out alternative a. Since the orientation of RR-3 in the maxicircle with respect to the HindIII site is known (Fig. 2), the direction of transcription must be as indicated in Figs. 2 and 11. The 9 S and 12 S RNA hybridization with the 24 kb-hybrid fragment (Fig. 8, lanes c-3 and c-4) confirms that they are coded by the region clockwise from D3. On the other hand, Tck-3 must reside (mainly) on the other side of D3 as deduced from the hybridization intensities. In the next section we will determine more precisely the position of these genes.

Construction of a Detailed Physical Map of the 9 S and 12 S RNA Region and the Genes Coding for Tck-1, Tck-2, and Tck-3

Since HindIII and BspI (isoschizomer of HaeIII) cleave close to the borders of the 9 S and 12 S RNA region at approximately 2-kb distance from each other (see Fig. 2), we have mainly used these enzymes to position the sites for other endonucleases in this maxicircle fragment. Figure 9 shows gels (panel I) of kDNA digested with Hinf1 (part A), TaqI (part B), and MboII (part C) in combination with BspI or HindIII. The corresponding autoradiograms, after hybridization with 9 S RNA, 12 S RNA, Tck-3, lambda·TbR-3, Tck-1, and lambda·TbR-2, are shown in panels II–VII, respectively. Results with MboI are shown in Fig. 10, lanes 4–7. On the basis of these data the
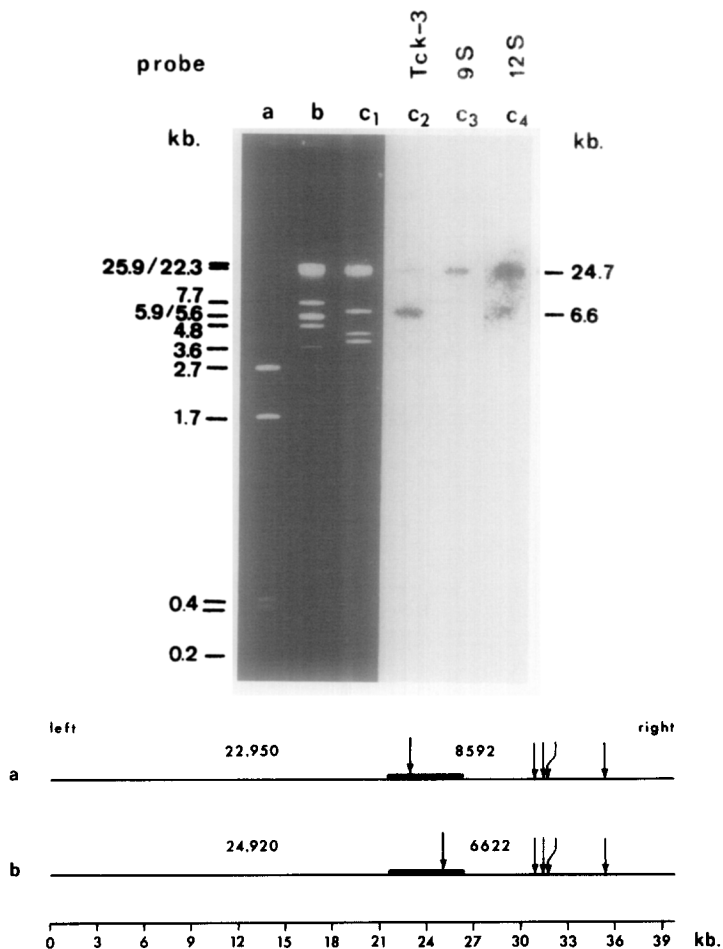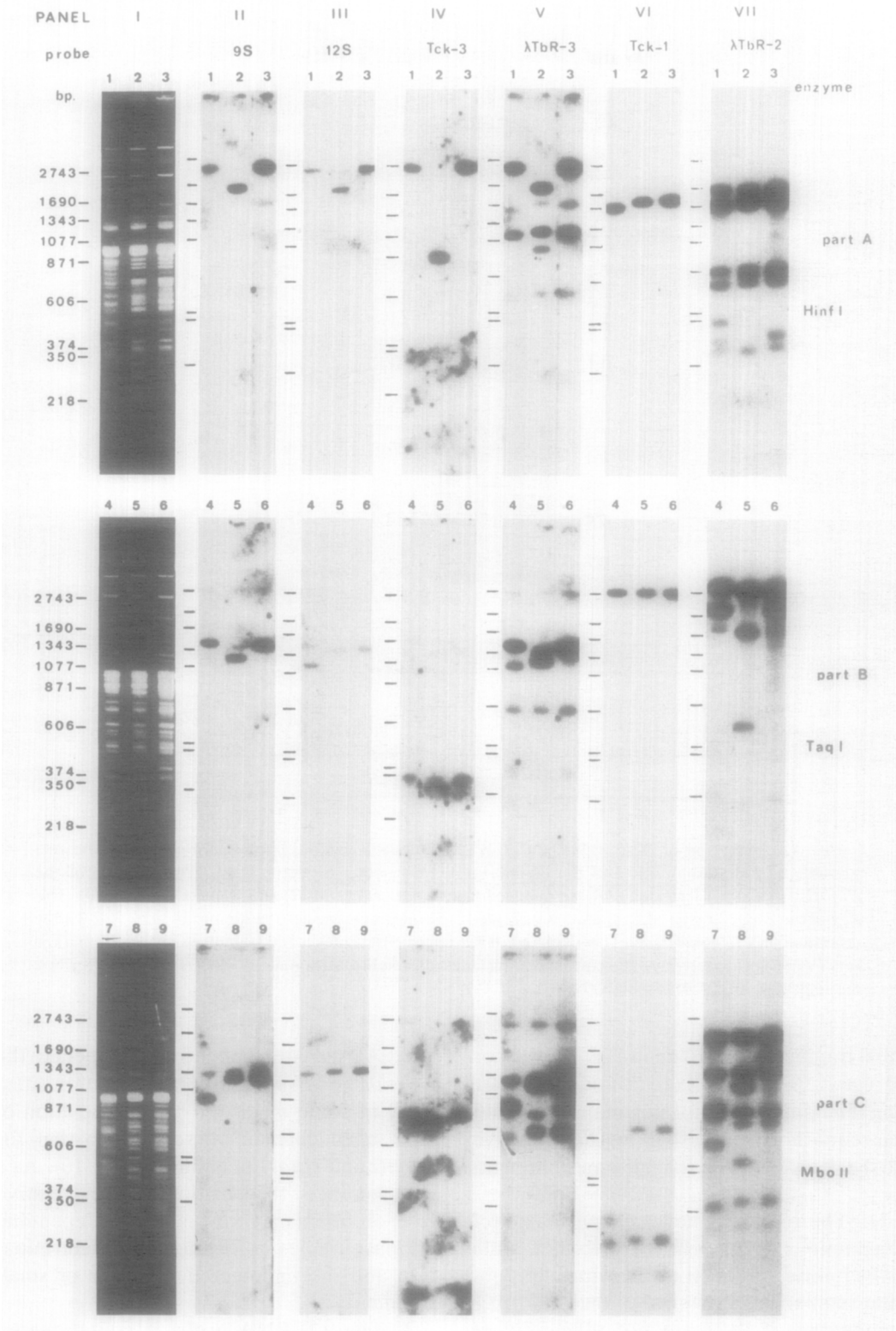
FIG. 8. Orientation of RR-3 in lambda·gt·*WES*–lambda·b vector. The bottom part gives the two possible orientations of RR-3 in the lambda·gt·*WES*–lambda·b vector DNA. The position of the *Hin*dIII sites in the lambda DNA are deduced from Leder *et al.* (1977) and Daniels *et al.* (1980). The size of the expected hybrid fragments is indicated. The 0.6% agarose gel containing lambda·TbR-3 after digestion with *Hin*dIII (lane c1) is depicted in the upper part, lanes a and b, molecular-weight marker sets IV and I, respectively. The corresponding lanes after hybridization with Tck-3, 9 S, and 12 S RNA are indicated as c2-c4, respectively.

final fragment map given in Fig. 11 was constructed. An Appendix to this paper describing the construction of this map is available on request. Here we shall restrict ourselves to the main conclusions and some comments.

