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The fragmented mitochondrial ribosomal RNAs of *Plasmodium falciparum* have short A tails

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ABSTRACT

The mitochondrial genome of *Plasmodium falciparum* encodes highly fragmented rRNAs. Twenty small RNAs which are putative rRNA fragments have been found and 15 of them have been identified as corresponding to specific regions of rRNA sequence. To investigate the possible interactions between the fragmented rRNAs in the ribosome, we have mapped the ends of many of the small transcripts using primer extension and RNase protection analysis. Results obtained from these studies revealed that some of the rRNA transcripts were longer than the sequences which encode them. To investigate these size discrepancies, we performed 3′ RACE PCR analysis and RNase H mapping. These analyses revealed non-encoded oligo(A) tails on some but not all of these small rRNAs. The approximate length of the oligo(A) tail appears to be transcript-specific, with some rRNAs consistently showing longer oligo(A) tails than others. The oligoadenylation of the rRNAs may provide a buffer zone against 3′ exonucleolytic attack, thereby preserving the encoded sequences necessary for secondary structure interactions in the ribosome.

INTRODUCTION

The mitochondrial genome of *Plasmodium falciparum* encodes very unusual highly fragmented rRNAs. Small (40–190 nt) regions of sequence similar to rRNAs are encoded in scrambled order by both DNA strands. These regions are well-conserved between *Plasmodium* species and correspond to highly conserved regions of rRNA from other species, suggesting that they may retain function despite their small size and extreme fragmentation (1–5). Small RNAs derived from the mitochondrial genome have been reported in *P.falciparum* (2,6), *Plasmodium gallinaceum* (3,7) and *Plasmodium yoelii* (4,5). In *P.falciparum*, a total of 20 such small RNAs have been found; 15 of these are similar to specific regions of rRNA sequence (2,6), further supporting a functional role for the fragmented rRNAs. The remaining five are also expected to be rRNA fragments but have not yet been demonstrated to correspond to specific regions of rRNA. Although transcripts containing more than one of the rRNA fragments can be detected (8), the fragments involved are always adjacent in the genome and often include fragments of both large (LSU) and small subunit (SSU) rRNA. Such larger transcripts are therefore presumed to be processing intermediates, consistent with the finding that *P.falciparum* mitochondrial transcription is polycistronic (8). The individual small transcripts are believed to associate *in trans*, presumably maintaining proper location and spacing between themselves by hydrogen bonding between the rRNA fragments and interactions between the RNAs and ribosomal proteins.

We are mapping the small transcripts to investigate the possible interactions between the fragmented rRNAs in the ribosome. Results obtained from RNase protection and primer extension studies showed that some of the rRNA coding sequences were smaller than their sizes estimated from RNA blots. Analysis of the 3′ end sequences of two of these transcripts, SSUA and RNA8, showed that each had additional A residues, and that the average oligo(A) tail length explained the size discrepancy. Analysis of a further 12 mitochondrial rRNA fragments, by 3′ RACE PCR analysis, has shown that all but one contain short stretches of non-encoded A residues, up to 21 nt long, at the 3′ end. RNase H mapping indicates that terminal A residues are also found on at least two of the six remaining rRNA fragments. This oligoadenylation may protect the 3′ ends of the small rRNAs from exonucleolytic degradation, preserving sequence needed for interactions to form and preserve ribosome structure.

MATERIALS AND METHODS

Parasites

The C10 line of *P.falciparum* was employed for these studies. Parasites were cultivated by the method of Trager and Jensen (9) and harvested by lysing infected erythrocytes with saponin, followed by washes with phosphate-buffered saline. Isolated parasites were quick-frozen in liquid N_2 and stored at –80°C for later use.

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors

RNA preparation and analysis

Total parasite RNA was prepared from mixed populations of parasites, generally 50% or more trophozoites, by an acid/guanidinium/phenol/chloroform protocol (10), as previously described (11). RNA blots were prepared electrophoretically from 12% polyacrylamide, 7 M urea gels and hybridized as previously described (6); probes are indicated in Table 1. RNase protection was performed as previously described (11), using a mixture of RNase A and RNase T1 to digest hybrids of total *P.falciparum* RNA with a radiolabeled *in vitro* transcript prepared from cloned mitochondrial genomic sequences. Primer extension was performed as previously described (12), employing an unlabeled oligonucleotide primer and Superscript reverse transcriptase (Life Technologies) in the presence of [^{32}P]dATP.

Table 1. Probes for RNA blots

gene	probe type	position[a]
SSUA	oligonucleotide	1919–1943
SSUB	oligonucleotide	431–452
SSUD	oligonucleotide	5395–5417
SSUE	*in vitro* transcript	1656–1675 rc
SSUF	oligonucleotide	5468–5489
LSUA	*in vitro* transcript	4957–5163
LSUD	*in vitro* transcript	5525–5967
LSUE	*in vitro* transcript	5525–5967
RNA1	oligonucleotide	554–577
RNA2	*in vitro* transcript	1658–1851 rc
RNA4	oligonucleotide	4526–4748 rc
RNA6	*in vitro* transcript	4790–4956 rc
RNA8	*in vitro* transcript	5894–5967
RNA9	*in vitro* transcript	2–130 rc
RNA10	oligonucleotide	644–665

[a]Position is given relative to GenBank entry M76611.
rc, reverse complement.

