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Sequence Identification of Cytochrome *b* in *Plasmodium gallinaceum*

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We have identified a gene that encodes the polypeptide cytochrome *b* in the avian malarial parasite *Plasmodium gallinaceum*. The gene containing the open reading frame was found to be located on a 6.2-kilobase multimeric extrachromosomal element. The amino acid translation from this gene demonstrated significant similarities to cytochrome *b* sequences from yeast, mammal, and fungus genomes. We present evidence that the *P. gallinaceum* cytochrome *b* transcript is part of a larger primary transcript from the element that is subsequently processed. The message for *P. gallinaceum* cytochrome *b* was found to be 1.2 kilobases in size. This is the first report identifying a mitochondrial nucleic acid sequence in malaria-causing organisms and suggests that a functional cytochrome system may exist in these parasites.

Until recently, very little was known about mitochondrial biogenesis and function during the life cycle of malarial parasites. *Plasmodium* species were thought to rely solely on glycolysis for the generation of ATP (36–38). The appearance of acristate mitochondria and the absence of a complete complement of functional Krebs cycle enzymes (6, 38) established the role of the mitochondria in the asexual parasite as an exclusive electron disposal mechanism for pyrimidine de novo biosynthesis (17, 20). On the basis of the sensitivity of the organisms to various mitochondrial inhibitors, recent reports have speculated on the possibility of an energy transport system in malarial parasites (7, 18). A classical functioning mitochondrion in *Plasmodium falciparum* has been implicated by the use of the cationic fluorescent dye rhodamine 123. This dye was used to demonstrate that the mitochondria in asexual parasites maintained a high transmembrane energy potential, indicating they are metabolically active (13). The enzyme cytochrome oxidase has been defined in *P. falciparum* and localized to the mitochondrial organelle (37).

The malarial mitochondrial genome has not been characterized. Three reports have identified a 20- to 30-kilobase (kb) closed circular extrachromosomal DNA in several species of *Plasmodium* (14, 25, 43). This DNA was classified as mitochondrial on the basis of its physical structure and subcellular fractionation. The partial sequence of a small-subunit mitochondrial rRNA has been mapped to this DNA molecule in *P. falciparum* (15). In most other systems, the mitochondrial genome consists primarily of closed circular species-specific molecules that encode several polypeptides and all tRNA and rRNA genes necessary for mitochondrial protein synthesis. These genomes operate independently of the nuclear genetic system and usually encode five main polypeptides (three subunits of cytochrome *c* oxidase, cytochrome *b*, and ATPase subunit 6) as well as a number of polypeptides within other unidentified open reading frames. In the mammalian system, these open reading frames have been found to encode several subunit polypeptides of NADH-coenzyme Q reductase (2, 10, 39).

One of the genes encoded by most mitochondrial systems corresponds to the polypeptide cytochrome *b*. Cytochrome *b* is one subunit of a large multimeric complex known as coenzyme QH_2-cytochrome *c* reductase. The cytochrome *b* protein is thought to play a role in the heme-binding activity of this mitochondrial complex (35, 42).

In this paper, we report the identification of the gene encoding the polypeptide cytochrome *b* from the avian malarial parasite *Plasmodium gallinaceum*. The sequence of this polypeptide demonstrates significant similarity to mitochondrial cytochrome *b* sequences from yeast (32), human (2), bovine (4), and *Aspergillus nidulans* (41) cells. This is the first identification of a mitochondrial nucleic acid sequence in a malarial parasite and suggests the existence of a cytochrome system in *Plasmodium* species. The cytochrome *b* gene from *P. gallinaceum* was found to be contained within a 6.2-kb multimeric DNA element that is conserved among a number of *Plasmodium* species (24). This conservation may reflect similar functional activities of cytochromes and electron transport within the mitochondria of various *Plasmodium* species.

MATERIALS AND METHODS

RNA preparation. Total *P. gallinaceum* RNA was prepared from approximately 5×10^8 zygotes (24). Parasites were lysed in a buffer containing 1% sarcosyl, 50 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8), 2 mM EDTA, and 100 mM NaCl (lysis buffer). After lysis, RNA was immediately extracted with phenol-chloroform (1:1) and repeatedly extracted until the interphase was clear. Total RNA was precipitated by the addition of 0.4 M sodium acetate and 2.5 volumes of ice-cold 100% ethanol. Poly(A) mRNA was isolated from total RNA by two passages over oligo(dT)-cellulose (5).

