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Complex Transcription from the Extrachromosomal DNA Encoding Mitochondrial Functions of *Plasmodium yoelii*

KATHLEEN SUPLICK, JOANNE MORRISEY, AND AKHIL B. VAIDYA*

Malaria Research Group, Department of Microbiology and Immunology, Hahnemann University, Philadelphia, Pennsylvania 19102-1192

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All tested members of genus *Plasmodium* contain tandemly arrayed, transcribed, extrachromosomal DNA with a unit length of 6.0 kb. This DNA contains two open reading frames with potential to encode cytochrome *c* oxidase subunit I (*cox1*) and cytochrome *b* (*cob*) as well as fragments of rRNA genes scattered on both strands. At least 10 discrete RNA molecules transcribed during erythrocytic stages of a rodent malarial parasite, *Plasmodium yoelii*, were recognized by the 6.0-kb DNA probes. The RNA molecules of 1.4 and 1.1 kb were identified as encoding *cox1* and *cob*, respectively. Primer extension and RNA sequencing were used to locate and characterize 5′ ends of these two RNAs, showing that an identical 12-nucleotide sequence, 5′-TATTTTT TGTTT-3′, was present at these positions. This sequence may act as a promoter or as an RNA processing signal. A stem-loop structure signifying a possible transcription termination was present at the end of the *cox1* open reading frame. At least six discrete RNA molecules of less than 250 nucleotides were recognized by different fractions of the 6.0-kb DNA. The largest of these, 200 nucleotides, was also characterized by primer extension and RNA sequencing. This molecule had a high homology to portions of the large-subunit rRNA domains IV and V. Other, small RNA molecules were recognized by regions of the 6.0-kb DNA that had homology to the highly conserved peptidyltransferase domain of large-subunit rRNA. These results show that the unusual compactly organized mitochondrionlike DNA of malarial parasites is transcribed in a complex pattern.

Malarial parasites, members of genus *Plasmodium*, belong to phylum Apicomplexa of kingdom Protista. Organisms within this phylum include many important pathogens, such as species of the genera *Toxoplasma*, *Babesia*, and *Eimeria*, in addition to *Plasmodium* species, all of which are intracellular parasites. They possess a complex array of organelles, some of which may assist in invasion of host cells; others have unknown functions. Among the organelles of *Plasmodium* species are mitochondria, whose contributions to the parasite physiology are not entirely clear. Because mammalian *Plasmodium* species rely mainly on glycolysis for energy generation and lack many citric acid cycle enzymes (22), mitochondria have been thought to act primarily as electron sink for dihydro-orotate dehydrogenase during pyrimidine biosynthesis (17). And yet, observations with rhodamine 123 staining (11, 24) and studies with mitochondrial inhibitors (4, 16) give a hint of organelles that may play a wider role in parasite biochemistry.

Until recently, not much was known about the organelle DNA of malarial parasites. A circular DNA molecule of about 35 kb was observed by electron microscopy and assumed to be the mitochondrial genome (12, 18, 28). This assumption appeared to be substantiated when genes encoding rRNA sequences resembling those of procaryotes and mitochondria were found within the 35-kb circular DNA of *Plasmodium falciparum* (15). However, through DNA sequencing of a 6.0-kb tandemly arrayed DNA of *Plasmodium yoelii*, we found that genes for the canonical mitochondrial proteins cytochrome *c* oxidase subunit I (*cox1*) and cytochrome b (*cob*) as well as portions of rRNA were present in this element (25). Furthermore, tandem arrays of the 6.0-kb sequence were detected in all tested species of malarial parasites (23, 26), and no significant cross-hybridization could be observed between the 6.0- and 35-kb DNA molecules (M. J. Gardner, J. E. Feagin, and A. B. Vaidya, unpublished data). Recently, M. J. Gardner, D. M. Williamson, and R. J. M. Wilson (Mol. Biochem. Parasitol., in press) have found sequences encoding proteins with homology to the β and β′ subunits of procaryotic RNA polymerase in the 35-kb circular DNA of *P. falciparum*. Thus, malarial parasites appear to possess two DNA molecules, each with a separate set of functions to encode organellar information.

Because organelle functioning could be a target for chemotherapeutic intervention by antimalarial agents, and because the plasmodial organelle genetic system appears to be so unusual, we are interested in studying the organization and expression of these genes. In this report, we describe transcripts originating from the 6.0-kb DNA of *P. yoelii*. A complex pattern of transcription was shown to result in at least 10 stable RNA molecules in erythrocytic stages of *P. yoelii*. Identification of the 5′ ends of *cox1* and *cob* mRNAs revealed common potential regulatory or processing signals. In addition, a short RNA molecule with partial rRNA-like sequence was also characterized.

MATERIALS AND METHODS

Experimental infections. Malaria infections were initiated by injecting 10^6 parasitized erythrocytes of lethal *P. yoelii* 17XL into 6- to 10-week-old BALB/c mice. Infections were monitored by enumerating parasitized erythrocytes in Giemsa-stained thin-blood films.

