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Molecular Cloning and Characterization of a cDNA for the Highly Conserved HMG-like Protein (Pf16) Gene of *Plasmodium falciparum*

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A cDNA clone (PfHB3-2-4) of 1538 bp corresponding to the highly conserved HMG-like protein (Pf16) was isolated. However, northern analysis suggests that the mRNA is about 2.2 to 2.3 kb. Analysis by RT-PCR indicated that the 0.6 to 0.7 kb sequence missing in the cDNA maps to the 3′ end, suggesting that the cDNA is terminated within the 26 adenosine residues that are in the middle of the Pf16 sequence. The most unique feature about this cDNA is the presence of two open reading frames (ORF), one from nucleotides 91 to 927 and the other starting from 1421. The second ORF corresponds to Pf16. Expression of the cDNA clones in *Escherichia coli* and translation in rabbit reticulocytes of RNA transcribed from the T7 promoter of the cDNA clones revealed that only the 3′ end Pf16 is translated from this mRNA. Further experiments with antisense oligonucleotides specific for Pf16 indicated that the Pf16 protein serves an important function in the life cycle of the parasite.

Plasmodium falciparum is the causative organism of cerebral malaria, the most lethal form of human malaria. Although a number of *Plasmodium falciparum* genes have been isolated and sequenced, there is little information on the organization, regulation of transcription, and RNA processing of these genes. Given the obstacles in chemotherapy and vaccine development posed by the complex life cycle of the parasite, and increasing evidence for the presence of stage-specific transcripts [1,2], defining the mechanisms underlying the regulation of gene expression and post-transcriptional processing during host-switching by the parasite could have important implications in the design of novel approaches for the treatment and prevention of malaria.

We have earlier cloned and characterized from *P. falciparum*, a novel gene (Pf16) coding for a protein of 147 amino acids [3]. This protein resembles the high mobility group (HMG) of proteins that are an important class of ubiquitous non-histone chromosomal proteins [4] thought to be involved in DNA replication, chromatin assembly and transcription [5]. Recent data show that several eukaryotic transcription factors, including RNA polymerase I transcription factor, hUBf [6], human T cell-specific transcription factor TCF-1α [7], and the testis determining gene SRY [8], have conserved DNA-binding motifs (called HMG-boxes) which correspond to a DNA-binding region of HMG1 protein.

In this paper, we describe the cloning and characterization of the cDNA encoding the Pf16 protein. Sequence analysis reveals the transcript to be continuous with its DNA and to possess large 5′ and 3′ untranslated regions. All plasmodial transcripts described earlier have long 5′ UTRs. Transcripts of the knob-associated histidine-rich protein and GP 130 have 5′ UTRs of 849 and 932 nucleotides, respectively [9,10] while the 5′ UTRs of actin I and II genes are 305 and 391 nucleotides long respectively [1]. To our knowledge, the 1420 nucleotide 5′ untranslated region of Pf16mRNA is the longest described to date.

MATERIALS AND METHODS

Cultivation of parasites. The origin of *Plasmodium falciparum* strains FCR3, HB3 and DD2, was described earlier [11]. These were grown and maintained as described [12].

Screening of P. falciparum cDNA library. A *P. falciparum* (HB3 strain) cDNA library was established in the phagemid vector, pcDNAII (Invitrogen). The 1.1 kb *Xba*I genomic fragment (Fig. 1A) containing the entire coding sequence of the Pf16 gene, was labeled by the random primer method [13] to high specific activity using

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[α-^{32}P]dATP ($\sim$ 3000 Ci/mmol, ICN, Irvine, CA). Hybridizations were carried out at 68°C in the hybridization buffer containing 0.9 M sodium chloride, 0.09 M sodium citrate, 0.1% SDS, 2× Denhardt's solution, and 100 μg/ml salmon sperm DNA. The filters were washed at 68°C three times, each for 15 minutes, with 2× SSPE (SSPE is 0.18 M NaC1, 10 mM NaH_2PO_4 pH 7.4 and 1 mM EDTA, pH 7.4) containing 0.1% SDS. Positive clones were characterized by restriction analysis. One of the clones (designated PfHB3-E) which had the largest insert ($\sim$ 0.8 kb) was gel-purified, labeled as described above and used to probe the cDNA library. The clone (designated PfHB3-2-4) with the largest insert at this round of screening was further characterized.