Gel electrophoresis, blotting, and hybridization. Gel electrophoresis of RNA was carried out in 1% agarose gels containing 20 mM Na_2HPO_4–NaH_2PO_4 (1× Northern buffer) and 6% formaldehyde. RNA samples were denatured by heating at 55°C for 15 min in a 20-μl volume containing 1× Northern buffer, 6% formaldehyde, and 50% formamide. RNA was transferred from agarose gels to nitrocellulose filters (Schleicher & Schuell, Inc.) overnight, using 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate). Northern (RNA) hybridizations were carried out in 50% formamide–10× Denhardt solution (1% Ficoll, 1% polyvi-

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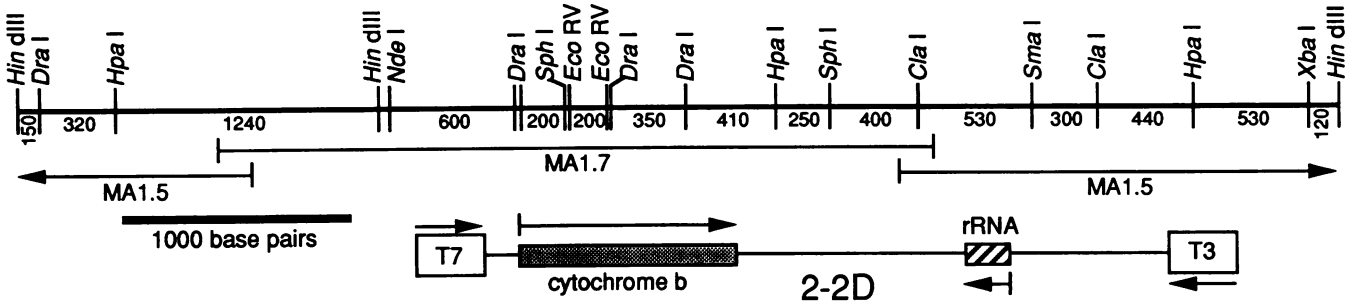

FIG. 1. Restriction map of *P. gallinaceum* cDNA clones. Restriction mapping of cDNA clone 2-2D (insert size of 2.7 kb) is shown in comparison with the *Plasmodium* DNA element map (24). Genomic clones are indicated as MA1.7 and MA1.5. Arrows indicate the proposed directions of transcription for both the cytochrome *b*-coding region (▩) and the region found to be homologous to mitochondrial large-subunit rRNA genes (▨). T7 and T3 promoter positions in relation to that of the 2-2D insert are also noted. Element unit size appears to be approximately 6.2 kb and is either tandemly repeated or circularly permuted.

nylpyrrolidine, 1% bovine serum albumin)–6× SSC–20 mM NaH_2PO_4–0.1 mg of herring sperm DNA per ml for 24 h at 42°C. Filters were washed once in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and twice in 0.1× SSC–0.1% SDS for 30 min each at 55°C, dried, and exposed to X-ray film.

Library synthesis and screening. A lambda gt11 *P. gallinaceum* cDNA library was prepared by the method of Gubler and Hoffman (19) as described by Neve et al. (31). After blunting and methylation of *P. gallinaceum* cDNA, *Eco*RI linkers (New England BioLabs, Inc.) were added, and DNA was ligated to *Eco*RI-digested, phosphatase-treated lambda gt11 arms (Stratagene). cDNA was packaged into bacteriophage lambda and amplified through *Escherichia coli* Y1088 as described by Huynh et al. (23). The *P. gallinaceum* cDNA library contained approximately 10^5 plaques, 60% of which contained inserts. Recombinant phage were plated at a density of approximately 500 plaques per 100-mm-diameter petri dish in LB agar containing 5 μM ampicillin, 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 0.05% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). After overnight growth, plaques were lifted to nitrocellulose filters, denatured with 0.5 N NaOH, and neutralized with 1.5 M Tris (pH 8). Baked filters were prehybridized for 4 h at 42°C in 50% formamide–10× Denhardt solution–5× SSC–0.1 mg of herring sperm DNA per ml. Hybridizations with radiolabeled probe were carried out for 12 to 24 h at 42°C. Filters were washed three times for 30 min each in 0.1× SSC–0.1% SDS at 55°C. Plaques that gave a positive signal were picked and screened twice on low-density plates. DNA was extracted from recombinant phage that remained positive and used to determine insert size or for subsequent subcloning.