3′ RACE analysis

Total RNA was treated with DNase I (Pharmacia) in the presence of RNAsin (Promega), following which a 3′ C tail was added with poly(A) polymerase (Life Technologies), as previously described (8). First strand cDNA was prepared using an oligo(dG) primer (CGGGATCCGGGGGGGGGGG) and Superscript reverse transcriptase (Life Technologies). The cDNA was then PCR amplified using *Tfl* DNA polymerase (Epicentre Technologies), with the oligo(dG) primer and a gene-specific 5′ primer (Table 2) for each rRNA fragment. Conditions were 2 min at 95°C, followed by four repetitions of 30 s at 55°C, 1 min at 72°C, 15 s at 95°C, then 29 repetitions of the same with the annealing temperature raised to 57°C, and finally 10 min at 72°C. The PCR products were cloned into the pCR2.1 vector (Invitrogen) and sequenced.

RNase H mapping

Total parasite RNA (3 µg) and 3.2 µM oligo(dT)$_{15}$ in H_2O were heated to 70°C for 5 min, then 42°C for 10 min. Digestion buffer (final concentration: 50 mM Tris pH 7.5, 8 mM $MgCl_2$, 64 mM KCl, 0.8 mM DTT) and 2 U of RNase H (Life Technologies) were added and the samples incubated at 37°C for 30 min. Samples were ethanol precipitated and resuspended in loading buffer prior to electrophoresis and blotting. Control samples were processed similarly but without the addition of oligo(dT).

Table 2. Gene-specific oligonucleotide primers for 3′ RACE PCR

gene	primer sequence	position[a]
SSUA	GCGACAGCGACCCTGACATACC	1977–1998 rc
SSUB	GTGTTCCACCGCTAGTGTTTGC	431–452 rc
SSUD	AAAGATGAAGGTCGTCGG	5428–5445 rc
SSUF	CAGTCGGTGCGAAGTCGTAACAT	5482–5501 rc
LSUA	TAGACCGAACCTTGGACTC	5123–5141 rc
LSUD	GGTTACAACACAAGTCACTG	5819–5838 rc
LSUE	CCCCATTGTCGCTAGTGT	5664–5681 rc
LSUF	GAAGGTTCGCCGGGGATAACAGG	1532–1554
LSUG	ATTGGAATGAGAGTTCACCGTTAGAAGC	341–368 rc
RNA2	GCGTTAACCTGTAGAGTTGAGATG	1707–1730
RNA3	CCATACAAGAGATCGCGTAC	1835–1854
RNA5	GGCTGAGCATGTAAATCCGATTG	4772–4794 rc
RNA7	GAGAGAATCCTCTTGGTAACTCAAC	5252–5275 rc
RNA8	AGTCGGATACAAGTTGAAACATCTC	5930–5954 rc

[a]Position is given relative to GenBank entry M76611.
rc, reverse complement.

RESULTS

Transcript mapping

The mitochondrial genome of *P.falciparum* encodes 20 small transcripts. Of these, 10 correspond to fragments of rRNA, based on their strong homology to highly conserved regions of other rRNAs. They have been named SSUA–SSUF and LSUA–LSUG based on the order in which they would be expected to occur in SSU or LSU rRNA, respectively (2). Three regions (SSUC, LSUB and LSUC) originally thought to be rDNA have since been found to lack mature transcripts (6) and are therefore considered erroneous identifications. An additional 10 transcripts were found by RNA blotting experiments (6) and have been identified with numbers (RNA1–RNA10). Five of these have been shown to correspond to specific regions of rRNA sequence (13) but the identity of the remaining small transcripts is not yet known. Until this issue is clear, we have elected not to rename the 15 identified transcripts to indicate the revised order of the rRNA fragments.

An oligonucleotide probe complementary to the SSUA rDNA sequence detects a transcript estimated to be 125 nt long (Fig. 1A). The broad appearance of the hybridizing band suggests minor heterogeneity in the size of the transcript. This rDNA sequence includes an 86 nt highly conserved region containing the 530 loop of SSU rRNA and additional flanking sequence (2). Primer extension from an oligonucleotide complementary to part of the highly conserved region indicated the location of the 5′ end of SSUA rRNA (Fig. 1B). An RNase protection assay yielded a protected product only 108 nt long (Fig. 1C, lane 3), however, instead of the 125 nt product expected based on the transcript size. This suggested that the SSUA coding sequence only partially overlapped the radiolabeled *in vitro* transcript used as the probe. However, the combined mapping data clearly indicated that the entire SSUA rRNA should have been protected (Fig. 1D). Similar size discrepancies were observed for RNA8 which has a transcript size of 115 nt on RNA blots (Fig. 2A). However, RNase protection gave only a 94 nt product (Fig. 2C). Primer extension