Nucleic acid isolation. Parasitized erythrocytes were collected from mice when parasitemias averaged 30%. Leukocytes were removed from the pooled blood by passage over columns of microcrystalline cellulose (14). Erythrocytes were lysed in phosphate-buffered saline (10 mM sodium

* Corresponding author.

phosphate, 150 mM sodium chloride [pH 7.4]) containing 0.1% glucose and 0.01% saponin. Parasites were separated from lysed erythrocytes by being pelleted at 11,000 × *g*. Parasites were subsequently lysed in 4 M guanidinium isothiocyanate solution (10). The lysate was layered over a 2-ml step gradient of 5.7 M cesium chloride and 3 ml of 3 M cesium chloride and was centrifuged for 20 h at 110,000 × *g*. RNA was recovered as a pellet at the bottom of the tube.

Northern (RNA) blotting. RNA samples were electrophoretically separated on 1.2% agarose containing formaldehyde and transferred to GeneScreen Plus (NEN Research Products) by standard Northern blotting procedures (2). To gain better resolution of the small-molecular-weight RNA molecules, RNA samples were run through 1.5-mm-thick 6% polyacrylamide gels containing 8 M urea in Tris-borate-EDTA buffer and then electroblotted onto GeneScreen Plus in 40 mM Tris hydrochloride (pH 8.0)–20 mM sodium acetate–2 mM EDTA at 0.3 V/cm for 16 h. The filters were prehybridized in 50% formamide–1 M NaCl–1% sodium dodecyl sulfate (SDS)–10% dextran sulfate for 6 h. The filters were then hybridized in the prehybridization buffer by the addition of 200 μg of yeast RNA per ml and 1×10^5 to 4×10^5 cpm of radiolabeled probe per ml (see below). Hybridization was done at 42°C, with constant agitation. After hybridization, the filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) at room temperature for 10 min, 2× SSC–1% SDS for 60 min at 60°C, and 0.1× SSC–0.1% SDS for 60 min at room temperature.

Probes. Complete digestion of the *P. yoelii* 6.0-kb DNA clone pPy5.8 with *Eco*RI, *Nde*I, and *Hin*dIII generated seven restriction fragments ranging in size from 280 to 1,700 nucleotides. These were given designations a to g (Fig. 1A). The restriction fragments were separated on a 6% polyacrylamide gel. Each fragment was cut from the gel and recovered by electroelution into dialysis bags. Restriction fragments were subsequently labeled with [α-^{32}P]dCTP, using the random-primer method (2), to a specific activity of 1×10^8 to 2×10^8 cpm/μg. pPy5.8 was similarly labeled after linearization of the plasmid by a restriction enzyme.

Primer extension and RNA sequencing. Three different primers were synthesized by standard phosphoramidite technology to characterize three different RNAs. T4 polynucleotide kinase was used to label 5′ ends of 50 to 80 ng of the primers with [γ-^{32}P]ATP. The labeled primers were adjusted to 2 M ammonium acetate and ethanol precipitated. The primers were annealed to 80 to 100 μg of total *P. yoelii* RNA in a solution containing 0.4 M NaCl and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4) by heating to 75°C for 10 min, followed by slow cooling to room temperature. The mixture was adjusted to 2 M ammonium acetate and precipitated with 10 volumes of ethanol. The precipitate was resuspended in 27.5 μl of a solution containing 50 mM Tris hydrochloride (pH 8.0), 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 mM KCl, 50 μg of bovine serum albumin per ml, and 25 U of RNAsin (Promega). For sequencing reactions, 5 μl each of the aforementioned suspensions was mixed with 3 μl of each of four separate dideoxy-deoxynucleoside triphosphate mixtures. Ratios of dideoxy- to deoxynucleoside triphosphates in the mixtures were 0.67 mM ddA:1.0 mM dA, 0.67 mM ddC:1.33 mM dC, 0.67 mM ddG:1.33 mM dG, and 0.67 mM ddT:1.0 mM dT. All other deoxynucleotide concentrations were 1.0 mM. The reactions were started by addition of 1.5 μl of avian myeloblastosis virus reverse transcriptase (7 U/μl) and incubated for 1.5 h at 42°C. At the end of incubation, 1 μl of calf liver tRNA (10 mg/ml) was added, followed by 100 μl of 2 M ammonium acetate and 110 μl of isopropanol. Precipitates were collected by centrifugation and washed three times with 70% ethanol. The reaction products were resuspended in formamide-dye-buffer mixture and electrophoresed in 6% polyacrylamide gel containing 7 M urea. Radioautography was done at −70°C. Parallel reactions with the same primers were done by using the dideoxynucleotide protocol of Sanger et al. (21) with single-stranded DNA templates, followed by electrophoresis and analysis as described above.

