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Genetics

Differential expression of mitochondrial genes between life cycle stages of *Trypanosoma brucei*

(kinetoplast DNA/transcription/RNA processing/differentiation)

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ABSTRACT **The mitochondrial respiratory system is differentially produced during the life cycle of the parasitic protozoan, *Trypanosoma brucei*. We have found 14 transcripts that are derived from a maxicircle (mitochondrial DNA) region that contains sequences homologous to cytochrome *c* oxidase subunits I and II, unassigned reading frame 1 from mitochondrial DNA of other organisms, and two other open reading frames. Ten of these transcripts occur as pairs that differ in size by ≈200 nucleotides. While most of these transcripts occur in both life cycle forms, four transcripts are differentially expressed. Of these, two are more abundant in bloodstream forms, one is more abundant in procyclic forms, and one is present in procyclic but not bloodstream forms. These results indicate that the differential production of the mitochondrial respiratory system in *T. brucei* entails regulation of mitochondrial transcriptional and/or posttranscriptional processes.**

Trypanosome mitochondria contain a DNA network called kinetoplast DNA (kDNA), which consists of about 5000 minicircles and 50 maxicircles in *T. brucei*. The 1-kilobase (kb) minicircles have not been found to be transcribed and have no known function (1, 2), although recent results suggest that minicircles from the related trypanosomatid *Crithidia fasciculata* encode protein products (3). The ≈20-kb maxicircles are transcriptionally active and have been equated to mitochondrial DNA from other organisms (1, 2, 4–8). Direct sequence analysis shows homology between maxicircle and other mitochondrial genes, confirming this relationship (7, 9–11; unpublished data).

Cytochromes and Krebs cycle enzymes are not detected in bloodstream trypomastigotes but are produced upon differentiation to procyclic trypomastigotes (1). Expression of the maxicircle seems essential for this differentiation because mutants devoid of kDNA (12) and species with natural deletions of maxicircle sequences (13) are unable to differentiate into procyclic forms. We wished to determine if this life cycle differentiation involves an altered state of mitochondrial gene expression. Previous studies demonstrated maxicircle transcription in both stages of the life cycle (4, 5). By a detailed analysis of the transcription of a 3.3-kb region of the *T. brucei* maxicircle, we found four transcripts that are differentially expressed in the two life cycle forms.

MATERIALS AND METHODS

Strains and Culture Conditions. *T. brucei brucei* clone IsTaR 1 of stock EATRO 164 was used in this study for both bloodstream and procyclic forms, the latter of which had been continuously cultured for 10–30 months. The trypanosomes were grown and isolated as described elsewhere (14), frozen in liquid nitrogen, and stored at −80°C.

Nucleic Acid Isolation. RNA was isolated as described by Ross (15), with the exception that ultracentrifugation was in a Beckman SW27.1 rotor at 20,000 rpm for 20–24 hr. Plasmid DNA and single-stranded phage M13 DNA were prepared as described by Birnboim and Doly (16) and Messing (17), respectively. Restriction fragments of pTKHR117 (5), a clone of the region between the second *Hin*dIII site and the third *Eco*RI site (H2R3 region; see map in Fig. 1), were isolated from gels as described (18). The M13 clones used as probes are described in detail elsewhere (11).

Gel Electrophoresis, Blotting, and Hybridization. RNA was electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde (19) and transferred to nitrocellulose filters (20). Plasmid probes were labeled by nick-translation (21). Phage M13 probes were prepared (17) by synthesizing the DNA strand (*i*) complementary to the insert using sequencing primer or (*ii*) complementary to M13 beyond the insert using M13 universal hybridization probe primer (Bethesda Research Laboratories). Prehybridization was at 42°C for 6–18 hr in 50% deionized formamide containing 5× NSPE buffer (0.9 M NaCl/50 mM NaH_2PO_4/5 mM Na_2EDTA, pH 7.4); 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone; 0.1% $NaDodSO_4$; and 100 μg of denatured salmon sperm DNA and 20 μg of poly(A) per ml. Hybridization was performed under the same conditions for 16–24 hr, followed by washes in 2× NSPE/0.1% $NaDodSO_4$ and 0.2× NSPE/0.01% $NaDodSO_4$; all washes were at 60°C.