An initial screening of the *P. gallinaceum* cDNA library with the genomic clone MA1.7 (24) yielded 102 positive clones. Since the library contains 6×10^4 total recombinants, this represented approximately 0.2% of the recombinant phage.

DNA sequencing. Recombinant phage were digested with the restriction enzyme *Eco*RI, and inserts were purified by gel electroelution. Inserts were subcloned into the *Eco*RI cloning site of the vector Bluescript (Stratagene) or pUC19. Nested deletions of Bluescript plasmids were created by using the exonuclease III-mung bean nuclease unidirectional deletion system (Stratagene) as specified by the manufacturer.

Single-stranded phage of nested deletion clones were prepared by using R408 helper phage according to protocols established by Stratagene. All sequencing reactions were performed by the dideoxy-chain termination method of Sanger et al. (34). Reaction condition were established by using either a ^{35}S sequencing kit (New England BioLabs) or Sequenase (United States Biochemical Corp.). All reactions were performed as specified by the manufacturers. Sequencing reactions were analyzed by using a 6% acrylamide–7.67 M urea–0.5× to 2.5× TBE gradient gel (1× TBE is 100 mM Tris, 80 mM boric acid, and 1 mM EDTA [pH 8.4]). Gels were run by using the Bio-Rad Sequigen system with either the 38- by 40- or 38- by 80-cm plates. Gels were electrophoresed at 100 W and 50 to 55°C for 2 to 6 h. Oligonucleotides synthesized as sequencing primers were made by using a model 8750 Biosearch DNA synthesizer.

T3 and T7 transcripts and hybridization. Plasmid templates for transcription were linearized by digestion with *Kpn*I for T7 transcripts and *Sac*I for T3 transcripts. [α-^{32}P]UTP-labeled transcripts were synthesized according to protocols established by Stratagene.

Prehybridization and hybridization of Northern filters with in vitro transcripts were carried out in 50% formamide–5× SSC–1× PE (1× PE is 50 mM HEPES [pH 7.5], 0.1% [wt/vol] sodium pyrophosphate, 1% SDS, 1% polyvinylpyrrolidine, 1% Ficoll, and 5 mM EDTA)–0.15 mg of denatured herring sperm DNA per ml at 50°C. Filters were washed twice in 2× SSC–0.1% SDS and twice in 0.1× SSC–0.1% SDS for 15 min each. All washings were carried out at 65°C.

RESULTS

Identification and characterization of *P. gallinaceum* cDNA encoding cytochrome *b*. Our original interest was to analyze highly expressed genes during the zygote stage of *P. gallinaceum*. Previous work (24) had identified a highly conserved, 6.2-kb extrachromosomal DNA element from *P. gallinaceum* that hybridized to multiple RNA transcripts. The purpose of these experiments was to isolate and analyze cDNA clones corresponding to these transcripts. Therefore, a *P. gallinaceum* zygote cDNA library was screened with the genomic DNA element clone MA1.7 (Fig. 1). From this screening several clones were identified, and recombinants were found to be multiple copies of phage with one of three insert sizes. The largest of the clones had an insert size of 2.7 kb; the other clones had insert sizes of either 1.6 kb or 500 bases. According to our DNA hybridization results, the multiple copies of each insert appeared to be identical.

Restriction mapping of clone 2-2D, which contained an insert of 2.7 kb, is shown in Fig. 1. Within the level of accuracy afforded by restriction mapping, this clone ap-