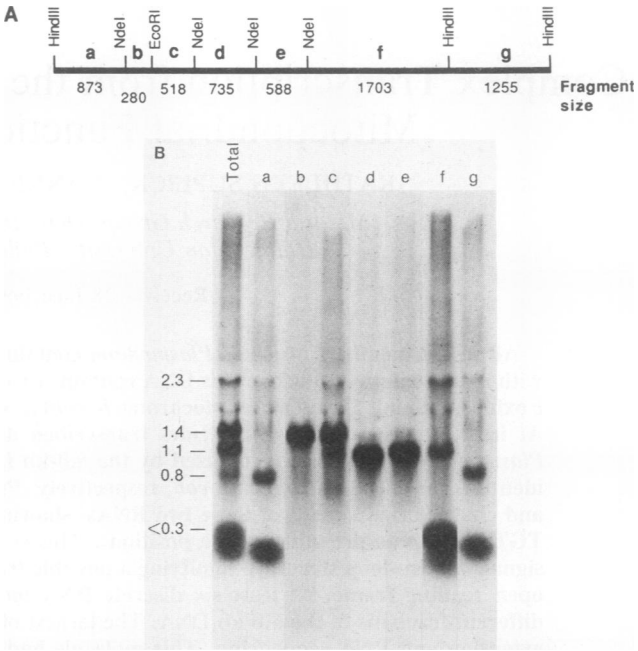

FIG. 1. Expression of discrete RNAs from various regions of the 6.0-kb DNA. (A) Restriction map and sizes (in nucleotides) of fragments generated by triple restriction digestion (*Hin*dIII, *Eco*RI, and *Nde*I) of the 6.0-kb DNA. The map is permuted for clarity; hence, the first nucleotide (and nucleotide 5952) lies in fragment f 452 nucleotides downstream of the *Nde*I site. (B) Northern blot hybridization results obtained by using the total 6.0-kb probe and probes synthesized from fragments a to g. Several parallel lanes containing 20 μg of total *P. yoelii* RNA each were subjected to formaldehyde–1.2% agarose gel electrophoresis and blotted onto nylon (GeneScreen Plus) membranes. Strips were cut from the membranes, prehybridized, and hybridized to the indicated probes. Radioautography was carried out for 18 to 36 h. The sizes (in kilobases) of RNA indicated at left were determined from the mobilities of rRNAs measured from ethidium bromide-stained marker lanes.

RESULTS

Transcription of at least 10 discrete RNA molecules from the 6.0-kb DNA. When a Northern blot of total RNA from erythrocytic stages of *P. yoelii* was probed with the 6.0-kb DNA clone pPy5.8, several discrete RNAs were recognized, ranging in size from less than 300 nucleotides to 2.3 kb (Fig. 1B, lane Total). In formaldehyde-agarose gels, the major RNA species were 2.3, 1.4, 1.1, and 0.8 kb long, along with a large smear of RNAs less than 300 nucleotides long. The smaller RNA molecules were better resolved by electrophoresis through 6% polyacrylamide gel containing 8 M urea, followed by electroblotting and probing with the 6.0-kb clone (Fig. 2, lane Total). Six major and several minor transcripts

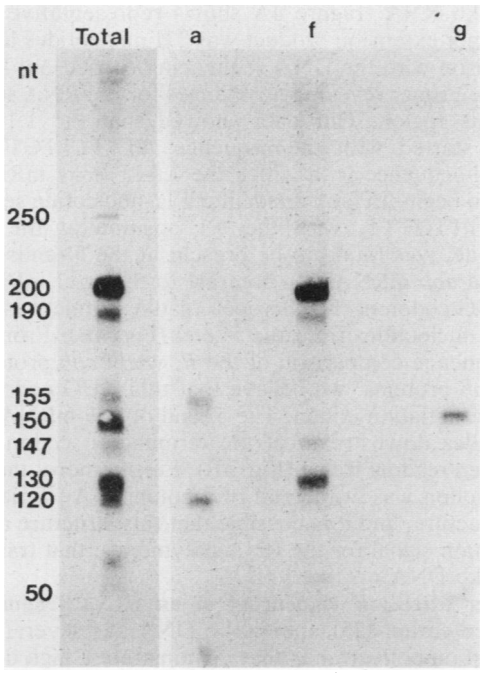

FIG. 2. Higher-resolution gel electrophoresis of the smaller RNAs recognized by the 6.0-kb probe. Total *P. yoelii* RNA (20 μg) was resolved by electrophoresis through 6% polyacrylamide containing 8 M urea and electroblotted to nylon membranes. Strips containing these RNAs were hybridized to probes from either the total 6.0-kb DNA (Total) or from fragments a, f, or g (see Fig. 1A), since these were the only fragments that recognized smaller RNA molecules in agarose gel-separated Northern blots (Fig. 1B). The radioautography was done without intensifying screens on Hyperfilm-βmax (Amersham Corp.) to maximize resolution. Sizes (in nucleotides [nt]) of the RNA band indicated at left were estimated from the mobility of end-labeled *Hpa*II-digested pBR322 and are therefore subject to slight error because of differences in the mobilities of RNA and DNA molecules.