RESULTS

Transcripts from the Maxicircle H2R3 Region. Several *T. brucei* maxicircle genes have been identified by sequence analysis; their locations are indicated on the abbreviated restriction map of the *T. brucei* 164 maxicircle (Fig. 1). These include the genes for the 12S and 9S rRNAs (6), apocytochrome *b* (7, 9), cytochrome *c* oxidase subunits I and II [CytOx I and II (acronyms COI and COII)] (10, 11), and three open reading frames (ORFs) with homology to unassigned reading frames (URFs) 1, 4, and 5 from other mitochondrial systems (refs. 10 and 11; unpublished data). The expanded map of the H2R3 region (Fig. 1) shows the positions of the genes for CytOx I and II and URF 1 as well as two ORFs that have not been identified with other mitochondrial genes (10, 11). The gene for CytOx I and ORF IA extend ≈1215 nucleotides (nt) and 1230 nt, respectively, into the adjacent maxicircle segments (ref. 10; unpublished data).

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Abbreviations: CytOx I and II (acronyms COI and COII), cytochrome *c* oxidase subunits I and II; H2R3, the region of maxicircle between the second *Hin*dIII and third *Eco*RI sites; kb, kilobase(s); kDNA, kinetoplast DNA; nt, nucleotide(s); ORF, open reading frame (not identified with genes from other mitochondrial systems); URF, unassigned reading frame (homologous to genes from other mitochondrial systems).

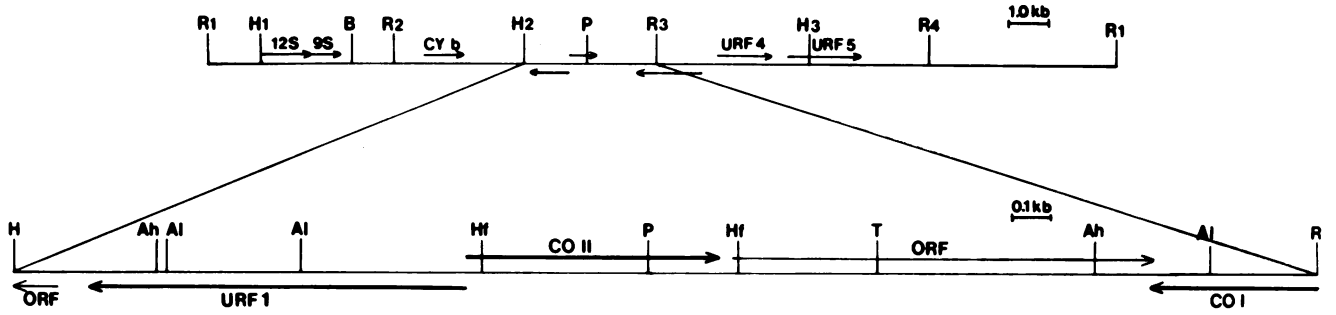

FIG. 1. Restriction map of the maxicircle of *T. brucei* 164. The upper map is a linearized map of the entire maxicircle, indicating selected restriction sites (5) and the location of the genes for the 12S and 9S rRNAs (6), apocytochrome *b* (CY b) (7, 9), and URF 4 and 5 (ref. 10; unpublished data). The lower map is an expansion of the H2R3 region, as cloned in pTKHR117, and shows additional selected restriction sites, and the genes for CytOx I (COI), CytOx II (COII), and URF 1, and two ORFs (10, 11) that are associated with transcripts (see text). The ORF adjacent to URF 1 is ORF IA, and that adjacent to COII is ORF IIA (nomenclature from ref. 11). Arrowheads denote the direction of transcription. Ah, *Aha* III; Al, *Alu* I; B, *Bam*HI; H, *Hin*dIII; Hf, *Hin*fI; P, *Pst* I; R, *Eco*RI; T, *Taq* I.

To characterize maxicircle transcription, we determined the number and location of transcripts from the H2R3 region. Probes prepared from pTKHR117 hybridized to 14 transcripts in RNA transfer blots (Fig. 2 *a–d*). RNAs of 2600, 1650, 1450, and 320 nt were detected by probe A (Fig. 2*a*). The fainter bands below 1450 nt were resolved as 1300 and 1100 nt on other RNA transfer blots. These and the 2600-nt transcript were also detected by probe B (Fig. 2*b*), but none of the above transcripts hybridized with probe C. The 2600-nt transcript is less abundant than the 1650-, 1450-, 1300-, and 1100-nt RNAs, based on the relative intensity of hybridization, and may be a precursor of those transcripts, as discussed below.