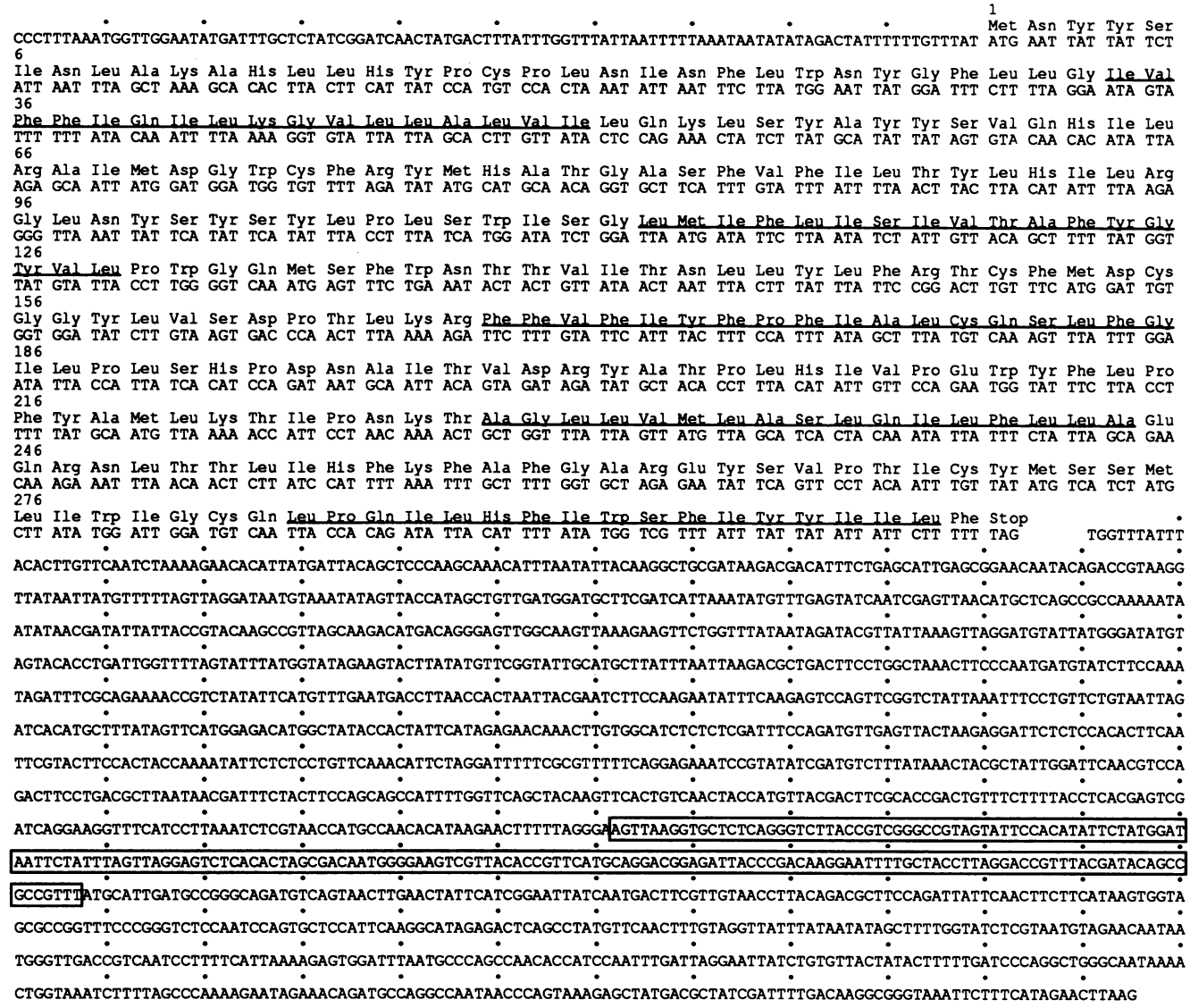

FIG. 2. Nucleotide sequence of the 2,690-bp insert from cDNA clone 2-2D. The long open reading frame and its predicted amino acid sequence are shown. Probable transmembrane sections are underlined and were determined by using the Intelligenetics SOAP program, which utilizes the method of Klein et al. (26). Boxed area indicates the region that shows significant nucleic acid homology to several mitochondrial large-subunit rRNA genes.

peared to map directly onto the genomic sequence derived for the repeated DNA element in *P. gallinaceum* (24).

Identification of the cytochrome *b*-coding sequence. The insert of clone 2-2D was sequenced by using the exonuclease III-mung bean nuclease unidirectional nested deletion system (Stratagene). The entire sequence was determined for both DNA strands. Oligonucleotides were synthesized and used as primers for regions not covered by a deletion clone.

The 2,690-bp sequence of clone 2-2D is presented in Fig. 2. Nucleotide base 1 is the 5′ *Eco*RI cloning site. The noncoding strand is shown; protein-coding sequences were translated by using the protozoal mitochondrial genetic code. This code recognizes TGA as tryptophan rather than termination, as is the case for the mitochondrial DNA of yeast (8), *Drosophila* (12), fungal (41), and mammalian (2) genetic systems. The sequence contains a single uninterrupted open reading frame (bases 103 to 1003) coding for a protein of 300 amino acids (36,000 daltons).