were recognized by this probe. The sizes of the major small RNAs were 200, 190, 155, 150, 130, and 120 nucleotides. To identify the regions of the 6.0-kb DNA that encoded these different sizes of RNA molecules, the 6.0-kb DNA was divided into seven fragments generated by triple digestions with *Eco*RI, *Hin*dIII, and *Nde*I (Fig. 1A). These fragments, named a to g, were used to probe Northern blots of *P. yoelii* RNA resolved by either formaldehyde-agarose or urea-polyacrylamide gel electrophoresis. The 1.4-kb RNA was recognized strongly by probes b and c and weakly by probes a and d; the 1.1-kb RNA was recognized strongly by probes d, e, and f; and the 0.8-kb RNA was recognized by probes a and g (Fig. 1B). Only fragments a, f, and g hybridized to the smaller RNA molecules, with probe a recognizing the 155- and 120-nucleotide RNAs, f recognizing 200-, 190-, and 130-nucleotide RNAs, and g hybridizing to the 150-nucleotide species (Fig. 2). The 2.3-kb RNA, on the other hand, was recognized by all of the fragments except b. This result may be due to the presence of more than one 2.3-kb transcript originating from the 6.0-kb DNA, or there may be complex splicing events involved in generation of the 2.3-kb RNA; on the other hand, it may merely represent nonspecific hybridization. A few minor larger RNAs appeared to comigrate with the parasite rRNAs, which may be due to fortuitous cross-hybridization because of the presence of rRNA-like sequences in some of the 6.0-kb fragments (see below). By using strand-specific RNA probes, we found that the 2.3-kb, 1.4-kb, 1.1-kb, 155-nucleotide, and 120-nucleotide RNA molecules were transcribed from one strand of the 6.0-kb DNA, whereas the 0.8-kb, 200-nucleotide, 190-nucleotide, 150-nucleotide, and 130-nucleotide RNA molecules were transcribed from the other strand (data not shown).

From the DNA sequence analyses of the 6.0-kb clone (25), we knew that there were two open reading frames with the potential to encode polypeptides homologous to the *cox1* and *cob* proteins. Hybridization patterns described above localized the 1.4-kb RNA as originating from the *cox1* open reading frame of 477 amino acids and the 1.1-kb RNA as originating from the *cob* open reading frame of 376 amino acids. A part of the DNA sequence of fragments recognizing the 0.8-kb RNA has an open reading frame with similarity to a portion of cytochrome *c* oxidase subunit III gene (*cox3*). However, cDNA sequencing will be required to establish the 0.8-kb RNA as the *cox3* mRNA. We were unable to assign any tentative functional role for the 2.3-kb RNA solely on the basis of Northern blot hybridization and DNA sequence.

5′ End of the 1.4-kb *cox1* mRNA. To localize the 5′ end of the 1.4-kb *cox1* mRNA, we carried out primer extension and RNA sequencing. A 24-nucleotide primer originating from fragment a, 5′-GATGAAGAGTATAATTCTGTACGT-3′, was synthesized from the DNA sequence of this region. This primer was used in dideoxynucleotide chain termination sequencing reactions, using either the cloned single-stranded DNA as a template and modified T3 DNA polymerase (Sequenase) as the enzyme or the total *P. yoelii* RNA as the template and reverse transcriptase as the polymerase. Such reactions were carried out and displayed by sequencing gel electrophoresis in five separate experiments. A representative result is shown in Fig. 3A. A single major primer extension product of 144 nucleotides was seen in primer extension of the RNA. (Bands larger than the major primer extension products in RNA reactions seen here may be due to binding of the primer to the 2.3-kb minor RNA or may represent spurious primer extensions.) Sequencing reactions showed the RNA sequence to be the same as the DNA sequence for this region.

The 5′ end of *cox1* mRNA was shown to contain the sequence ATTTTTTGTTT. Interestingly, the first methionine codon for this mRNA was predicted to occur at codon position 62 of the *cox1* open reading frame (Fig. 3B); since RNA sequencing did not show any posttranscriptional changes for this region, assignment of the initiation codon for *cox1* was difficult. One possibility is that the AUG codon at position 62 acts as the initiation codon. However, the first 61 amino acids encoded by this RNA have substantial homology to the first hydrophobic domain of *cox1* polypeptides from a variety of species, as determined by FASTA (20) analysis. This region is predicted to form the first hydrophobic transmembrane domain of the *cox1* protein, a polypeptide with a total of 12 predicted transmembrane domains (27). Another possibility is that a codon other than AUG acts as an initiator. In any case, N-terminal sequencing of the *cox1* polypeptide will be needed to resolve this question.

5′ End of the 1.1-kb *cob* mRNA. A 28-nucleotide primer from fragment d with the sequence 5′-GAATACAAGTGAT GCACCAGTAGCGTGC-3′ was synthesized and used in primer extension reactions to localize the 5′ end of the 1.1-kb *cob* mRNA. Both *P. yoelii* 6.0-kb clone DNA and total parasite RNA were used as templates as described above for