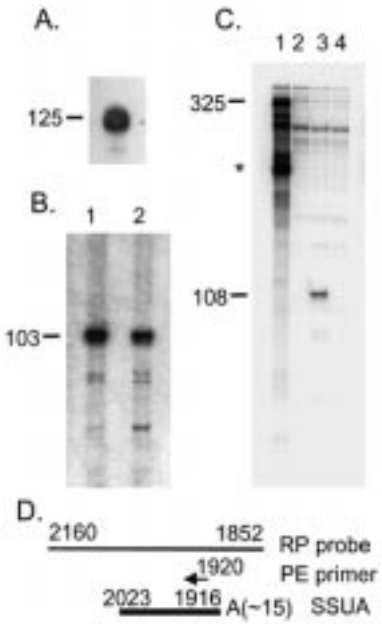

Figure 1. Mapping the *P.falciparum* SSUA rRNA. Total *P. falciparum* RNA from mixed erythrocytic stage harvests was analyzed by RNA blotting (A), primer extension (B) and RNase protection (C) to map the SSUA transcript. (**A**) An aliquot of 3 µg of RNA was electrophoresed on a denaturing 12% acrylamide, 7 M urea gel, transferred by electroblotting to nylon membrane, and probed with a radiolabeled oligonucleotide (5′-CGAACGCTTT-TAACGCCTGACATGG) complementary to part of SSUA. (**B**) An aliquot of 3 µg of RNA was hybridized with the same oligonucleotide and extended, at different temperatures, with reverse transcriptase. Products were electrophoresed on a 12% acrylamide, 7 M urea sequencing gel. Lane 1, 48°C; lane 2; 55°C. (**C**) RNA was hybridized with a radiolabeled *in vitro* transcript complementary to SSUA sequences, digested with RNase A+T1, and electrophoresed on a 12% acrylamide, 7 M urea sequencing gel. The hybridizing RNAs were: lane 1, *in vitro* transcript complementary to the probe; lane 2, no input RNA; lane 3, *P.falciparum* RNA; lane 4, *Trypanosoma brucei* RNA. The product marked with an asterisk in lane 1 of (C) appears to be artifactual; the 325 nt product in that lane is the size expected for accurate protection of the probe. The size exceeds the 308 nt *P.falciparum* portion of the probe due to protection of 17 nt of vector sequence by the *in vitro* complement RNA. Sizes for (A–C) are given in nt and were estimated from a ladder of *in vitro* transcripts (A, C) or radiolabeled *Hin*fI restriction fragments from ϕX174 DNA (B). (**D**) Schematic representation of the probes and mapping data. The location of the 5′ and 3′ ends (5′ only for oligonucleotide) are given relative to GenBank entry M76611. RP, RNase protection; PE, primer extension.

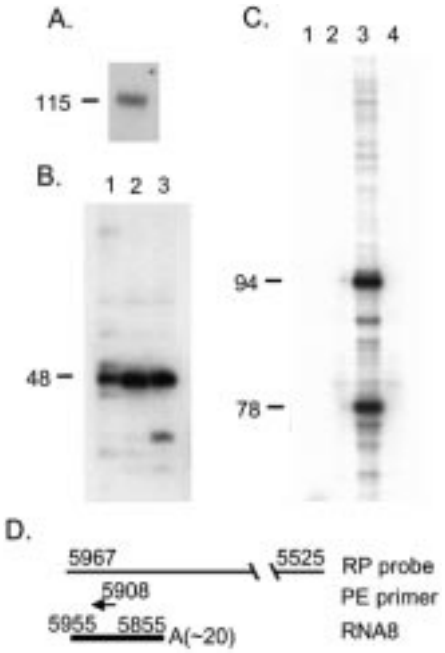

Figure 2. Mapping the *P.falciparum* RNA8 transcript. Total *P.falciparum* RNA from mixed erythrocytic stage harvests was analyzed by RNA blotting (**A**), primer extension (**B**) and RNase protection (**C**) to map the RNA8 transcript. Details are as given in Figure 1, with the following exceptions. The oligonucleotide used for the experiments shown in (A) and (B) was 5′-GCTCCATTCAAGGCATAGAGACTC. The temperatures for the primer extensions in (B) were 42, 50 and 55°C in lanes 1–3, respectively. In (C), the protected product in the positive control (lane 1) is not seen as it is 443 nt long and thus outside the portion of the gel shown here. The 94 nt product corresponds to RNA8 while the 78 nt product corresponds to LSUD, which is located immediately 3′ of RNA8.

(Fig. 2B) indicated the location of the RNA8 5′ end, showing that the probe used for RNase protection should have protected the entire RNA8 transcript (Fig. 2D).