Sequence comparison. The protein-coding sequence of 2-2D was compared with other known eucaryotic protein sequences contained in the Intelligenetics Swisspro data bank. Comparisons were carried out by using the Lipman-Pearson algorithm (29), with a k-tuple value of 1 and the distance parameter set to 2 amino acids. The result of this comparison indicated a significant similarity between the *Plasmodium* sequence and protein sequences corresponding to cytochrome *b* from a number of species. In Fig. 3, the amino acid sequences of mitochondrial cytochrome *b* for yeast, bovine, human, and *A. nidulans* genomes are compared with the *Plasmodium* sequence. The extent of overall amino acid homology between *Plasmodium* species and all other species is calculated to be approximately 47%. In comparison, the cytochrome *b* sequence for *Saccharomyces cerevisiae* has 50% homology with the cytochrome *b* sequence from bovine cells (4).

The *Plasmodium*-encoded protein was found to be slightly

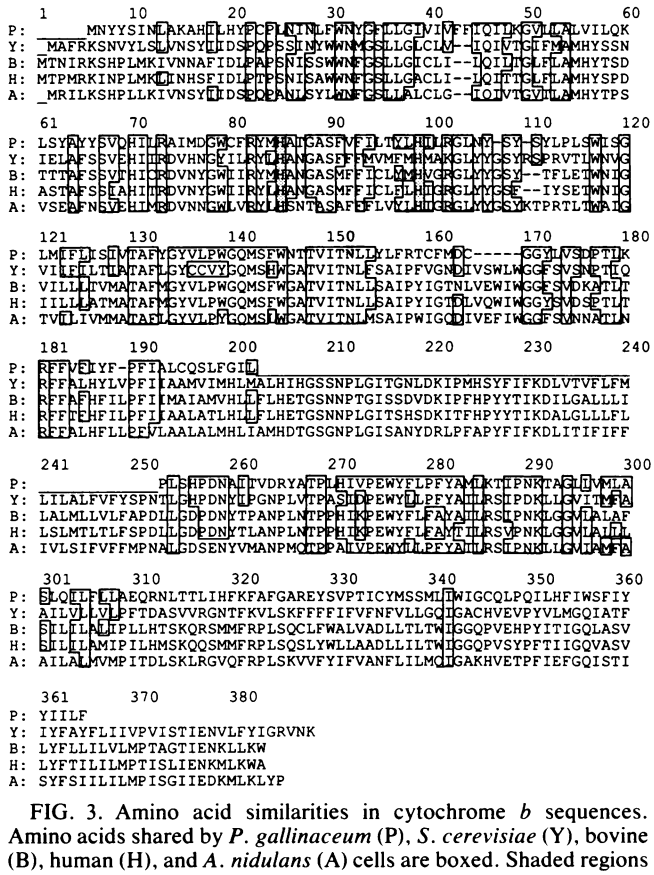

FIG. 3. Amino acid similarities in cytochrome *b* sequences. Amino acids shared by *P. gallinaceum* (P), *S. cerevisiae* (Y), bovine (B), human (H), and *A. nidulans* (A) cells are boxed. Shaded regions represent amino acids thought to play a role in cytochrome *b* functional activity. A gap (residues 202 to 250) was introduced in the *Plasmodium* sequence for optimal alignment.

smaller than other cytochrome *b* proteins at both the amino and carboxy ends (Fig. 3). In addition, there is a region within the yeast, mammalian, and fungal proteins that seemed to be absent from the *Plasmodium* sequence (residues 202 to 250). Two of the four invariant histidine residues proposed as the heme-binding sites in cytochrome *b* and the chloroplast b_6-f complex (35, 42) were present in the *Plasmodium* sequence (His-85 and His-99). The other two histidine molecules (His-186 and His-200) would be located near the break in the *Plasmodium* cytochrome *b* polypeptide and may reflect membrane folding patterns slightly different from those of yeast, mammalian, and fungal cytochrome *b* polypeptides. The *Plasmodium* sequence contained seven other histidine molecules that may participate in the binding of heme. In addition, the basic amino acids (Arg-82, Arg/Lys-102, and Arg-181) that are thought to play a role in the formation of salt bridges with the propionate side chains of the protohemes (35, 42) were also present in the *Plasmodium* sequence.

A comparison of the hydropathy plots for yeast, human, *A. nidulans*, and *Plasmodium* sequences demonstrated a high degree of similarity (Fig. 4). This similarity may reflect the fact that most of the substitutions in the *Plasmodium* protein sequence are conservative substitutions of the Ile-Leu-Val-Ala type. The protein-coding region of *Plasmodium* species is predicted to be an integral membrane protein with five membrane-spanning regions (Fig. 4). The amino acid proline, which produces bends in a polypeptide sequence, was found 14 times in the *Plasmodium* sequence. Of these 14 prolines, 10 were found to be conserved in other species, thus allowing some preservation of secondary structure.