DNA sequencing. All nucleotide sequencing was performed by the dideoxy chain termination method [14] using Sequenase, version 2.0 (USB, Cleveland, Ohio) and denatured double-stranded DNA templates or single-stranded M13mp18 and M13mp19 recombinant phage templates. Oligonucleotide primers were derived from the known sequence of the cDNA inserts or were vector based and flanked the cloning sites used. The DNA sequence of both strands was determined.

RT-PCR. Reverse-transcriptase polymerase chain reaction was carried out as follows. Approximately 2 μg of total RNA isolated from *P. falciparum* (DD2 strain) was used as a template with 400 units of M-MLV reverse transcriptase (BRL) for 1 hour at 37°C in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3mM $MgCl_2$, 10 mM dithiothreitol, and dNTPs at 200 μM each in a total volume of 50 μl and 100 ng of primer b (Fig. 1A). The reaction products were phenol-chloroform extracted, ethanol precipitated and resuspended in 50 μl TE. 5 μl of this served as a template for the PCR reaction for which **a** and **b** were the left and right primers, respectively (Fig. 1). The conditions for amplification were 95°C, for 2 minutes, 50°C for 3 minutes, 68°C for 3 minutes for 35 cycles. PCR products were visualized directly in ethidium-bromide stained agarose gels.

In vitro transcription. RNA was synthesized from the cDNA clone PfHB3-2-4 and the complete genomic clone Pf16-E [3] both of which have their inserts in the correct orientation. For synthesis of run-off transcripts of defined size, the double-stranded DNA templates were digested to completion with *Bam*H1, which cleaves on the 3′ side of the insert, phenol-chloroform extracted and precipitated by ethanol. *In vitro* transcription reactions using T7 RNA polymerase were carried out using the Ambion Megascript T7 kit (Ambion, Inc., Austin, TX) following the manufacturer's recommendations. The transcripts were analyzed by fractionating aliquots on 1.2% agarose-formaldehyde gels.

In vitro translation. mRNAs obtained in the *in vitro* transcription experiments were translated using the Ambion Retic Lysate In Vitro Translation (IVT) kit essentially as recommended by the manufacturer. The [^{35}S]-methionine labeled translation products were analyzed on 10-18% gradient SDS-polyacrylamide gels. The gels were fixed, dried and exposed to Fuji X-ray film at room temperature.

Northern blot analysis. Total RNA was isolated from *P. falciparum* blood-stage parasites ($\sim$ 50% trophozoites) by the guanidinium thiocyanate/cesiumchloride method [15]. Two μg each total RNA were fractionated on 1.2% agarose-formaldehyde gel, electroblotted onto nylon membrane, and probed with the 1.1 kb *Xba*I fragment (Fig. 1A) at 55°C in hybridization buffer containing 50 mM PIPES, pH 6.5, 100 mM NaCl, 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, pH 7.0 and 0.5% SDS. The blots were washed at 55°C with buffer containing 150mM sodium chloride, 15mM sodium citrate and 5% SDS, and exposed to X-ray sensitive film (Fuji) in the presence of intensifying screens at −70°C.

Expression of the Pf16 cDNA clones from the T7 promoter. As described earlier, the cDNA clones are in an expression vector downstream of the T7 promoter. The DNA from the PfHB3-E and PfHB3-2-4 clones were transferred into *E. coli* HB101 harboring the T7RNA polymerase containing plasmid pGP-1-2 [16]. Cloning of the 1.1 kb *Xba*I fragment (containing the Pf16 coding sequences) in the pGEM-3Z vector, and the introduction of this DNA into *E. coli* HB101 cells was described earlier [3]. ^{35}S-labeling was done essentially as described [16]. Briefly, the cells, grown at 30°C in minimal M9 medium with all the amino acids except methionine and cysteine, were induced to synthesize T7 RNA polymerase by shifting the temperature to 42°C followed by the addition of 200 μg/ml rifampicin. The cultures were shifted back to 30°C and pulsed with 60-80 μCi ^{35}S-Translabel ($\sim$ 1100 Ci/mMole, ICN) for 5 minutes. The cells were fractionated into membranes and inclusion bodies as described [17] and aliquots were analyzed on 10-18% gradient polyacrylamide gels [3]. Gels were fixed, dried and exposed to X-ray film (Fuji).