Probe B hybridized to four other RNAs: 1900-, 1700-, 900-, and 750-nt transcripts. The 900-nt transcript appears similar in abundance to the 1650-, 1450-, 1300-, and 1100-nt transcripts, whereas the others are less abundant. Probe C hybridized to these and to 1350- and 1150-nt RNAs (Fig. 2*c*). These latter transcripts reproducibly were found to be slightly larger than the 1300- and 1100-nt transcripts that hybridized with probe B. The 1350- and 1150-nt transcripts are distinct from the 1300- and 1100-nt transcripts because, as

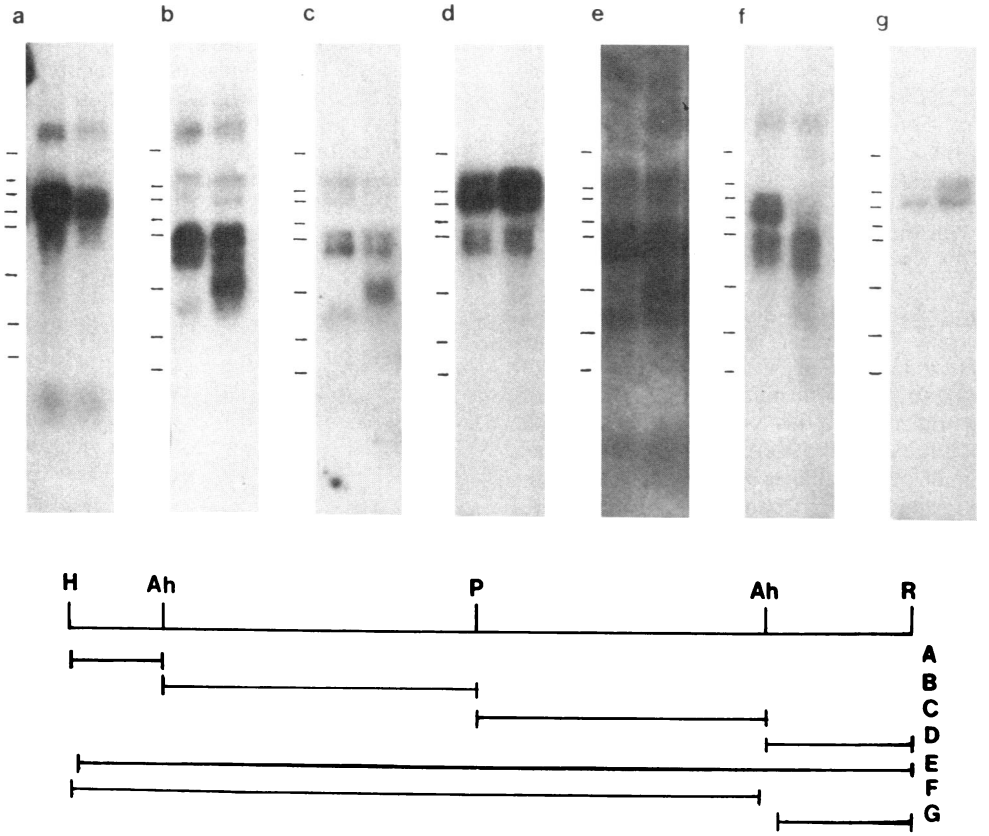

FIG. 2. Localization of transcripts of *T. brucei* maxicircle segment H2R3. (*Upper*) RNA transfer blots were prepared and hybridized as described in *Materials and Methods*. (*a–g*) The left-hand lane in each is bloodstream RNA, and the right-hand lane is procyclic RNA. The same amount of total RNA (10 μg) was loaded in each lane. Rehybridization of filters with a nuclear cDNA gave signals of similar strength for bloodstream and procyclic lanes. The filters were probed with nick-translated restriction fragments from plasmid pTKHR117 (*a–d*) or with subfragments cloned into phage M13 labeled with probe primer (*e* and *f*) or sequencing primer (*g*) as described. (*Lower*) Locations of the probes (A to G) corresponding to blots *a–g*; the restriction sites indicated correspond to those in Fig. 1. Positions of size markers (denatured pBR322 restriction fragments) are indicated: 2.32, 1.81, 1.63, 1.44, 1.31, 0.91, 0.66, and 0.52 kb.

discussed below, they are transcribed from the opposite DNA strand. The 1900- and 1700-nt transcripts that hybridized with probes B and C may be precursors of the 1350- and 1150-nt and the 900- and 750-nt transcripts, as discussed below. Probe D hybridized with 1900-, 1700-, 1350-, and 1150-nt RNAs (Fig. 2*d*). The 1900- and 1700-nt transcripts that hybridized with probe D are distinct from those revealed with probes B and C because the two sets of 1900- and 1700-nt RNAs are transcribed from different DNA strands, as discussed below. Three transcripts previously mapped to the H2R3 region were estimated to be 1.30, 1.17, and 0.98 kb (5); these correspond, respectively, to the 1350- and 1300-, the 1150- and 1100-, and the 900-nt transcripts.