As in the yeast gene for cytochrome *b*, the *Plasmodium* nucleic acid sequence was particularly A+T rich. The A+T content of yeast cytochrome *b* is 73% (32). The *Plasmodium* open reading frame was also 73% A+T.

A nucleic acid sequence search of the entire 2-2D sequence with the GenBank data base was completed by using the Bionet Fasta program. This search resulted in the identification of statistically significant homologies between several cytochrome *b* DNAs and the 2-2D open reading frame as well as a region on the opposite strand which indicated homology with various mitochondrial large-subunit rRNA genes. The yeast (*Saccharomyces pombe*) mitochondrial cytochrome *b* gene demonstrated 59% identity with 2-2D nucleotide residues 50 to 660, and the *Drosophila yakuba* cytochrome *b* gene showed 57% similarity with residues 50 to 1020. On the opposite strand of the cytochrome *b* open reading frame was identified a region (Fig. 2, residues 2035 to 2223) that demonstrated 73% identity over 188 nucleotides with the maize mitochondrial 26S rRNA gene (11). The *Tetrahymena* mitochondrial 21S rRNA gene (21) had 72% identity within this same region.

Transcriptional mapping of cytochrome *b* message. When 2-2D plasmid probes were used against Northern blots of *P. gallinaceum* RNA, at least two transcripts could be detected: a transcript of 1.2 kb as well as one or more small transcripts of less than 300 bp (Fig. 5, lane 1). The large message detected with the 2-2D insert is presumed to encode the *Plasmodium* cytochrome *b* polypeptide. As predicted from the open reading frame analysis of this polypeptide, the RNA transcript would have to be at least 1 kb long.

To identify transcripts that were generated from either strand of the 2-2D insert, the insert was cloned into the vector Bluescript, and RNA transcripts were synthesized off opposite strands by using the T3 and T7 promoters (see Fig. 1 for directions of the T3 and T7 transcripts). These transcripts were then used as probes against *P. gallinaceum* RNA blots (Fig. 5, lanes 2 and 3). With the T3 promoter used for RNA synthesis, at least three transcripts were detected (Fig. 5, lane 2): one of approximately 5 kb, one of 1.2 kb, and a third of less than 300 bp. When RNA probes were synthesized by using the T7 promoter, transcripts of approximately 5 kb, 1.8 kb, and less than 300 bp were detected.

DISCUSSION

Mitochondrial genomes have been extensively studied in recent years. The entire sequence, organization, and biogenesis of mitochondrial DNA from a number of higher eucaryotes have been completely determined (3, 10). In most systems, the mitochondrial genome operates autonomously from the nuclear system. Replication, transcription, and translation of a select set of mitochondrial polypeptides occur only within the mitochondrion.

One of the polypeptides encoded by the mitochondrial genome is the cytochrome *b* subunit of coenzyme QH_2-cytochrome *c* reductase. The amino acid sequence and structure of this polypeptide are very well conserved among a number of species. In this paper, we have presented evidence that the sequence for cytochrome *b* in malarial parasites is located within the 6.2-kb DNA element previously described (24). This polypeptide is translated by utilizing a codon usage similar to that in other mitochondrial genetic systems. Like cytochrome *b* from other organisms, the *Plasmodium* cytochrome *b* is predicted to be a trans-