1. The only way to visualize maxicircle fragments smaller than the 1-kb minicircles is by hybridization. It is, therefore, possible that small fragments (smaller than 200 bp) go undetected if they are not efficiently retained on the nitrocellulose filter.

2. All sites are mapped with respect to the *Hin*dIII and *Bsp*I (*Hae*III) recognition sites. Only in some instances has the position of sites been determined relative to that for *Eco*RI and *Hap*II in addition (Fig. 10). As a consequence, the position of sites relative to each other may vary slightly, depending on inaccuracies in fragment size determination and the undetected presence of small fragments.

3. The 9 S RNA probe is contaminated with the comigrating R-2b RNA (cf. Fig. 3,

panels II and III) and contains in addition traces of degraded 12 S RNA (see e.g., panel II of Fig. 9, lanes 3, 7–9).

4. The *Taq*I map contains some "gaps" indicated in Fig. 11 by a question mark. This is due to the presence of clusters of *Taq*I sites, resulting in many small fragments that could not be located precisely as explained in the Appendix.

5. The exact order for the three small *Mbo*II fragments in the gene coding for Tck-1 was not established and the order given in Fig. 11 is arbitrary (see Appendix).

The map of Fig. 11 allows a fairly accurate determination of the borders of the single genes for 9 S and 12 S RNA. The 12 S gene should start just beyond D3 and end close to N4. The space between the 9 S and 12 S genes should be less than 350 bp since the 9 S hybridization stops before or close to the unique *Bam*HI site.

An interesting point following from the map is the hybridization of Tck-3. As explained in the Appendix, the Tck-3 hybridization extends to the left of *Mbo*II site N2 and *Mbo*I site M7 and to the right somewhere between M8 and D3: the *Taq*I fragment Q10Q11 (1170 bp), as well as the *Mbo*I fragment M8M9 (2300 bp), give clear hybridization (see Fig. 10, parts A and B). In some experiments a very weak hybridization is even found with a region beyond D3 (see Fig. 8); in the cDNA itself, however, the *Hin*dIII site is absent (data not shown). This demonstrates that the Tck-3 insert (460 bp) hybridizes with a region of approximately 800 bp. This is illustrated for instance by the hybridization of Tck-3 to the *Mbo*I digest (Fig. 10, part B, lane 4). Main hy-

bridization is found with the 640-bp fragment (M7M8-7); weaker but distinct hybridization is observed with M8M9-3 (2300 bp) and M6M7-8 (460 bp). The most likely interpretation is that the gene from which the cDNA is derived contains intervening sequences that are removed from the primary transcript during processing. A preliminary map of the Tck-3 insert (Fig. 11, bottom line) shows that one *Mbo*I and probably one *Taq*I site are missing: one of the two *Taq*I sites in the cDNA is very close to the border and the region on the left-hand side (about 40 bp) is too short to form stable hybrids under the conditions used, especially when the GC linker is subtracted (approximately 20 bp in other inserts from the same bank). The most logical interpretation is, therefore, that *Taq*I sites Q8 and Q10 are conserved, but that the middle Q9 site is absent in the cDNA. Similar results were obtained with *Mbo*I (*Sau*IIIA) for which two sites were expected in the cDNA whereas only one was found (Fig. 11). On the basis of these preliminary data it is not possible to localize the genomic region(s) that is (are) absent in the cDNA. On the other hand, all results with Tck-1 (and also 9 S and 12 S RNA) are compatible with continuous transcripts from these genes.

DISCUSSION

Our hybridization studies on kDNA transcription in *T. brucei* have confirmed that the maxicircle is the genetically active component in the kDNA network and have led to the identification of at least eight discrete maxicircle transcripts, which are indicated on the partial transcription map of Fig. 2.