The above results suggest that the 2600-, 1650-, and 1450-nt RNAs and one set of the 1900- and 1700-nt RNAs are transcribed from sequences extending over the *Hin*dIII and *Eco*RI sites, respectively, into the adjacent maxicircle regions. This was confirmed by using those segments as probes of RNA transfer blots (unpublished data). We sometimes observed two additional faint bands in long exposures of autoradiograms. One, an 1850-nt transcript, was detected with probes near the *Hin*dIII site, and the other, a 1350-nt transcript, was detected with probes near the *Eco*RI site. Both also appeared to extend into the adjacent maxicircle regions (unpublished data).

The strands from which each of the above RNAs is transcribed were identified by using single-stranded phage M13 probes. A probe that hybridized to transcripts from the CytOx II coding strand (see Fig. 1) revealed the 1900-, 1700-, 1350-, 1150-, 900-, 750-, and 320-nt transcripts (Fig. 2*e*). A probe corresponding to all but the *Eco*RI end of the CytOx I coding strand (see Fig. 1) hybridized to the 2600-, 1650-, 1450-, 1300-, and 1100-nt transcripts (Fig. 2*f*). As discussed above, the 1350- and 1150-nt and the 1300- and 1100-nt transcripts mapped to different regions of H2R3. Transcription from opposite DNA strands confirms that these similarly sized transcripts are distinct. An M13 probe corresponding to the *Eco*RI end of the CytOx I coding strand revealed 1900- and 1700-nt transcripts (Fig. 2*g*), indicating that these transcripts are distinct from the less-abundant 1900- and 1700-nt transcripts seen with the CytOx II coding strand (Fig. 2*e*). Table 1 summarizes the hybridization pattern of the H2R3 region transcripts.

To examine the possibility that some of the transcripts described above may be nuclear transcripts with some homology to maxicircle sequences, we hybridized H2R3 probes to RNA prepared from a mutant that is devoid of kDNA (12). No hybridization to this RNA was detected with any H2R3 probes, but a normal pattern of hybridization was seen when a trypanosome tubulin probe was used (Fig. 3). Thus, all of the transcripts described above appear to be derived from the H2R3 region of the maxicircle.

Correspondence of Transcripts with H2R3 Genes. Ten of the transcripts described above map as pairs, differing in size by 150–200 nt, to the five putative genes in the H2R3 region (see Fig. 1). An *Alu* I restriction fragment that is contained within the 960-nt URF 1 coding sequence hybridized principally to the 1300- and 1100-nt transcripts (Fig. 4*a*). Faint hybridization to the 2600-nt putative precursor transcript and to 1850- and 1650-nt RNAs also was detected. A *Hin*dIII/*Aha* III fragment that overlaps the URF 1 coding sequence and ORF IA hybridized to 2600-, 1650-, and 1450-nt transcripts as well as to the 1300- and 1100-nt transcripts (Fig. 2*a*). Probes from the adjacent region of the maxicircle that are contained within ORF IA hybridized to the 2600-, 1650-, and 1450-nt transcripts (unpublished data). Thus, the 1300- and 1100-nt and the 1650- and 1450-nt transcripts are derived from the URF 1 coding sequence and ORF IA, respectively, and the 2600-nt transcript may be a precursor of both transcript pairs. The significance of the 1850- and 320-nt transcripts from this region remains unknown.