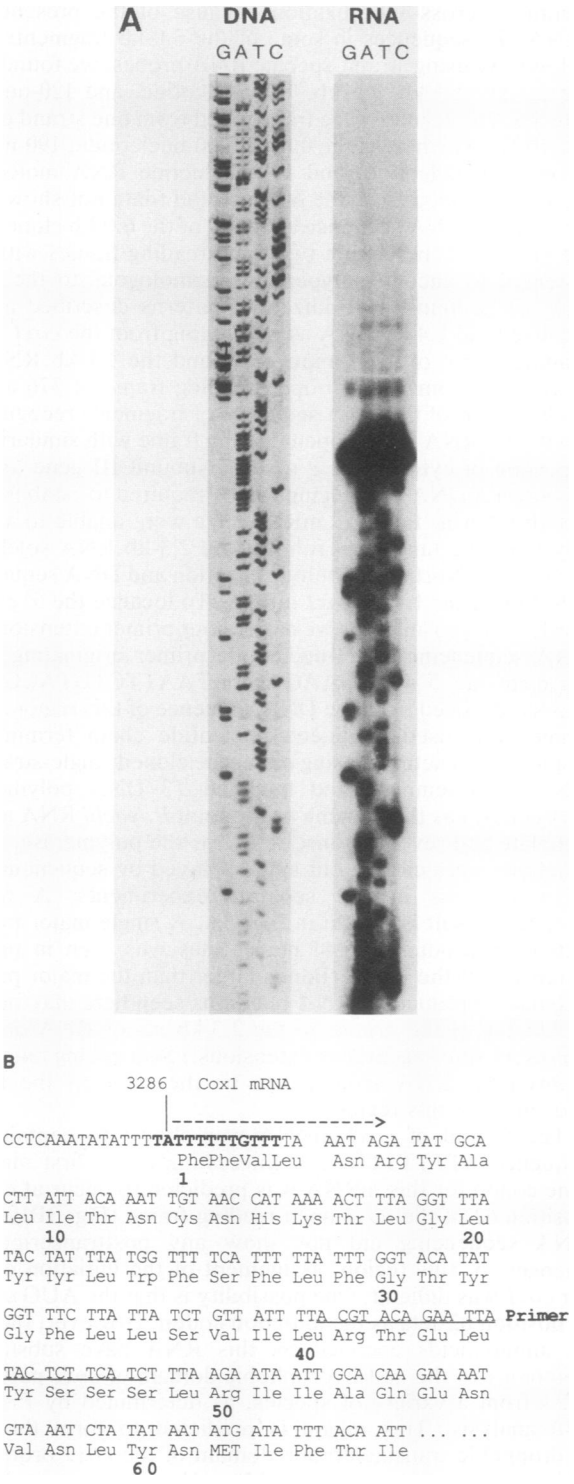

FIG. 3. Determination of the 5′ end of the *cox1* mRNA. (A) A 24-mer synthetic oligonucleotide from fragment a (Fig. 1A) was used in a primer extension sequencing reaction as described in Materials and Methods. Templates used were either single-stranded DNA containing the 6.0-kb clone (rescued as single-stranded phage particles) or total *P. yoelii* RNA. The DNA reactions contained ^{35}S-labeled dATP, whereas the RNA reactions contained 5′-^{32}P-labeled primers. The major primer extension product in RNA reactions was 144 nucleotides long. (B) DNA sequence around the 5′ end of *cox1* mRNA. The 5′ end of the mRNA at nucleotide position 3286 is indicated by a vertical line with an arrow showing the direction of transcription. A 12-nucleotide sequence shared by 5′ ends of *cox1* and *cob* mRNAs is in bold type. The position of the primer is indicated by underlining. Note that the first methionine codon occurs at amino acid position 62 in the *cox1* open reading frame.

the 1.4-kb RNA. Figure 4A shows representative results. The primer extension product was 271 nucleotides long, and comparison with the DNA sequencing carried out by using the same primer revealed no changes for the RNA sequence from this region. Our data showed that the 1.1-kb *cob* mRNA started with the sequence ATTTTTTGTTT, the identical sequence with which the 1.4-kb *cox1* mRNA was shown to begin. In fact, an identical 12-nucleotide sequence, TATTTTTTGTTT, with the +1 position at the second nucleotide, was found to be present at the 5′ ends of both *cox1* and *cob* mRNAs. In contrast to the *cox1* mRNA, the first AUG codon of the *cob* open reading frame was located only 13 nucleotides from the 5′ end (Fig. 4B). From amino acid sequence comparison of the *P. yoelii cob* protein with other *cob* proteins, we believe that this AUG codon is the probable initiation codon. The 5′ end of *cob* mRNA was 14 nucleotides downstream of the termination codon for the *cox1* open reading frame (Fig. 4B). Furthermore, this termination codon was at the end of a potential A+T-rich stem-loop structure, and it is possible that this structure acts as a termination signal for the RNA polymerase that transcribes the 6.0-kb DNA.