3′ RACE analysis

Mitochondrial transcripts, including rRNAs, from a variety of organisms have been shown to be altered by RNA editing, either by addition/deletion or modification of nucleotide sequence (14,15). In order to assess whether similar mechanisms might explain the observed size discrepancy, and to specifically map the 3′ end of the SSUA transcript, we employed 3′ RACE PCR analysis to obtain the rRNA sequence (16). This procedure commonly uses oligo(dT) as a cDNA primer, as a first step to mapping the 3′ end of mRNAs. To map the SSUA rRNA end, which we presumed lacked polyadenylation, we first enzymatically added C-tails to total RNA and then employed oligo(dG) to prime cDNA synthesis. Following PCR with oligo(dG) and an SSUA-specific primer, the product was cloned and sequenced. No differences were seen between the cloned PCR products and the DNA sequence, except at the 3′ end. There, to our surprise, the cDNA sequence revealed the presence of non-encoded A residues between the C-tail and the encoded sequence. Four clones were compared: each has a tail of A residues, ranging from 7 to 18 nt in length (Table 3). This oligo(A) tail is presumably responsible for the ~15 nt size discrepancy between the RNA blot and RNase protection results.

We also performed 3′ RACE analysis for RNA8. Seven clones were sequenced and all were found to be oligoadenylated (Table 3). There is an A residue in the genomic sequence at the site in the RNA where the oligo(A) tail is added. We cannot distinguish whether the first A in the oligo(A) tail is encoded by the DNA or added by the polyadenylation machinery. One of the clones has an 8 nt oligo(A) tail but the others range from 14 to 20 nt. The RNA8 coding sequence was estimated to be 94 nt by RNase protection. However, primer extension and RACE PCR indicate that the length of the encoded portion of RNA8 is 101 nt. The reason for this small size difference is uncertain. One possibility is that the RNase protection results reflect breathing and cleavage of the hybrid which ends with a stretch of eight As and Us. Taken together, these data suggest that, as with SSUA rRNA, the addition of the oligo(A) tail is responsible for the majority of the size discrepancy for RNA8.