Table 1. Transcripts from maxicircle H2R3 region

Transcript size, nt	Gene/ORF	Hybridization pattern: H2R3 fragment A	B	C	D	Strand I	II
2600		+	+			+	
1900*	CytOx I				+	+	
1700	CytOx I				+	+	
1900			+		+		+
1700			+		+		+
1650†	ORF IA	+				+	
1450†	ORF IA	+				+	
1350	ORF IIA			+	+		+
1150	ORF IIA			+	+		+
1300	URF 1		+			+	
1100	URF 1		+			+	
900‡	CytOx II		+		+		+
750	CytOx II		+		+		+
320		+					+

Transcript sizes were estimated by comparison to denatured pBR322 restriction fragment markers. The data from Fig. 2 are summarized in the hybridization pattern columns with a + denoting hybridization between the indicated probes and transcripts. Transcripts which appear in pairs (see text) are bracketed. The genes to which pairs of transcripts map (see text) are indicated. The low-abundance 1850- and 1350-nt transcripts described in the text from the ORF IA and CytOx I gene regions, respectively, are not listed.
*Greater in abundance in procyclic RNA.
†Greater in abundance in bloodstream RNA.
‡Absent in bloodstream RNA.

Most of the 629-nt coding sequence for CytOx II is contained within a *Hin*fI restriction fragment that overlaps ORF IIA (Fig. 1). This probe hybridized strongly to the 900- and 750-nt transcripts and less strongly to 1900-, 1700-, 1350-, and 1150-nt transcripts (Fig. 4*b*). Strong hybridization to the 1350- and 1150-nt transcripts was seen when a *Taq* I/*Alu* I

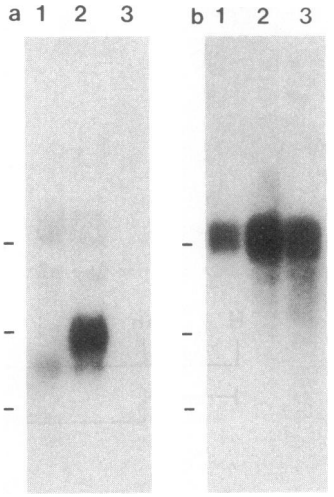

FIG. 3. Transcripts hybridizing to H2R3 probes are mitochondrially coded. RNA transfer blots were prepared and hybridized as described for Fig. 2, with the addition of a lane of 10 μg of RNA prepared from a mutant devoid of kDNA. Lanes: 1, bloodstream RNA; 2, procyclic RNA; 3, mutant RNA. (*a*) Probe is a subfragment of the *Hin*fI restriction fragment that contains most of the CytOxase-encoding sequence. (*b*) Probe is a genomic trypanosome tubulin clone, pTbαβT-1 (22). Other H2R3 probes also failed to hybridize with the mutant RNA (unpublished data). Size markers, pBR322 restriction fragments, are indicated: 1.63, 0.91, and 0.52 kb.

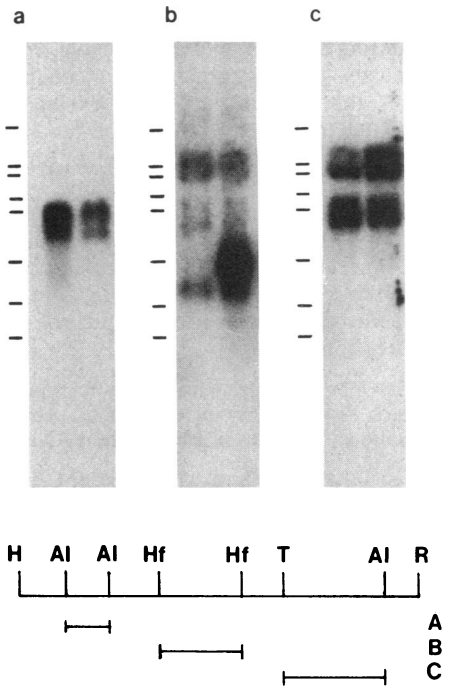

FIG. 4. Correspondence of transcripts with H2R3 genes. (*Upper*) RNA transfer blots were prepared and hybridized as described for Fig. 2. The lane of procyclic-form RNA in *a* is underloaded; other hybridization data indicated that the 1300- and 1100-nt transcripts were present in similar abundance in bloodstream and procyclic RNAs. The size markers are as indicated in Fig. 2. (*Lower*) Locations of probes A–C (nick-translated plasmid fragments) corresponding to blots *a*–*c*. The restriction sites correspond to those in Fig. 1.