Effect of anti-sense oligonucleotides on parasite infection. The sense oligonucleotide corresponding to nucleotide sequence from 1418 to 1438 in Fig. 2 (5′-GTG ATG ATG AAG AGG GGG ACT-3′) and the antisense oligonucleotide (5′-CGT CCC CCT CTT CAT CAT CAC-3′) for the same region were synthesized as phosphorothioate nucleotides (Oligos Etc., Inc., Wilsonville, OR). The nonspecific oligonucleotide used was totally unrelated to the Pf16 gene and was kindly given to us by the same company. *P. falciparum* FCR3 or HB-3 strains were grown in human red blood cells as described [12] and synchronized by sorbitol and treated with the oligonucleotides at the indicated concentrations. On day 3, smears were prepared and counted for schizonts and rings. Percent inhibition of schizonts to rings for each oligonucleotide was calculated by taking untreated control as 100%.

RESULTS

Molecular characterization of the Pf16 cDNA clones. A schematic representation of the structures of the Pf16 partial cDNA clones (PfHB3-E and PfHB3-2-4) and their topographical relationship to the corresponding genomic DNA is shown in Fig. 1 (A and B). The complete nucleotide sequence of PfHB3-2-4 and the predicted amino acid sequences of its ORFs are shown in Fig. 2. A comparison of the amino acid sequences of the Pf16 protein deduced from the cDNA and genomic clones is depicted in Fig. 1 (D). The 781 bp insert of PfHB3-E was sequenced and compared with the sequence of the genomic *Xba*I fragment, described by us earlier [3]. The cDNA shows an ORF beginning at the ATG initiation site corresponding to the ATG of genomic Pf16 and terminating abruptly at the 24th dA residue in the 26 nucleotide stretch of dA within the Pf16 coding region. Between these two points, the two sequences show near-perfect homology, except for the differences shown in Fig. 1 (D). The cDNA sequence upstream of the coding region up to the *Xba*I site shows 98% homology to the genomic DNA sequence, indicating that the transcript is co-linear with this DNA. Although the Pf16 gene itself is very highly conserved amongst different *Plasmodium* species [3], the small differences between the genomic DNA and cDNA sequences could reflect differences in strains, as the genomic DNA corresponds to *P falciparum* FCR 3, whereas the cDNA was derived from *P. falciparum* HB3.

Using the labeled $\sim$ 0.8 kb insert of PfHB3-E as a probe, the clone (designated PfHB3-2-4) containing 1.5 kb insert was obtained. Sequence analysis revealed it to be longer towards the 5′ end, but very similar at its