Primer extension sequencing of an rRNA fragment. As published earlier (25), the 6.0-kb DNA has several regions scattered on both strands that demonstrate a high degree of sequence homology to some of the most highly conserved regions of rRNA. We are interested in knowing whether such regions are transcribed and, if so, what their structure and function may be. To begin such analysis, we have characterized the region of 6.0-kb DNA with the longest sequence homology to rRNA by primer extension and sequencing. A 36-nucleotide primer from fragment f with the sequence 5′-TTCTATTTATAGGAGTCTCACACTAGC GACAATGG-3′ was synthesized for this purpose. The choice of primer for the rRNA region had to be made carefully because of relatively high homology of parts of these regions with other rRNAs, including those encoded by the nucleus. In some of our experiments, certain synthetic primers have given extension products from the much more abundant nuclear-coded rRNAs. The primer mentioned above hybridized to the 200-nucleotide RNA recognized by fragment f (data not shown). Primer extension sequencing reactions are shown in Fig. 5A; the major extension product was 128 nucleotides long. Both DNA and RNA sequencing reactions gave identical sequences. The sequence at the 5′ end of this RNA was quite different from that of the *cox1* and *cob* mRNAs. We do not know where the 3′ end of this RNA is located, but knowing that the primer recognized the 200-nucleotide RNA, and assuming that this 200-nucleotide RNA is colinear with the DNA sequencing, we can approximately place the endpoints of this RNA molecule. This RNA, then, has a high degree of homology to portions of domains IV and V of the large-subunit rRNA (19) (Fig. 5B and C). This RNA molecule can potentially form hydrogen-bonded helical structures typical of these domains by *trans* association with other small RNA molecules potentially encoded from separate regions of the 6.0-kb DNA, specifically by nucleotides 2776 to 2859, and those complementary to nucleotides 1535 to 1590. (All nucleotide positions refer to the sequence with accession number M29000 in GenBank.)

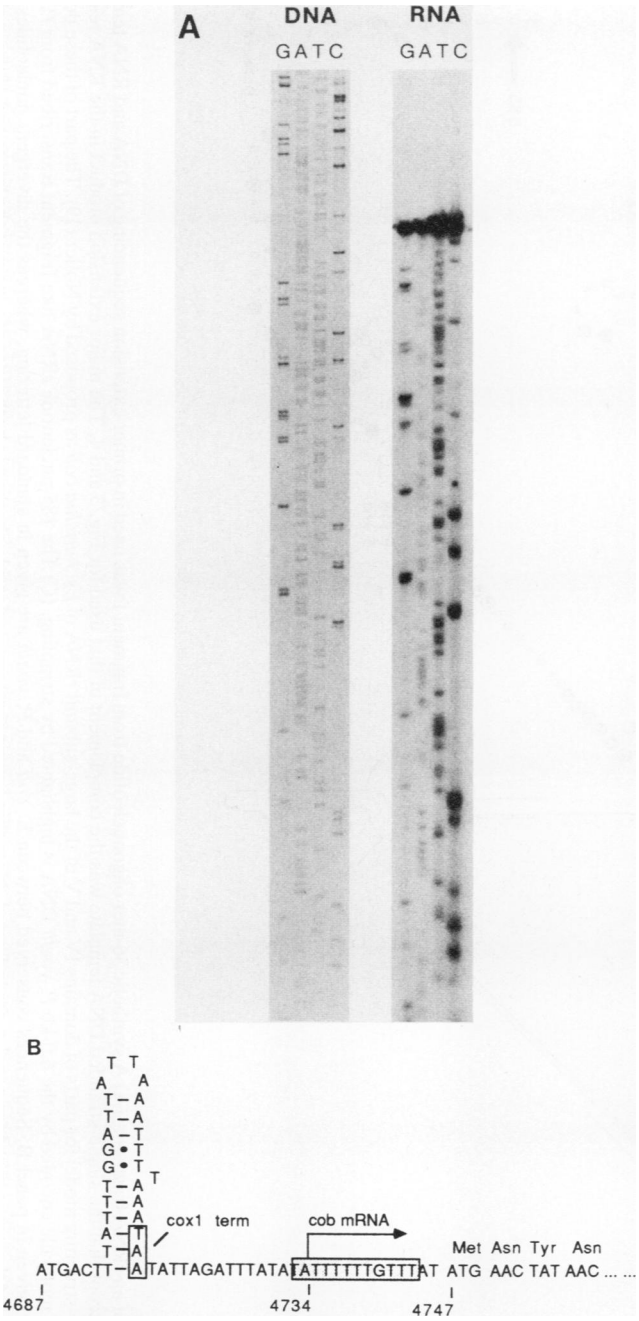

FIG. 4. Determination of the 5′ end of the *cob* mRNA. (A) A synthetic 28-mer primer from fragment d (Fig. 1A) was used in primer extension sequencing reactions as described in the text. The DNA and RNA templates were the same as in Fig. 3. The major primer extension product was 271 nucleotides long, and no changes in the RNA sequence were observed. (B) DNA sequence around the 5′ end of the *cob* mRNA. The 5′ end of the mRNA at nucleotide position 4734 within the strippled box is indicated. A potential A+T-rich stem-loop structure precedes the 5′ end of *cob* mRNA, and at the end of the stem-loop structure is the TAA termination codon for the *cox1* open reading frame. The first methionine codon for the *cob* open reading frame starts 13 nucleotides away from the 5′ end of the *cob* mRNA.

In fact, Northern blot hybridization experiments have identified several small RNA molecules from the regions of the 6.0-kb DNA (Fig. 2) with potential to encode rRNA fragments. We do not yet know whether *trans*-associated ribosomes with mini-rRNAs exist in *Plasmodium* species or, if they do, what their physiological roles may be.