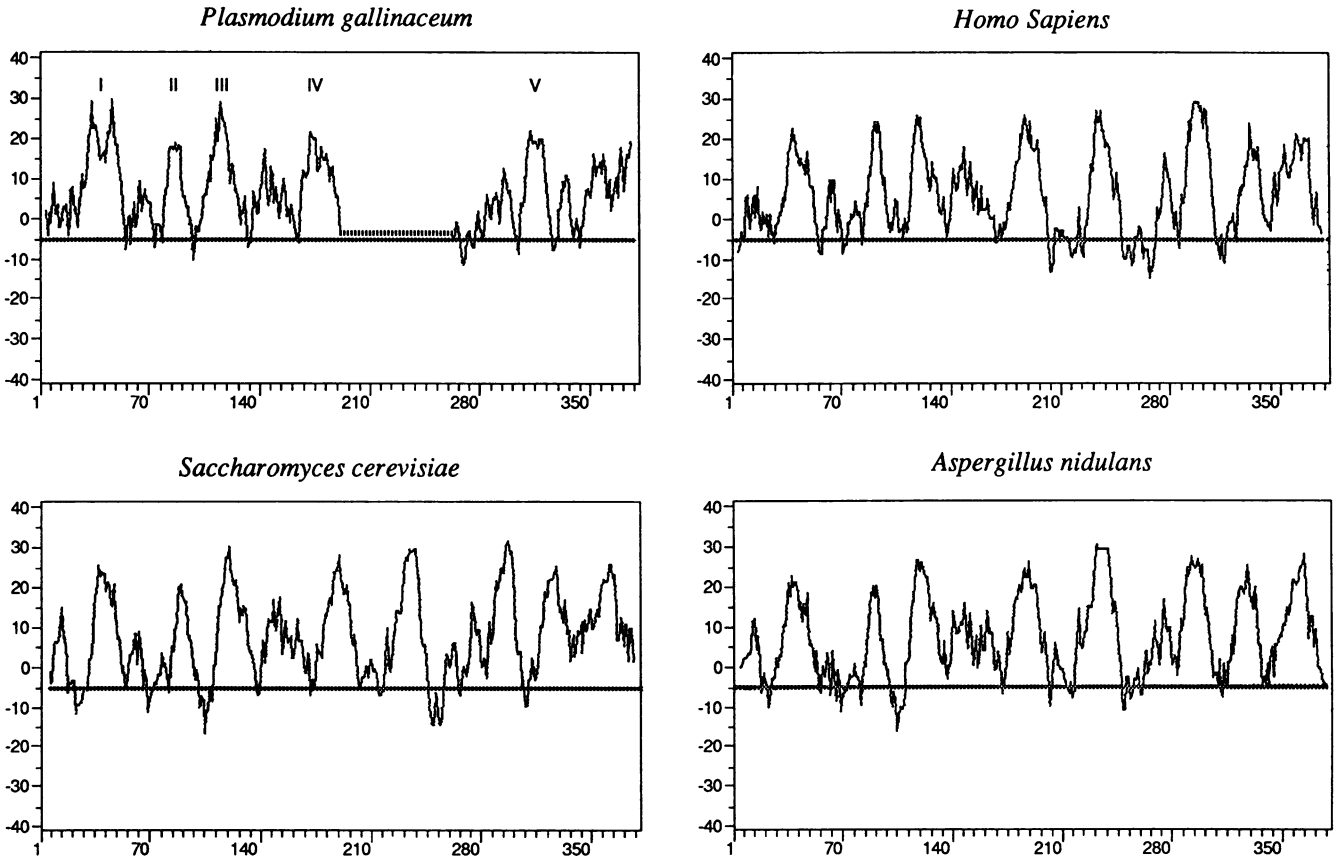

FIG. 4. Comparison of cytochrome *b* hydropathy plots. Hydropathy plots for cytochrome *b* sequences from *P. gallinaceum*, *S. cerevisiae*, human, and *A. nidulans* cells are illustrated for comparison. Plots were generated by using the Intelligenetics SOAP program, which employs the method of Kyte and Doolittle (27). A gap (|||) was introduced in the *Plasmodium* sequence to allow a more meaningful comparison. The predicted transmembrane regions (see Fig. 2) are indicated as I, II, III, IV, and V.

membrane molecule, with five membrane-spanning segments. *Plasmodium* cytochrome *b* is found to be somewhat smaller than other cytochrome *b* polypeptides (36,000 daltons versus approximately 42,000 daltons) and lacks one transmembrane region in comparison with yeast, mammalian, and fungal sequences. It is interesting that in spinach chloroplast the cytochrome *b* protein is split into two subunits (42). The spinach chloroplast b_6-f complex contains two polypeptides, one of 23,000 daltons which contains two heme-binding sites and another of 17,000 daltons that is of unknown function. This split of the chloroplast b_6-f complex occurs in approximately the same region as the deletion in the *Plasmodium* sequence. This particular region of the *Plasmodium* protein may not be essential for functional activity in the malarial system. In the yeast, mammalian, and fungal proteins, this region is predicted to be a membrane-spanning region with no functional binding sites (35, 42). Several other amino acid residues thought to be involved in cytochrome *b* heme-binding activity are conserved in the *Plasmodium* sequence.