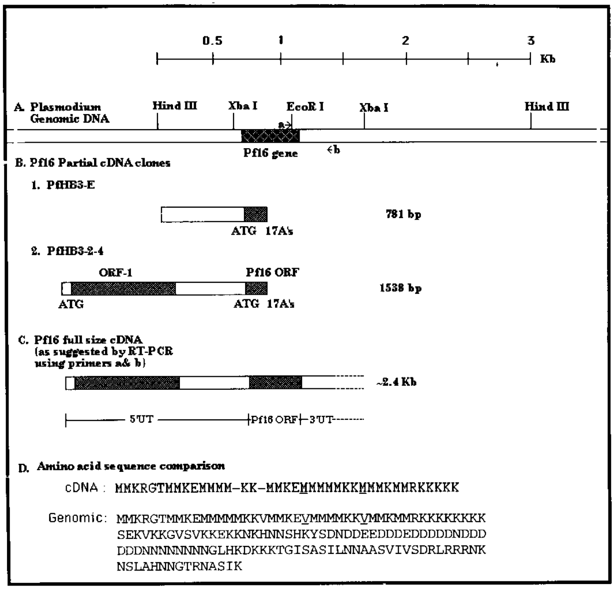

FIG. 1. Scheme depicting the molecular clones used in this work. (A) Restriction map of the 3 kb genomic DNA clone encoding Pf16 which was originally isolated by immunoscreening of *Plasmodium falciparum* FCR/3 genomic library as described earlier [3]. Solid box represents the coding region for Pf16. **a** and **b** represent the forward and reverse primers used in the RT-PCR. 5′-CATGGAATGGATCTG-TAATCCATT-3′ and 5′-CAATGCTGCTAGTGTTATTGTTTC-3′, respectively. (B) Schematic representation of the structures of the Pf16 partial cDNA clones, showing their topographical relationship to the genomic DNA. (C) Pf16 full-length cDNA as suggested by RT-PCR using primers **a** and **b.** Details are described in the Materials and Methods. (D) Comparison of the predicted amino acid sequences of the Pf16 protein from the cDNA clone and the genomic clone. Gaps have been inserted to maximize identity between the sequences and the variant amino acids have been underlined.

3′ end to the PfHB3-E cDNA. At its 3′ end, the cDNA terminated abruptly at the 17th nucleotide residue of the 26 dAs, which is a part of the coding sequence (PfHB3-E stopped at the 24th dA). It is possible that the premature termination of the 3′ end of the cDNA clones at different points in the 26 dA stretch, results from a priming artifact of oligo dT during cDNA synthesis. On its 5′ side, it is longer by 0.7 kb and surprisingly codes for another large potential ORF. This ORF is 837 bases long, coding for a putative protein of 279 amino acid residues with a predicted molecular size of ~ 31 kDa. (Fig 2). Relative to the pf16 ORF, this ORF is in the +1 frame. The two ORFs are separated by 475 bases of noncoding sequence. It is interesting to note that the cDNA sequence begins with a string of 59 Ts. However, we do not know whether this polydT stretch of sequence is the result of a cloning artifact.

Northern blot analysis and RT-PCR of RNA. Hybridization of RNA from three different strains of *P falciparum*, using the very well conserved genomic Pf16 DNA as probe, showed a ~ 2.2 kb mRNA to be the likely Pf16 mRNA species, as this signal was common to both strains examined (Fig 3A). Sequence comparisons strongly suggest that the Pf16 mRNA is continuously transcribed from its genomic DNA. Although, results of Northern blot analysis using the *Eco*R1-*Xba*1 probe indicate that the transcript extends beyond the poly dA stretch in the coding region, we decided to use the RT-PCR method to provide additional evidence. Prim-

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   1 tttttttttttttttttttttttttttttttttttttttttttttatttttttttttttta   60

  61 tcttcttatccttttatccttttaagaaagatgagtgaagcacttgatattgataatgca  120
                                     MetSerGluAlaLeuAspIleAspAsnAla

 121 aaggcaaaaataggtttagtattttcttactggaagaaggttgctaataatgatttttct  180
     LysAlaLysIleGlyLeuValPheSerTyrTrpLysLysValAlaAsnAsnAspPheSer

 181 aagtgtagcatatttgtgtgttatcaggaaaatctagtaaagatgagaatgccactata  240
     LysCysSerIlePheCysValLeuSerGlyLysSerSerLysAspGluAsnAlaThrIle

 241 caagaacaatttcagatgtggttaacaggttatcaattgactgaaacatttttgggttt  300
     GlnGluGlnPheGlnMetTrpLeuThrGlyTyrGlnLeuThrGluThrPhePheGlyPhe

 301 ttaaaaaatagtgaacgcatattaattttaacaagtgataagaagaagagattttacaa  360
     LeuLysAsnSerGluArgIleLeuIleLeuThrSerAspLysLysLysArgPheLeuGln

 361 ccattgttagataatattaagaatgtagatgtattagaaagaagtaatgataatacatca  420
     ProLeuLeuAspAsnIleLysAsnValAspValLeuGluArgSerAsnAspAsnThrSer

 421 aattttgagaatataaaatctacaattgaatcgacaaattgtgatgaaattgctttatta  480
     AsnPheGluAsnIleLysSerThrIleGluSerThrAsnCysAspGluIleAlaLeuLeu