Table 3. *Plasmodium falciparum* mitochondrial rRNA 3′ end sequences

SSUA	*GTATTATCCA*	*TCCATGTCAG*	*GCGTTAAAAG*	*CGTTCGTTCT*	*TATAGTGTAG*
	GTATTATCCA	TCCATGTCAG	GCGTTAAAAG	CGTTCGTTCT	AAAAAAAAAAAAA
	GTATTATCCA	TCCATGTCAG	GCGTTAAAAG	CGTTCGTTCT	AAAAAAAAAAAAAAAAAA
	GTATTATCCA	TCCATGTCAG	GCGTTAAAAG	CGTTCGTTCT	AAAAAAAAAAAAA
	GTATTATCCA	TCCATGTCAG	GCGTTAAAAG	CGTTCGTTCT	AAAAAAA
SSUB	*TTGTTTCATT*	*TGATAGTAAA*	*CACTATACCT*	*TACCAATCTA*	*TTTGAACTTG*
	TTGTTTCATT	TGATAGTAAA	CACTATACCT	TACCAATCTA	
	TTGTTTCATT	TGATAGTAAA	CACTATACCT	TACCAATCTA	
	TTGTTTCATT	TGATAGTAAA	CACTATACCT	TACCAATCTA	
	TTGTTTCATT	TGATAGTAAA	CACTATACCT	TACCAATCTA	
SSUD	*GACGTCAGGA*	*AGTCCTGGAC*	*GTTGAATCCA*	*ATAGCATTGA*	*TTAAAAGACA*
	GACGTCAGGA	AGTCCTGGAC	GTTGAATCCA	ATAGCATTGA	AAA
	GACGTCAGGA	AGTCCTGGAC	GTTGAATCCA	ATAGCATTGA	AA
	GACGTCAGGA	AGTCCTGGAC	GTTGAATCCA	ATAGCATTGA	
SSUF	*AACATGGTAG*	*TTGACAGTGA*	*ACTTGTAGCT*	*GAACCAAAAA*	*TGGCTGCTGG*
	AACATGGTAG	TTGACAGTGA	ACTTGTAGCT	GAACCAAAAA	
	AACATGGTAG	TTGACAGTGA	ACTTGTAGCT	GAACCAAAAA	AAAAAA
	AACATGGTAG	TTGACAGTGA	ACTTGTAGCT	GAACCAAAAA	AAAAAA
	AACATGGTAG	TTGACAGTGA	ACTTGTAGCT	GAACCAAAAA	AAA
LSUA	*TAGACGGTTT*	*TCTGCGAAAT*	*CTATTTGGAA*	*GATATATCAT*	*TGGGAAGTTT*
	TAGACGGTTT	TCTGCGAAAT	CTATTTGGAA	GATATATCAT	AAAAAAAAAAAAA
LSUD	*TGATGAATAT*	*TTCAAGTTAC*	*TGACATCTGC*	*CCGGCATCAA*	*TGATAAACGG*
	TGATGAATAT	TTCAAGTTAC	TGACATCTGC	CCGGCATCAA	AAA
	TGATGAATAT	TTCAAGTTAC	TGACATCTGC	CCGGCATCAA	AA
LSUE	*GCCCGACGGT*	*AAGACCCTGA*	*GCACCTTAAC*	*TTCCCTAAAA*	*GTTCTTATGT*
	GCCCGACGGT	AAGACCCTGA	GCACCTTAAC	TTCCCTAAAA	AAAAAAAAAAAAAAA
LSUF	*TTGGCACCTC*	*CATGTCGTCT*	*CATCGCAGCC*	*TTGCAATAAA*	*TAATATCTAG*
	TTGGCACCTC	CATGTCGTCT	CATCGCAGCC	TTGCAATAAA	AAA
	TTGGCACCTC	CATGTCGTCT	CATCGCAGCC	TTGCAATAAA	AAAAA
	TTGGCACCTC	CATGTCGTCT	CATCGCAGCC	TTGCAATAAA	AAAAAA
LSUG	*AGAACGTCTT*	*GAGGCAGTTT*	*GTTCCCTATC*	*TACCGTTTTA*	*TCTTTGCATG*
	AGAACGTCTT	GAGGCAGTTT	GTTCCCTATC	TACCGTTTTA	AAAAAAAAAAA
	AGAACGTCTT	GAGGCAGTTT	GTTCCCTATC	TACCGTTTTA	AAAAAAAAAAAA
RNA2	*TGAGATGGAA*	*ACAGCCGGAA*	*AGGTAATTTT*	*ACGCCCTTAA*	*CGTAAAGATC*
	TGAGATGGAA	ACAGCCGGAA	AGGTAATTTT	ACGCCCTTAA	AAAAAAAAAA
	TGAGATGGAA	ACAGCCGGAA	AGGTAATTTT	ACGCCCTTAA	AAAAAAAAAAAAAAA
	TGAGATGGAA	ACAGCCGGAA	AGGTAATTTT	ACGCCCTTAA	AAAAAAAAAAAAAAAAAAAA
RNA3	*CTGTGAGGAA*	*ACTACATTAA*	*AGGAACTCGA*	*CTGGCCTACA*	*CTATAAGAAG*
	CTGTGAGGAA	ACTACATTAA	AGGAACTCGA	CTGGCCTACA	AAAAAA
	CTGTGAGGAA	ACTACATTAA	AGGAACTCGA	CTGGCCTACA	AAAAAAAA
	CTGTGAGGAA	ACTACATTAA	AGGAACTCGA	CTGGCCTACA	AAAAAAAA
	CTGTGAGGAA	ACTACATTAA	AGGAACTCGA	CTGGCCTACA	AAAAAA
RNA5	*TTACAGTATC*	*AATCGGATTT*	*ACATGCTCAG*	*CCGCCAAAAA*	*CTATAACGAT*
	TTACAGTATC	AATCGGATTT	ACATGCTCAG	CCGCCAAAAA	AAAAAAAAAA
	TTACAGTATC	AATCGGATTT	ACATGCTCAG	CCGCCAAAAA	AAAAAA
RNA7	*AATCGAGAGA*	*GATTCCATTA*	*GTTGTCTCTA*	*TGAATAGTGG*	*TTATAGCCAT*
	AATCGAGAGA	GATTCCATTA	GTTGTCTCTA	TGAATAGTGG	AAAAAAAAAAAAAAA
	AATCGAGAGA	GATTCCATTA	GTTGTCTCTA	TGAATAGTGG	AAAAAAAAAA
	AATCGAGAGA	GATTCCATTA	GTTGTCTCTA	TGAATAGTGG	AAAAAAAAAAAAAAA
RNA8	*CCCGGGAAAC*	*CGGCGCTTCC*	*ATTTATAAGA*	*AGTTAAATTA*	*CTGGAAGCGT*
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAAAAAAAA
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAAAAAAAAAAA
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAAAAAAAAAAA
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAAAAAAAAAAA
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAAAAAAAAAAAAA
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAAAAAAAAAAAAA
	CCCGGGAAAC	CGGCGCTTCC	ATTTATAAGA	AGTTAAATTA	AAAAAAA

The top sequence (in italics) for each rRNA fragment corresponds to the genomic DNA sequence while the sequences below are of the 3′ RACE products. The amount of encoded sequence shown was arbitrarily limited to the 3′-most 40 encoded nt plus the oligo(A) tails.