restriction fragment that is largely contained within the 1038-nt ORF IIA was used as a probe (Fig. 4*c*). These data and those presented above (Fig. 2) indicate that the 900- and 750-nt RNAs are transcribed from the coding sequence for CytOx II, whereas the 1350- and 1150-nt transcripts are from ORF IIA. The 1900- and 1700-nt transcripts that hybridized with the *Hin*fI probe (Fig. 4*b*) comigrated with the 1900- and 1700-nt RNAs that were detected by the *Taq* I/*Alu* I probe (Fig. 4*c*). The latter probe overlaps the 3′ end of the 1650-nt coding sequence for CytOx I (Fig. 1). A probe representing the coding strand for CytOx I in this region hybridized to 1900- and 1700-nt transcripts (Fig. 2*g*), as did probes from the adjacent maxicircle segment (unpublished data). This indicates that these transcripts correspond to the coding sequence for CytOx I. The similarly sized transcripts seen with the *Hin*fI fragment are potential precursors for the transcripts from the CytOx II gene and ORF IIA.

Based on the data presented above and the nucleotide sequence of the H2R3 region (10, 11), we constructed a schematic transcription map (Fig. 5). The positions assigned to the five transcript pairs in the map closely match the five putative genes with sequences in this region. In each case both members of the pair are large enough to contain the coding sequence. The H2R3 region appears to be completely transcribed on one strand or the other, with multiple transcripts mapping to the same DNA segment.

Differential Expression of Maxicircle Transcripts. To assess the role of differential expression of the mitochondrial genome in differential production of the mitochondrial respiratory system during the life cycle of *T. brucei*, we compared the H2R3 transcripts present in bloodstream and procyclic forms. While differential transcription of mitochondrial genes could account for the differential production, previous studies (4, 5) indicate that this is not the case. Both reported less abundant maxicircle transcripts in bloodstream

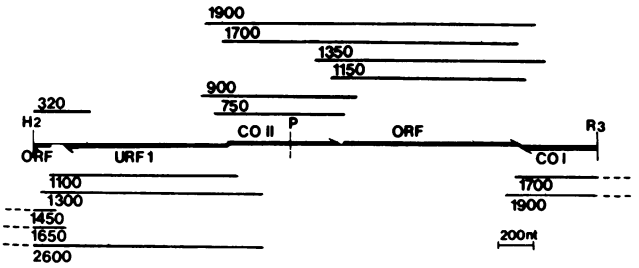

FIG. 5. Transcription map of the H2R3 region. The coding sequences of the H2R3 region genes are indicated, as in Fig. 1. Approximate positions for the transcripts were determined from the mapping experiments. Transcripts that extend into adjacent maxicircle regions are indicated by dotted lines. The low-abundance 1850- and 1350-nt transcripts described in the text from the ORF IA and CytOx I gene regions, respectively, are not shown.

than procyclic RNAs, with no specific differences between the two life cycle stages. We found that, while each of the genes in the H2R3 region is transcribed in both life cycle stages, specific transcripts differed greatly in their abundance between bloodstream and procyclic forms. Comparison of Figs. 2 and 4 shows that the intensity of hybridization was similar between bloodstream and procyclic RNAs for all but four transcripts. Thus, most H2R3 transcripts are present in similar amounts in these two life cycle stages.

In contrast to the similarity in abundance of many H2R3 transcripts between bloodstream and procyclic forms, the abundance of specific transcripts differed strikingly between these two stages. The most dramatic example is the prominence of the 900-nt CytOx II transcript in procyclic RNA and its absence, within our limits of detection, from bloodstream RNA (Fig. 2 *b*, *c*, and *e*; Fig. 4*b*). Similarly, the 1900-nt CytOx I transcript is more abundant in procyclic than bloodstream RNA (Fig. 2 *d* and *g*; Fig. 4*c*). Conversely, the 1650- and 1450-nt transcripts of ORF IA are more abundant in bloodstream than in procyclic RNA (Fig. 2 *a* and *f*). All of these cases of differential expression of specific maxicircle transcripts have been confirmed with several different RNA transfer blots, using different RNA preparations. Only minor variations were seen in the relative abundance of the various transcripts.

DISCUSSION

The African trypanosome has the ability to regulate the production of its mitochondrial respiratory system during its life cycle. While most components of this respiratory system are encoded by the nucleus, several essential components are encoded by mitochondrial DNA in all organisms examined. The studies presented in this paper indicate that the differential production of the mitochondrial respiratory system in *T. brucei* involves differential expression of mitochondrial genes.