 481 aaagataaggatgcaacaggttcgtttttgaaaactgttatgattttattaagacgtta  540
     LysAspLysAspAlaThrGlySerPhePheGluAsnCysTyrAspPheIleLysThrLeu

 541 aataaaaaagaaatggatgtaaataataatataaaggaattattaaattttcgttcagat  600
     AsnLysLysGluMetAspValAsnAsnAsnIleLysGluLeuLeuAsnPheArgSerAsp

 601 acagatatgaaaattcaaaaaagtggtagtgatatagcttgtattatccttaaaagtatt  660
     ThrAspMetLysIleGlnLysSerGlySerAspIleAlaCysIleIleLeuLysSerIle

 661 ttgattactactattgaaaatgctttagataatgaagaatttgaaagtcatgataaaata  720
     LeuIleThrThrIleGluAsnAlaLeuAspAsnGluGluPheGluSerHisAspLysIle

 721 aaagaaaaagctttaaaatttatggataataaaaagtgtgtaatgaaattaaaagataaa  780
     LysGluLysAlaLeuLysPheMetAspAsnLysLysCysValMetLysLeuLysAspLys

 781 ttaaaagtagatattgttgaaatagatgttatatatagtaatgtacagagtggtaataat  840
     LeuLysValAspIleValGluIleAspValIleTyrSerAsnValGlnSerGlyAsnAsn

 841 tttacattaacttataaaaatagtaatgataaaaattatttatcgcaaaatgaaggaact  900
     PheThrLeuThrTyrLysAsnSerAsnAspLysAsnTyrLeuSerGlnAsnGluGlyThr

 901 atacttgttcggtgtcggtttaaaatataaagaattatgttgtaatataacaaggactct  960
     IleLeuValArgCysArgPheLysIleEnd

 961 cttattaaacgcaaggacacaacataaagaattatataactttactatatctatagaaaa 1020

1021 atacattataaaagaatgcctaaaagttggtaccaacttttctagtgtttataaaaaaca 1080

1081 ttagaatatgtaaaagaacataaaaaggaatataaaaccttaagtaatattcaaatagaa 1140

1141 aattattttgttaaatgtattggtcatataataggtattgaatttattgataaagaatac 1200

1201 ttaataatagaaagtaaccatcaaggaaaaatacaaaaaaacacctcatacaatttatct 1260

1261 gtgggttttgaaaatcttcaaggtctagagaaaaataaatttgctatttggattagtgat 1320

1321 accatatgtattgatgataatgaagatgtaattgtattaacagatgcaattagtaaggaa 1380

1381 attaataccatatcatgcgaattagaagataccaaaagtgatgatgaagagggggacgat 1440
                                          MetMetLysArgGlyThrMe

1441 gatgaaagagatgatgatgatgaaaaagatgatgaaagagatgatgatgatgatgaaaaa 1500
     tMetLysGluMetMetMetMetLysLysMetMetLysGluMetMetMetMetMetLysLy

1501 gatgatgatgaagatgatgagaaaaaaaaaaaaaaaaa   1538
     sMetMetMetLysMetMetArgLysLysLysLysLys
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FIG. 2. Nucleotide and deduced amino acid sequence of the Pf16 cDNA (PfHB3-2-4). The Pf16 ORF begins at position 1421 and ends abruptly at the 17th A in the 26 A stretch encoding the polylysine region in the gene. Located upstream is a putative ORF of 279 amino acids extending from nucleotide position 91 through 927.

ers for the RT-PCR were designed to amplify a 326 bp region downstream of the poly d(A) region. Primers **a** and **b** (see Fig. 1A) are located at nucleotide positions 485-508 and 788-811, respectively, downstream from the 5′ *Xbal* site. While the left primer corresponds to the coding region, the right primer corresponds to sequences in the potential 3′ UT region (230 bases past the TAA stop codon). As shown in Fig. 3B, the 326 bp product of RT-PCR (lanes 2 and 3) is the exact size obtained when a DNA-PCR is performed on the genomic fragment (lane 4). Significantly, this product was not obtained (Lane 1) when reverse transcriptase was omitted from the reaction, thus ruling out the fortuitous amplification of DNA contaminating the RNA sample. The specificity of the amplified products was further confirmed by Southern hybridization using the *Eco*RI-*Xba*I fragment as probe (data not shown). The results of RT-PCR show convincingly that the naturally-obtained Pf16 transcript, unlike the cDNA clones, actually extends for at least 230 bases beyond the stop codon, as the 3′ untranslated region of the Pf16 gene.