FIG. 9. Construction of a detailed physical map of the regions containing the genes for 9 S and 12 S RNA and Tck-1 and Tck-3 for the enzymes *Hin*fI, *Mbo*II, and *Taq*I. kDNA (0.5 μg) was digested with: lanes 1–3, *Hin*fI; 4–6, *Taq*I; 7–9, *Mbo*II in single (lanes 3, 6, and 9) and double digestion with *Bsp*I (lanes 1, 5, and 7) and *Hin*dIII (lanes 2, 4, and 8). Three parallel gels with slight variations in DNA migration were used. After transfer of the denatured DNA to nitrocellulose filters hybridization was carried out with the six ^{32}P-labeled probes indicated. Panel II, 9 S RNA; panel III, 12 S RNA; panel IV, Tck-3; panel V, lambda·TbR-3; panel VI, Tck-1; panel VII, lambda·TbR-2. The filters of panel II were reused for hybridization with lambda·TbR-3 (panel V), after removal of bound radioactivity by low-salt washing.
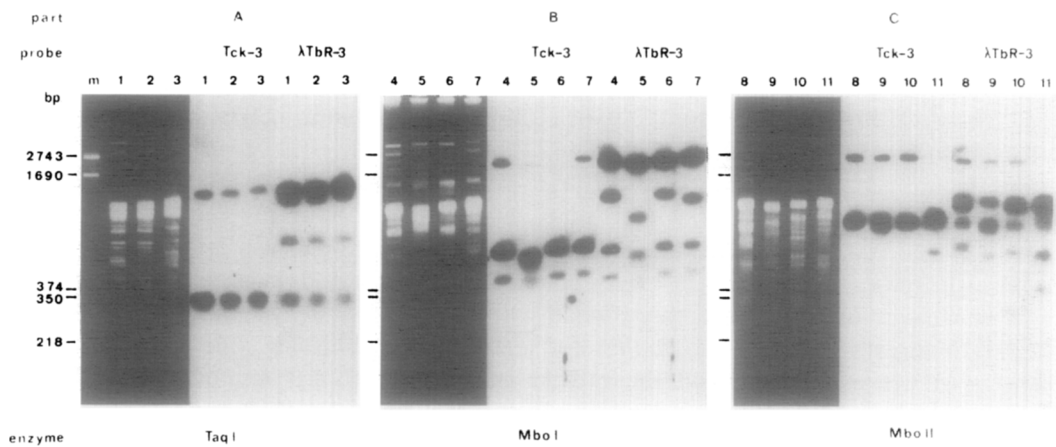
FIG. 10. Construction of a detailed physical map for RR-3 for the enzymes MboI, MboII, and TaqI. kDNA (0.3 μg) was digested with: lanes 1-3, TaqI; 4-7, MboI; 8-11, MboII in single digestions (lanes 1, 4, and 8) and in combination with BspI (lanes 5 and 9), HindIII (lanes 6 and 10), EcoRI (lanes 3, 7, and 11), and HapII (lane 2). The corresponding hybridizations with ³²P-labeled Tck-3 and lambda·TbR-3 are indicated. Marker set III is electrophoresed in lane m.

The 9 S and 12 S RNAs Are Mitochondrial rRNAs

The two major RNA species detected in RNA and DNA blots are the equivalent of the 9 S and 12 S RNAs discovered by Simpson and Simpson (1978) in the kinetoplast fraction of *Leishmania tarentolae*. The proposal that they represent mitochondrial rRNAs (Borst and Hoeijmakers, 1979a) is now supported by the following findings:

1. The 9 S and 12 S RNAs are the only major large RNAs found in trypanosome mitochondria. In all organisms analyzed thus far the major mitochondrial RNAs are the rRNAs.

2. The 9 S and 12 S RNAs are found in approximately equimolar amounts (see Fig. 3, panel III, lane 3).

3. Their sequence and size are strongly conserved in trypanosome evolution. Cheng and Simpson (1978) report no apparent differences between the RNAs from *Leishmania tarentolae* and *Phytomonas davidi*. We have found that the 9 S and 12 S RNAs of *T. brucei* and *C. luciliae* readily cross-hybridize and have the same size (unpublished). Similar results were recently obtained for *T. brucei* and *L. tarentolae* by

A. M. Simpson and L. Simpson (personal communication).

4. In contrast to the other maxicircle RNAs, they are not selectively bound to oligo(dT), indicating the absence of long poly(A) tails.