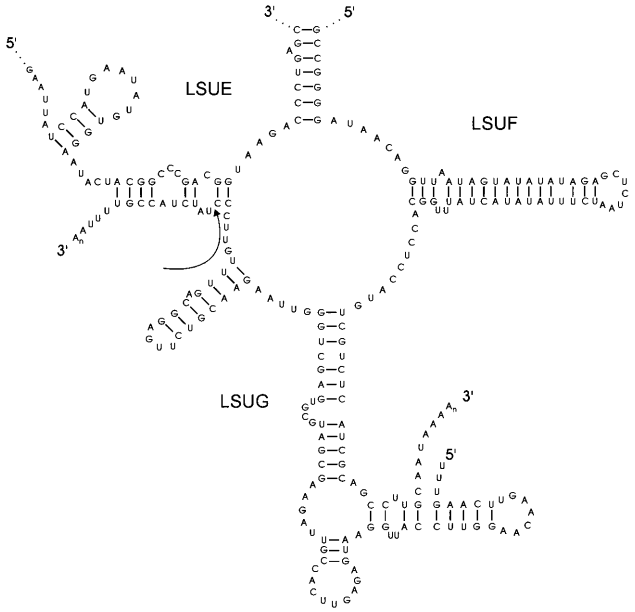

Figure 3. Predicted secondary structure interactions between LSUG and LSUE. A consensus secondary structure, based on comparisons of sequences from three phylogenetic domains and two organelle types, is shown for all of LSUG and parts of LSUE and LSUF. The site previously predicted (8) to be the 3′ end of LSUG is shown with an arrow.

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5′ LSUG
TTTGAACTTG AACAAGGTTC CATTGGAATG AGAGTTCACC GTTAGAAGCG    50
.......... .......... .......... .......... ..........    50
......T... ......A... .......... .......... ..........    50

                                                LSUG 3′ truncation
ATGCGTGAGC TGGGTTAAGA ACGTCTTGAG GCAGTTTGTT CCCTATCTAC   100
.......... .......... .......... .......... ..........   100
.......... .......... .......... .......... ..........   100

 LSUG 3′
CGTTTTATCT TTGCATGGAT GATGATACGT TAAGTTCTAT GAAAAA-TTTA 150
........T. A..T.....G .....A.... .......... ......G.... 151
.......... ...T....T. .........- .......... ......G.... 150
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Figure 4. Comparison of LSUG rDNA sequences between *Plasmodium* species. The *P.falciparum* (GenBank™ accession no. M76611) LSUG rDNA sequence, with some 3′ flanking sequence, is aligned with the corresponding sequences from *P.vivax* (GenBank™ accession no. AF055587) (18) and *P.yoelii* (GenBank™ accession no. M29000) (4). The latter two sequences are identical to the *P.falciparum* sequences except where indicated; – indicates introduction of a gap to maintain alignment. The 5′ and 3′ ends of the transcript are indicated in bold, with the numerals directly above the terminal nucleotides. The CCC which marks the truncation site is underlined.

We have expanded 3′ RACE analysis to look at an additional 12 of the small mitochondrial RNAs. All but one (SSUB) have non-encoded A residues at the 3′ end of at least some of their transcripts (Table 3). In general, transcripts for each gene appear to have a characteristic oligo(A) tail length. For example, there are only a few A residues added to the SSUD and LSUD rRNAs while the oligo(A) tail length on SSUA, RNA2, RNA7 and RNA8 rRNAs averages around 15 residues.

Four clones of SSUB rRNA in the current analysis were all found to lack additional A residues. In a prior analysis of polycistronic transcription of the *P.falciparum* mitochondrial genome (8), 3′ RACE analysis of the SSUB transcript showed three different 3′ ends. One of these corresponds to the 3′ end seen in these four clones while the others were 1 and 3 nt further upstream, respectively, and may represent 3′ end nibbling; no added A residues were seen on any of these clones. Thus in two independent experiments, the SSUB transcript has been shown to lack the extra A residues which are found on the 13 other RNAs examined by RACE analysis.

Data on the 3′ end of the LSUG rRNA has also been previously published, from a single clone (8). We obtained two additional 3′ RACE clones in this study (Table 3), both of which are 14 nt longer than the first clone. The newly determined 3′ end for LSUG is predicted to interact with LSUE, forming a helix in the peptidyltransferase domain of large subunit rRNA (Fig. 3). In a comparison of LSU rRNA sequences from three phylogenetic domains and two organelle types (17), the precise sequence of this helix is not highly conserved but there is strong structural conservation. The predicted structure of the LSUG/LSUE helix is a good, though not perfect, match. In addition, the LSUG sequence is perfectly conserved between *P.falciparum* (2), *Plasmodium vivax* (18), and *P.yoelii* (4) except for two mismatches, both near the 5′ end. In contrast, the sequence immediately downstream of the longer LSUG rDNA has seven mismatches within the first 25 nt (Fig. 4). The position of this abrupt change favors the LSUG 3′ end indicated by our current data. The previously observed LSUG 3′ RACE product may have resulted from inappropriate priming with oligo(dG) at a CCC triplet (Fig. 4), the site at which the transcript was previously reported to end (8). This could have occurred during either cDNA synthesis or PCR amplification, both of which employed an oligo(dG) primer.