The *Plasmodium* cytochrome *b* polypeptide maps onto a 1.2-kb RNA transcript (Fig. 5, lanes 1 and 2). In addition, it appears that *P. gallinaceum* also synthesizes transcripts off the opposite strand of the DNA element (Fig. 5, lane 3). These transcripts may correspond to large-subunit rRNA molecules, as indicated from the nucleic acid homology search. In the blots probed with the T7 and T3 RNA transcripts (Fig. 5, lanes 2 and 3), several bands are present

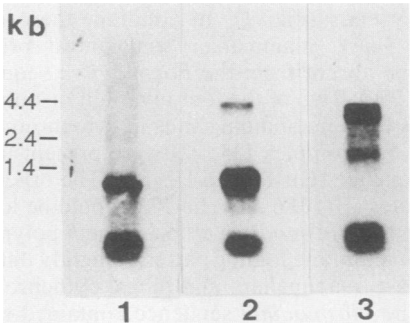

FIG. 5. Northern blots of *P. gallinaceum* DNA element transcripts. In lane 1, 5 μg of total *P. gallinaceum* RNA was probed with nick-translated plasmid 2-2D. In lanes 2 and 3, 5 μg of total *P. gallinaceum* RNA was probed with transcripts synthesized by using the T3 or T7 RNA polymerase promoter of the Bluescript vector (Stratagene). Lane 2 show the result of probing *P. gallinaceum* Northern blots with RNA transcripts synthesized by using the T3 promoter and the noncoding strand of the 2-2D insert as template. This transcript hybridizes to a 1.2-kb message that encodes cytochrome *b*. Lane 3 shows the result of Northern blots probed with transcripts generated by using the T7 promoter and the coding strand of the 2-2D insert. This transcript recognizes a message of 1.8 kb and could encode a large-subunit rRNA gene. The larger (approximately 5 kb) and smaller (less than 300 bp) bands in lanes 2 and 3 are believed to be primary transcripts and processing products from the element. Molecular weight markers were from Bethesda Research Laboratories, Inc.

which are not evident in the blot probed by using plasmid probes (Fig. 5, lane 1; bands at 1.8 and 5 kb). We believe that this is due to the higher specific activity of the RNA probes compared with the nick-translated 2-2D plasmid probe. The band present at about 1.8 kb, which we feel represents a large-subunit ribosomal gene sequence, may not be evident in the plasmid-probed blots because of the relative abundance of cytochrome *b* sequence (approximately 1 kb) in relation to putative ribosomal sequence (200 bp). The 5-kb band that is present in both the T3 and T7 lanes is not always detected in Northern probing and may represent the primary transcript from the DNA element. The extent of processing within any batch of RNA probably determines how much of this transcript is present. The small (less than 300 bp) sequences could correspond to processed products or tRNA structures.

Several investigators have reported the presence in various malarial species of a closed circular DNA molecule 20 to 30 kb in size (14, 25, 43). This closed circular DNA was identified as mitochondrial on the basis of its physical structure and subcellular fractionation. In addition, a partial sequence of a *P. falciparum* small-subunit mitochondrial rRNA has been mapped to this molecule (15). It has been well documented that certain mitochondrial systems consist of large closed circular DNA molecules as well as a number of small polydisperse circular and linear DNAs (33). These mitochondrial episomes have a variety of functions and appear to be extremely species specific. The *Plasmodium* DNA element is thought to be a similar type of molecule (24). We have proposed a model which suggests that the mitochondrial genome in malarial parasites is composed both of large closed circular DNAs and smaller episomal molecules. These different mitochondrial DNA species may act independently or as an organized unit. The genes encoding other mitochondrion-associated polypeptides may be located on the smaller 6.2-kb DNA element and the large closed circular 20- to 30-kb DNA molecule. Expression of these genes from either DNA molecule may be dependent on stage-specific requirements.

The identification of cytochrome *b* from a malarial parasite confirms previous speculation regarding the presence of a functional cytochrome system (22, 40). Malarial parasites were originally thought to derive essentially all energy requirements through the Embden-Meyerhof pathway (38). *Plasmodium* species apparently lack most of the enzymes required for the citric acid cycle (6, 38), and the mitochondria of some intraerythrocytic parasites appear to have an acristate morphology (1, 28, 30). The malarial mitochondrion was originally thought to be involved only in pyrimidine de novo biosynthesis (17, 20). The presence of cytochrome *b* in *P. gallinaceum* would serve to explain the sensitivity of the malarial parasite to mitochondrial inhibitors and antibiotics that are thought to have their sites of action in the mitochondria (7, 9, 16, 18). The identities of the other components of the coenzyme QH_2-cytochrome *c* reductase complex have not yet been determined. A much more detailed biochemical and physiological investigation will be required to elucidate the mechanism involved in energy transport in malarial parasites.

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