5. There is only one gene for 9 S RNA and one for 12 S RNA and these are adjacent and encoded by the same strand (Figs. 2 and 11). Whether the two RNAs are synthesized from a common precursor remains to be determined.

6. We have provided Mr. Ian C. Eperon (M.R.C., Cambridge, U. K.) with the DNA segment containing the 9 S and 12 S genes and he has found that this segment shows homology with the rRNAs of human mitochondria and *E. coli* (personal communication).

We conclude from these data that the 9 S and 12 S RNAs are the major RNAs of the mitochondrial ribosome. With an apparent combined size of 1080 + 590 = 1670 nucleotides they are the smallest rRNAs known in nature, even much smaller than the mitochondrial rRNAs of *Drosophila* (Klukas and Dawid, 1976) and vertebrates (Dawid, 1972). This raises the possibility that the trypanosomal mitochondrial ribosome has unusual

direction of transcription

R_2R_3-1 R_3R_1-3 R_1R_2-2

B Bam HI
D Hind III (Bsp I)
E Hae III (Bsp I)
M Mbo I (Sau 3A)
N Mbo II
Ps Pst I
Q Taq I
R Eco R I
Y Hap II

a hybridization with Tck-3
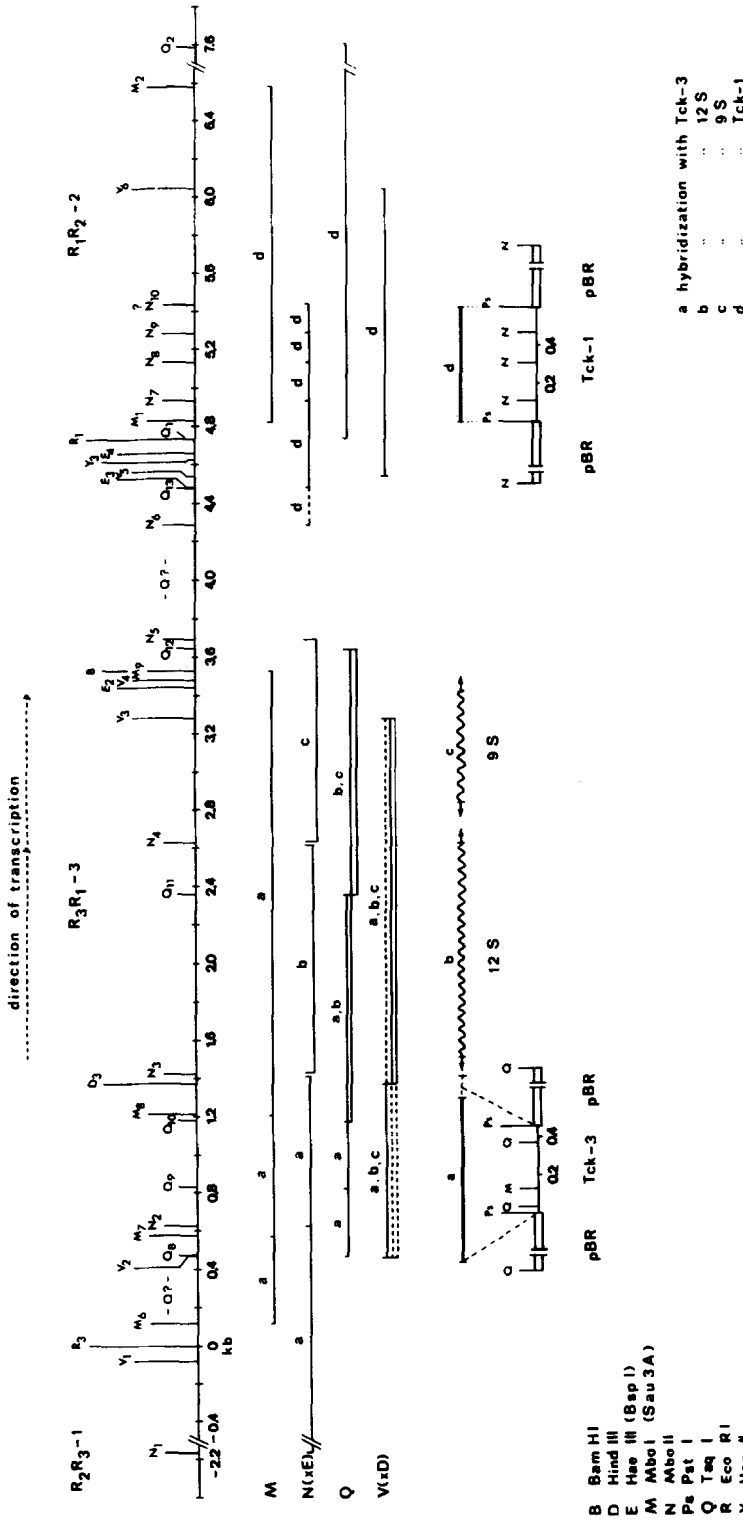b " " 12 S
c " " 9 S
d " " Tck-1