RNase H mapping

We have employed RNase H mapping to assess the extent of oligo(A) tails on the remaining small RNAs from the mitochondrial genome. RNase H cleaves the RNA strand of RNA:DNA hybrids, consequently incubation of total RNA with oligo(dT) in the presence of RNase H removes poly(A) tails (19). It may also cut within transcripts at runs of A residues. This poses potential problems for assessment of *P.falciparum* transcripts, since the genes are quite A+T-rich. However, the mitochondrial genome has a significantly lower A+T content (68%) than the nuclear genome (82%) and the rRNA fragments are even less A+T-rich than the mitochondrial genome as a whole. None of the small RNAs contain more than six A residues in any 10 nt run and only once are the six As consecutive. It seemed likely that interaction of oligo(dT) with such short regions would not be stable enough to allow RNase H cleavage. We also expected that oligo(dT) would not bind the shortest oligo(A) tails well. The RNase H assay was therefore expected to detect significant oligo(A) tails but not necessarily shorter ones.

We tested the ability of the RNase H mapping approach to detect the rRNA oligo(A) tails using rRNAs for which we have 3′ RACE data. Probing blots of RNA treated with RNase H in the presence and absence of oligo(dT) shows that the SSUA, RNA2 and RNA8 transcripts are decreased in size by ~10–20 nt (Fig. 5), consistent with the average length of oligo(A) tail observed in their 3′ RACE products (Table 3). All of these transcripts exhibit heterogeneity of size both before and after RNase H treatment. Heterogeneity after treatment could be due to heterogeneity at the 5′ end or to variability in the number of A residues removed on individual transcripts. Primer extension for SSUA and RNA8

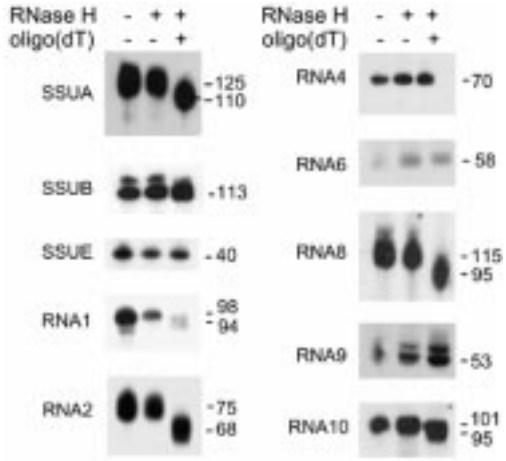

Figure 5. RNase H analyses of *P.falciparum* mitochondrial RNAs. Total *P.falciparum* RNA that was untreated and treated with RNase H in the presence or absence of oligo(dT) was electrophoresed on a denaturing 12% acrylamide, 7 M urea gel, transferred by electroblotting to nylon membrane, and probed with radiolabeled oligonucleotide or *in vitro* transcript probes (Table 1). Sizes are indicated in nucleotides and were estimated from a ladder of *in vitro* transcripts.

(Figs 1B and 2B) produces a single strong product, indicating that variation in A residues removed is the more likely explanation. This variation probably reflects the site of hybridization of the oligo(dT) 15mer in these longer A tails. In contrast to the transcripts with comparatively long A tails, no change is observed for the SSUB transcript (Fig. 5) which lacks an oligo(A) tail (Table 3). Applying this analysis to the small RNAs which for which we have no 3′ RACE data, we found that RNA1 and RNA10 transcripts decrease by ~5–10 nt, suggesting they have intermediate length oligo(A) tails. As with SSUA, RNA2 and RNA8, there is some size heterogeneity in the RNase H-treated RNA1 and RNA10 transcripts; this may reflect hybridization efficiency of these shorter (A) tails with oligo(dT)$_{15}$. SSUE, RNA4, RNA6 and RNA9 transcripts (Fig. 5) all remain the same size after RNase treatment in the presence of oligo(dT), suggesting that oligo(A) tails for these RNAs are short or lacking.

In addition to the major hybridizing band, the probes for both SSUB and RNA9 also hybridized to a slightly larger, less abundant band. The precise relationship of these transcripts to the mature rRNAs is uncertain but the larger product may be an RNA processing intermediate. The 5′ end of SSUB is 15–20 nt downstream of the estimated 3′ end of RNA1, based on primer extension and RNA blotting experiments (data not shown). The larger transcript may thus reflect an SSUB precursor transcript from which the short intergenic sequence has not yet been removed.

Search for RNA processing signals

We have examined the sites for oligo(A) addition to see if there is a shared characteristic among the small RNAs that might be a polyadenylation signal sequence. Of the 13 oligo(A)-tailed RNAs for which we have specific sequence information (Table 3), 10 have an A as the last encoded nucleotide before the tail. It is interesting to note in this regard that the most common sequence at the site of polyadenylation in eukaryotic mRNAs is CA (20). The preference for a terminal A in the encoded sequence of the rRNAs may reflect a role in recognition of processing sites, needed to produce the mature rRNAs from the polycistronic mitochondrial transcript. In the mitochondrial genomic sequence, the residue just 3′ of the A is a T in nine cases; this too may contribute to processing site recognition. However, the terminal A is clearly not sufficient for a polyadenylation signal since the SSUB transcript also ends at an A but is not oligoadenylated. No other characteristics are shared by as many of the transcripts.