The H2R3 region of the *T. brucei* maxicircle contains five ORFs that probably correspond to functional genes. Three of these ORFs have sequence homology to the CytOx I, CytOx II, and URF 1 mitochondrial genes from other organisms (10, 11) and are probably their functional equivalents. We have been unable to detect homology between the two other ORFs in this region and sequences from mitochondrial DNA of other nonkinetoplastid organisms. However, the presence of transcripts corresponding to these two ORFs suggests that they have an as yet unknown function.

The data reported here and summarized in Fig. 5 confirm and extend previous data showing that the sizes of maxicircle transcripts exceed the single-strand coding capacity of their respective maxicircle regions (4, 5, 8). Two phenomena are responsible for this finding: potential precursor transcripts

and paired transcripts for a single gene. The size and map location of the 2600-nt transcript that overlaps the URF 1 gene and ORF IA and the 1900- and 1700-nt transcripts that overlap the CytOx II gene and ORF IIA suggest that they may be precursors that are processed to the smaller RNAs. The pairs of transcripts may arise by differential transcription start and/or stop sites or, as seems more likely, by post-transcriptional modifications distinct from the large precursor processing, as discussed below.

Several possibilities could account for the size differences between the members of each pair of transcripts. These include removal or addition of internal sequences, differential processing, terminal sequence removal or addition, or some combination thereof. It is unlikely that the smaller transcript arose by removal of intron sequences from the larger transcript since *T. brucei* maxicircle genes do not appear to contain introns (refs. 6–11; unpublished data). No precedent exists for internal sequence addition. The possibility that precursor transcripts could be differentially processed to produce the transcript pairs remains open.

Alternatively, terminal sequences might be added to or removed from one member of each transcript pair. Two clear possibilities exist for addition of sequences to the transcript termini: 5′ addition of a spliced leader sequence and 3′ polyadenylylation. While many nuclearly encoded mRNAs in *T. brucei* have a 35-nt spliced leader sequence at their 5′ ends (23, 24), it is not known if this sequence or an analogue occurs on mitochondrial mRNAs. Polyadenylylation of mammalian mitochondrial mRNAs has been estimated to be 50–60 nt long (25), and a similar estimate has been made for several *T. brucei* maxicircle transcripts (7). This is insufficient to account for the size difference between members of a pair. Furthermore, in preliminary experiments, we found that both transcripts of the CytOx I and II genes bound to oligo(dT)-cellulose (unpublished data). Combinations of the above possibilities or other factors may account for the size difference between members of transcript pairs. Since the members of each pair differ by a relatively constant size (Table 1), the mechanism responsible for this phenomenon may be a general feature of maxicircle gene transcripts.

Either one or both members of each transcript pair may be functional. The simplest situation is that the larger member of each pair is the functional mRNA. This would be consistent with the presence or increased abundance of the larger transcript of the CytOx I, CytOx II, and apocytochrome *b* (unpublished data) genes in procyclic compared to bloodstream forms. It would also account for the presence of a fully functional mitochondrial respiratory system in procyclic but not bloodstream forms. However, some transcript pairs have both members present in similar abundance. Furthermore, ORF IA has both transcript pair members in greater abundance in bloodstream compared to procyclic forms, implying that both may be functional.

Multiple transcripts from the same gene have been described in a number of systems. Two mouse α-amylase mRNAs exhibit 5′ heterogeneity in noncoding sequences and direct tissue-specific synthesis of α-amylase (26). The secreted and cytoplasmic forms of yeast invertase result from two mRNAs, only one of which contains 5′ signal sequences (27). Similarly, immunoglobulin μ heavy chain mRNAs exhibit 3′ heterogeneity in coding sequences, which result in secretion or membrane localization (28, 29). The maize chloroplast ribulose-1,5-biphosphate carboxylase gene has two mRNAs that are differentially regulated during light-induced development of the chloroplast (30). These examples of multiple mRNAs from a single gene being used to fine-tune expression of that gene suggest that maxicircle transcript pairs may play a similar role to effect the differentiation between bloodstream and procyclic forms.

To cope with the varied and often hostile environments encountered during the course of its life cycle, *T. brucei* has developed unusual genetic regulatory mechanisms. This study indicates that the production of the mitochondrial respiratory system during the course of the life cycle is regulated in part by controlling the level of specific mitochondrial DNA transcripts. The mechanisms controlling these transcript levels are unknown. They may operate at the level of transcription, transcript processing, transcript degradation, or some combination of these factors. It seems likely that different regulatory events, at least in degree, are involved in generating the various kinds of differential transcript expression.

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