FIG. 11. A detailed physical map of the maxicircle fragment RR-3 and the region hybridizing with Tck-1. The position of the restriction cleavage sites and the genes for the 9 S and 12 S RNA species and Tck-1 and Tck-3 are deduced from the experiments presented in Figs. 9 and 10. The argumentation on which the map is based can be found in the Appendix. The regions hybridizing with each probe are indicated below the map. The arrows show the direction of transcription of the 9 S and 12 S RNA genes as determined in Figs. 7 and 8. The physical maps for Tck-1 and Tck-3 are based in part on the digests presented in Fig. 6 and in part on unpublished experiments. Uncertain sites are indicated by a question mark. The designation of sites is explained under Materials and Methods.

properties (e.g., a very high protein/RNA ratio) and this would explain why we and others have been unable to isolate it thusfar and why we have been unable to find evidence for mitochondrial protein synthesis in *Crithidia* using classical inhibitors of mitochondrial translation like chloramphenicol, ethidium, or spiramycin (Kleisen and Borst, 1975).

There are two other unusual features of the 9 S and 12 S rRNAs: the large RNA is 5' to the small one, an arrangement also recently found in *Leishmania* mitochondria (L. Simpson, personal communication), but not in animal mitochondria or elsewhere in nature (Wolstenholme *et al.*, 1979) (disregarding the unusual situation in *Saccharomyces* and *Tetrahymena* mtDNA, where the two genes are far apart (Sanders *et al.*, 1975; Goldbach *et al.*, 1978b)). Another unusual feature is the high AU content of close to 80 mol% (I. C. Eperon, personal communication).

The Minor RNAs

Simpson and co-workers (Simpson and Lasky, 1975; Simpson *et al.*, 1976) have reported that 9 S and 12 S RNA from *Leishmania* stimulated protein synthesis in a wheat germ system. Moreover, in hybridization experiments a large segment of the maxicircle was found to hybridize with both of these RNAs (Simpson and Simpson, 1978; Masuda *et al.*, 1979). Both results can be explained by our observation that at the position of the 9 S and 12 S two other RNA species migrate that will contaminate 9 S and 12 S RNA preparations purified on size. We think that these minor RNAs are mitochondrial messengers or mRNA precursors, because this could explain both the *in vitro* experiments of Simpson and co-workers and the binding to oligo(dT), which suggests the presence of poly(A) tails.