DISCUSSION

Our analysis of the 3′ ends of the *P.falciparum* mitochondrial rRNAs revealed the unexpected presence of non-encoded A residues at the 3′ end of most, but not all of them. These tails are unlikely to be experimental artifacts from the C-tailing, cDNA synthesis or PCR steps of 3′ RACE analysis, since RNase H mapping indicates the presence of terminal A residues on the native RNAs. The size differences in transcripts before and after RNase H digestion in the presence of oligo(dT) correspond generally to the length of non-encoded A residues found on 3′ RACE clones. These points strongly support the presence of A-tailed rRNAs in *P.falciparum* mitochondria. At least some of the *P.yoelii* mitochondrial rRNAs are found in oligo(dT)-bound RNA, suggesting they also carry oligo(A) tails (21). Similarly, some but not all of the rRNA fragments from the mitochondrion of *Theileria parva*, a related parasite of cattle, are retained on an oligo(dT) column (22), indicating the likely presence of oligo(A) tails.

Among other roles, polyadenylation of RNAs affects their stability. Polyadenylation of eukaryotic cytoplasmic mRNAs increases their stability and stimulates their translation (23,24). Recent reports indicate that at least some prokaryotic (25) and chloroplast (26) mRNAs also become polyadenylated. In contrast to eukaryotic polyadenylation, bacterial and plastid poly(A) tails do not confer stability but rather mark the mRNAs containing them for degradation. The role of polyadenylation in mitochondria has not been studied. However, mammalian mitochondrial mRNAs carry poly(A) tails ~50 nt long (27–29) and those of mitochondrial mRNAs from kinetoplastid protozoa are 100–200 nt long (19,30,31). Thus in mitochondria, like eukaryotes, polyadenylation is common. This ubiquity suggests that mitochondrial polyadenylation is unlikely to trigger degradation as reported for prokaryotes and chloroplasts. Indeed, polyadenylation of mammalian mitochondrial mRNAs in some cases creates the translational stop codon and is thus critical for successful protein synthesis (32,33).

Short oligo(A) tails have been previously reported from mammalian (up to five A residues) and mosquito (36 A residues) mitochondrial rRNAs (34–36). As with the mitochondrial mRNAs, the function, if any, of these tails is unknown. They may simply represent a lack of specificity in the mitochondrial polyadenylation machinery, so that any available 3′ end is a substrate for poly(A) polymerase. This would be consistent with their lack of an obvious shared sequence to specify a poly(A) addition site, a characteristic shared by the *P.falciparum* mitochondrial rRNAs. In the same vein, trypanosome mitochondrial rRNAs have oligo(U) tails, presumably added by the very active organelle terminal uridylyl transferase (37). We similarly do not know whether addition of A residues to *P.falciparum* mitochondrial rRNAs has functional significance. The finding that each rRNA fragment has a characteristic range of oligo(A) tail length,

with variation in size among the different fragments, indicates that there is some level of discrimination between the transcripts. While this could reflect a functional role, it may also be due to differences in the relative accessibility of the 3′ ends of different transcripts to the polyadenylation machinery or to exonuclease activity. The relative stability of a hairpin in viral sequences has been shown to influence polyadenylation efficiency of viral RNAs (38). The *P.falciparum* mitochondrial rRNAs may well exhibit differences in the stability of their secondary structures which influence their polyadenylation. The minor variability in oligo(A) tail length of different transcripts from the same rDNA suggests that there is no absolute requirement for a specific length of tail on individual rRNAs.

The small rRNA fragments are hypothesized to come together *in trans* to form a functional mitochondrial ribosome (1,2,5,6). We cannot yet rule out the possibility that there is a subset of the small RNAs that lack oligo(A) tails and that these are the ones that assemble into ribosomes. However, this would have to be a very small subset since the RNase H mapping experiments demonstrate for several of the rRNAs that there are virtually no transcripts lacking an oligo(A) tail. If the oligo(A) tails of the *P.falciparum* mitochondrial rRNAs have a role, what might it be? One possible role would be participation in the assembly process or in maintaining the proper three-dimensional structure of the ribosome. However, predictions of the potential secondary structures of the fragmented rRNAs have thus far revealed no obvious interactions for the added A residues (J.E.Feagin, unpublished results). A second, and perhaps more likely, possibility is that addition of extra A residues to the 3′ ends of the fragmented rRNAs protects the encoded sequences from exonuclease action. The variation in oligo(A) tail length among transcripts of the same rRNA and the apparent minor nibbling at the 3′ end of some SSUB transcripts may in part reflect this. If A tails protect the rRNAs, one might expect all the rRNA fragments to have them, which is clearly not the case. However, it may be that the structural characteristics which make some rRNA 3′ ends inaccessible to poly(A) polymerase also serve to provide a degree of exonuclease protection, making oligoadenylation less critical for those transcripts. Protection from nucleases may be a particularly important consideration for these fragmented rRNAs. Their structure is expected to depend considerably on intermolecular hydrogen bonding between short regions of the rRNA fragments (2,6). Considering the points above, it is likely that the oligo(A) tails are at least tolerated in the ribosome and their presence may preserve the sequences required for proper assembly and/or interaction in the ribosome.

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