DISCUSSION

In this report, we have described complex transcription from one of the two organellelike DNAs of malarial parasites. We have shown that discrete regions of the 6.0-kb DNA are transcribed into at least 10 stable RNA molecules and that both strands of the DNA are transcribed. A transcription map (Fig. 6) shows that transcription appears to occur in a nonoverlapping manner. Although it is not clear where the transcription starts, *cox1* and *cob* mRNAs have a common sequence at their 5′ ends. It has been shown in mitochondrial transcription systems of *Saccharomyces cerevisiae* (3, 13), *Xenopus laevis* (6), and mammals (7–9) that the promoters, consisting of 8- to 11-nucleotide sequences, are present very close to the 5′ ends of primary transcripts. By analogy, therefore, if the 1.4- and 1.1-kb mRNAs are primary transcripts, the sequence TATTTTTTGTTT may contain within it a mitochondrial promoter for *P. yoelii*. It is interesting that an identical sequence is also present in a *Plasmodium gallinaceum* 6.0-kb element at the beginning of the *cob* gene (1). Furthermore, a slightly divergent sequence has also been identified at the beginning of *cox1* and *cob* genes of *P. falciparum* (J. Morrisey and A. B. Vaidya, unpublished results). On the other hand, the 5′ end of the 200-nucleotide rRNA-like transcript did not show any similarity to this sequence. Hence, it is difficult to state where transcription initiates within the two strands of the 6.0-kb DNA. One possibility is that the tandem organization of the 6.0-kb DNA molecules permits synthesis of large primary transcripts overlapping adjacent repeating units concomitant with rapid processing to generate stable RNA molecules. Another possibility is that there are multiple promoters and terminators within the 6.0-kb DNA molecule generating multiple primary transcripts, some of which require further endonucleolytic processing and others of which do not. If the latter is true, the 6.0-kb DNA may contain highly compact transcription units.

We have not detected any tRNA-like genes or transcripts in the 6.0-kb DNA; however, the rRNA-like sequences were clearly present. These sequences have a bizarre organization, reminiscent of what has been observed in *Chlamydomonas reinhardtii* mitochondrial DNA (5). The rRNA-like regions of the 6.0-kb DNA show more than 95% conservation among the three diverse species of *Plasmodium* (*P. yoelii*, *P. falciparum*, and *P. gallinaceum*) from which we have sequence information (1, 23, 25; Morrisey and Vaidya, unpublished data). Hence, there appears to be evolutionary pressure to maintain the sequence and, by implication, the function of these fragmented rRNA domains. What this function could be is not clear. If these domains contribute to the formation of some functional ribosomes, the remainder of the rRNA domains will have to be derived from sequences other than those specified by the 6.0-kb DNA. These are intriguing problems indeed.

The 6.0-kb DNA is one of two molecules of *Plasmodium* species encoding organelle functions. At present, there are many unknown aspects regarding the 6.0- and 35-kb DNA molecules. We do not yet know the intracellular location of the 6.0- and 35-kb DNA molecules. Although we can detect