It is remarkable that the RNAs found thus far are so small. Mitochondrial precursor RNAs or mRNAs in yeast are much longer (Grivell *et al.*, 1979; Van Ommen *et al.*, 1979)

and the minimally required coding sequence for, e.g., subunit I of cytochrome oxidase and the apocytochrome *b* protein in *Neurospora* and yeast mitochondria exceeds our longest maxicircle transcript (Borst and Grivell, 1978; Werner and Bertrand, 1979). It is possible that longer transcripts have been missed in our experiments. Their concentration could be below our detection level; they could have been lost specifically during isolation or they could be coded for by the maxicircle fragment RR-1, which has resisted cloning in *E. coli* (Brunel *et al.*, 1980). The latter possibility is not unreasonable because maxicircle transcripts longer than 12 S RNA have recently been found in *Leishmania* (A. M. Simpson and L. Simpson, personal communication). Alternatively, the genes for the larger mitochondrial proteins might reside in the nucleus (like the gene for the DCCD-binding protein in *Neurospora* (Sebald *et al.*, 1977)) or the protein corresponding to subunit I of oxidase and apocytochrome *b* could be smaller or absent in trypanosomes.

From the hybridization data of RR-3 (see Fig. 2) it seems that this maxicircle segment is crowded with genes, a situation that also is found in *Leishmania* (L. Simpson, personal communication). The added molecular weights of all hybridizing RNAs (4170 nucleotides) plus the Tck-3 region (at least 700 bp) even exceed the size of RR-3 (4700 bp). Moreover, this active region extends in RR-2, because the R-2b gene is situated very close to the border with RR-3 (Fig. 11). It is possible that some of these transcripts are precursor or degradation products of others. The presence of interrupted genes on the maxicircle is suggested by the hybridization with Tck-3 (Fig. 11), for which we were unable to detect the corresponding transcript, probably because it is present in very low concentrations. However, the possibility has to be ruled out that rearrangements have occurred during construction of the cDNA hybrid and subsequent growth in *E. coli*, although analysis of 31 (VSG) cDNA clones from the same bank has provided no

indications for such artifacts (Hoeijmakers *et al.*, 1980). The data for the other genes studied (9 S, 12 S RNA and Tck-1) are all compatible with a continuous coding sequence; small intervening sequences could obviously have been missed, however.

No maxicircle tRNAs have shown up in our hybridizations. This is not due to inefficient binding of tRNA-sized molecules to the filter; a 73-bp DNA marker is clearly visible on the autoradiograms (see Figs. 3 and 5). However, it is possible that our hybridization conditions were too stringent for efficient 4 S hybridization since analogous experiments with yeast mitochondrial tRNAs are carried out under conditions equivalent to 9–10°C below our hybridization temperature.

Mitochondrial Biogenesis

The repression of mitochondrial respiration and oxidative phosphorylation in the bloodstream form was shown to correlate with a 5- to 10-fold decrease in the level of maxicircle transcripts. From the RNA blots (Fig. 5) we conclude that all transcripts are affected to approximately the same extent. This cannot readily account for the differential repression of mitochondrial enzymes, the respiratory chain being completely absent, whereas an oligomycin-sensitive ATPase (Opperdoes *et al.*, 1976) which presumably requires mitochondrially synthesized subunits like in yeast (Borst and Grivell, 1978), is still present. There may be, therefore, an additional control at the level of mitochondrial translation or membrane assembly.

Minicircle Transcription

As in our previous studies with *Crithidia* we have not found RNA complementary to the minicircles. In contrast, Fouts and Wolstenholme (1979) have recently reported the detection of a 240-nucleotide RNA, homologous to the heavy strand of the minicircles in *Crithidia acantocephali*. This RNA seemed to be present in the kinetoplast frac-

tion as well as in the cytoplasm in the same (high) concentrations. Such an RNA should have been readily detectable in our experiments if present in *T. brucei* or *C. luciliae*. We cannot account for this discrepancy. Nevertheless, we think that most of the data now available make it unlikely that minicircles code for an essential protein or structural RNA. The high-sequence heterogeneity of minicircles in some species (Steinert *et al.*, 1976; Steinert and Van Assel, 1980; Donelson *et al.*, 1979) and the high rate of sequence evolution of this DNA (Borst and Hoeijmakers, 1979b; Borst *et al.*, 1980b) and the recent results of sequence analysis of minicircles (Chen and Donelson, 1980) support this view. Why trypanosomes have minicircle networks remains to be determined.

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