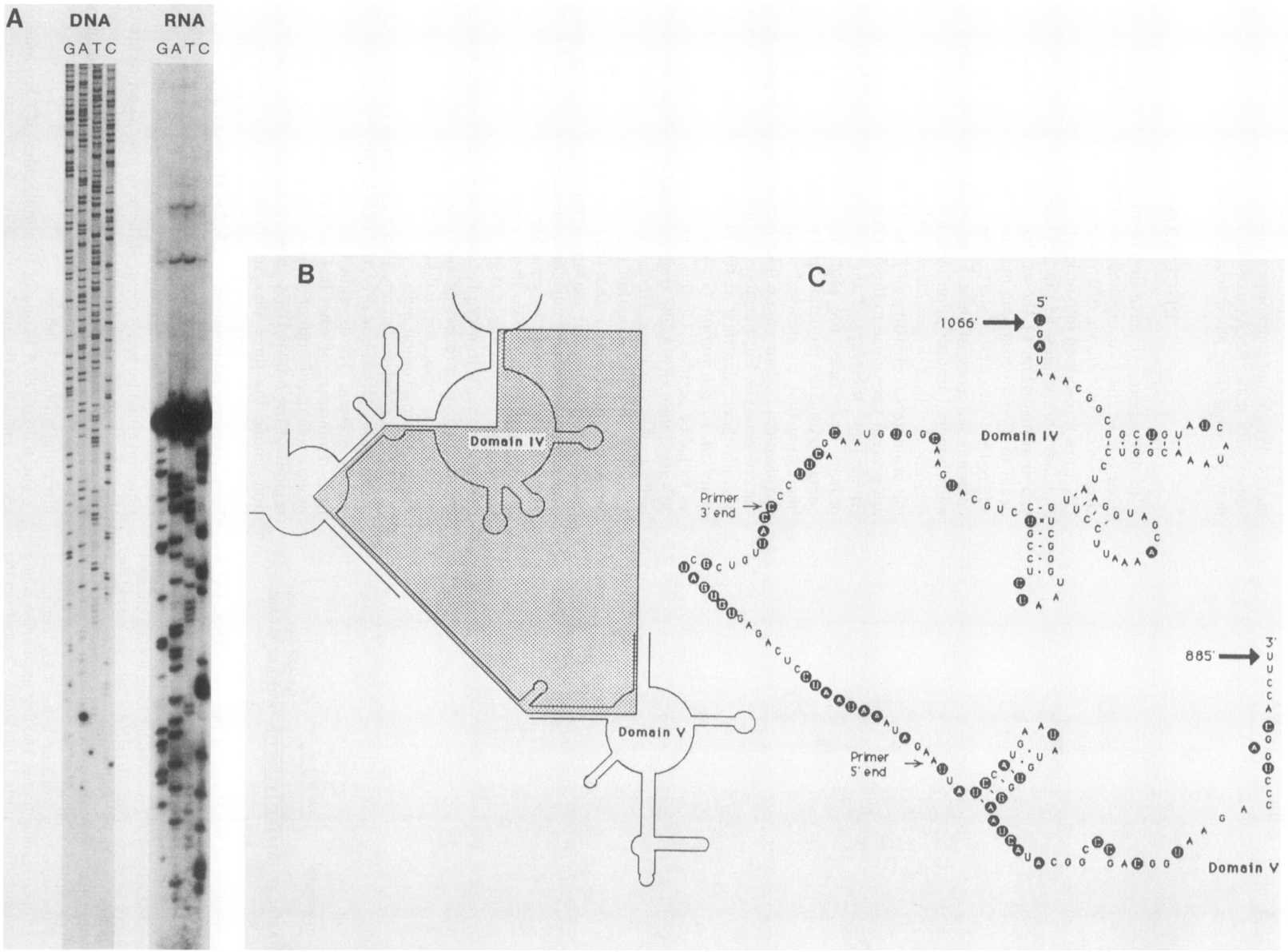

FIG. 5. Determination of the 5′ end of an rRNA fragment. (A) A synthetic 36-mer oligonucleotide from fragment f was used in primer extension sequencing of DNA and RNA templates as described in the text and for Fig. 3 except that the single-stranded DNA template was the complement of that used for Fig. 3 and 4. The major extension product in the RNA reactions was 128 nucleotides long. (B) Secondary-structure model for parts of domains IV and V of the large-subunit rRNA of *Escherichia coli* as proposed by Noller (19). The part of these domains represented by the 185-nucleotide RNA molecule encoded by the 6.0-kb *P. yoelii* DNA is highlighted by strippling. (C) The 185-nucleotide rRNA-like fragment transcribed from *P. yoelii* 6.0-kb DNA folded according the model given in panel B. Sequences conserved between *E. coli* and *P. yoelii* are given in standard lettering, whereas the divergent nucleotides are in negative contrast. The 5′ and 3′ positions of the primer used for panel A are indicated. Note three compensatory nucleotide changes in both sides of a stem at the lower end of the molecule.

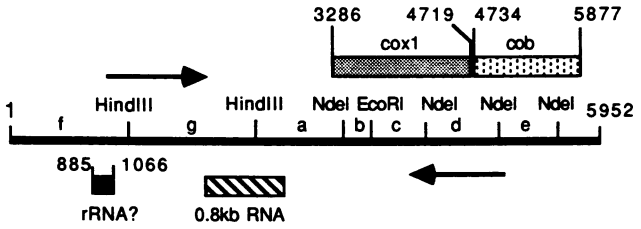

FIG. 6. Transcription map of the 6.0-kb DNA of *P. yoelii* encoding mitochondrial functions. The numbers represent nucleotide positions in the published sequence. The arrows indicate the direction of transcription; the upper strand is transcribed toward the right, and the lower is transcribed toward the left. The 5′ ends of *cox1* and *cob* mRNAs were experimentally determined. The 3′ ends of these mRNAs are not exactly located; hence, positions of the termination codons in the open reading frames are given. The 5′ end of an rRNA-like fragment was from Fig. 5, and the 3′ end corresponds to the region where homology to rRNA breaks down. The position of 0.8-kb RNA is approximate.

the 6.0-kb sequences in crude cytoplasmic fractions of *P. yoelii*, we also detect these molecules in nuclear fractions, since clean separation of mitochondrial and nuclear fractions is not possible (Morrisey and Vaidya, unpublished data). The 35-kb DNA, on the other hand, contains two copies of rRNA genes as well as genes encoding eubacteriumlike RNA polymerase subunits (Gardner et al., in press). These characteristics are more reminiscent of a chloroplast DNA than of a mitochondrial DNA. The *cox1* and *cob* genes, the usual mitochondrial functions, are present on the 6.0-kb DNA and not on the 35-kb DNA. The codon usages of the 35-kb DNA and the 6.0-kb DNA sequences are quite distinguishable (Gardner et al., in press; A. B. Vaidya et al., unpublished data). Furthermore, the rRNA sequences encoded by the 6.0- and the 35- DNA molecules are substantially divergent in both organization as well as in composition (Gardner et al., unpublished data). All of this information tempts us to speculate that the 6.0-kb molecule is a highly derivatized mitochondrial DNA, that the 35-kb molecule is a highly derivatized chloroplast DNA, and that these molecules are expressed in separate compartments. By extension, this speculation could also be applied to other organisms of phylum Apicomplexa. It will be important to investigate these suggestions further because, if true, they may affect our views of the evolutionary position and modes of control of these important pathogens.

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