Protein expression studies. In order to demonstrate the authenticity of the Pf16 cDNA clone and the translatability of the Pf16 transcript, we have expressed the Pf16 protein in both prokaryotic and eukaryotic systems. The cDNA clones PfHB3-E and PfHB3-2-4 encode for truncated Pf16 proteins of 41 and 39 amino acid residues, respectively. The predicted molecular size of these polypeptides is ~ 4.5 kDa. The full-length Pf16 protein expressed from the genomic clone migrated as a 14 kDa protein [3].

As shown in Fig. 3C, the Pf16-E clone makes a protein of ~ 14 kDa which, as described by us earlier [3], is mostly localized in the inclusion bodies (Lane 3). Clone Pf16-C, having its insert in the opposite orientation, does not make this protein (data not shown). PfHB3-E (Fig. 1B) makes a truncated protein of ~ 5 kDa (Lane 4). Interestingly, unlike the full size protein, this protein is not in the inclusion bodies, suggesting that COOH-end is involved in aggregation. *E. coli* expression studies were also carried out with the PfHB3-2-4 clone (Fig. 1, B2) to examine if the large putative ORF, upstream of the Pf16 ORF, translates into protein. However, no such protein (predicted molecular size of ~ 31 kDa) was made (data not shown).

Given the fact that deletion and rearrangement of *P. falciparum* DNA, possibly resulting from the extreme A + T content of its genome occurs in some *E. coli* hosts [18], we used another host, *E. coli* BL21 to try and express the upstream ORF. No protein corresponding to this ORF was made (data not shown). In order to examine the possibility of this ORF being translated in an eukaryotic translation system, we first synthesized the sense strand RNA from the PfHB3-2-4 clone using T7 RNA polymerase. As a control, we also obtained the *in vitro* transcript from the genomic clone

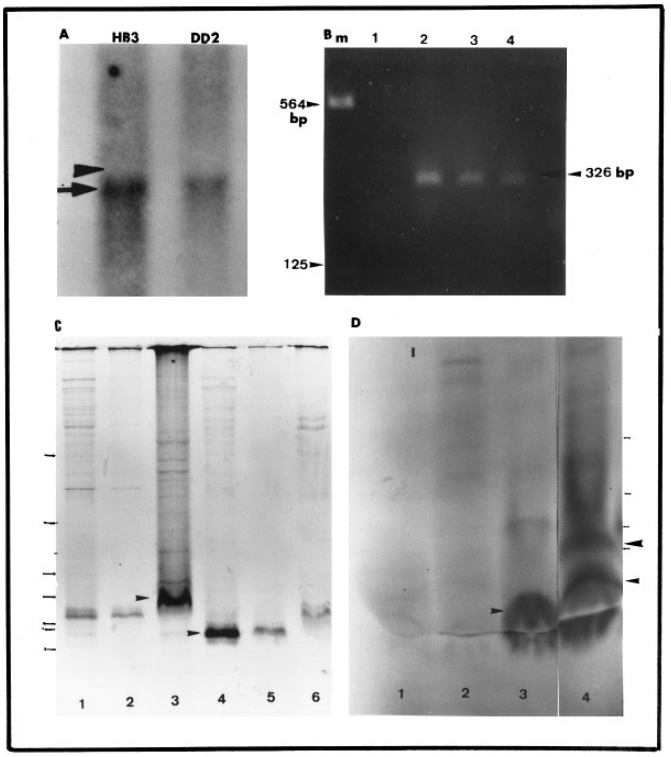

FIG. 3. (A) Northern blot analysis of *Plasmodium falciparum* (blood-stage) total RNA. RNA was isloated from blood-stage parasites (~ 50% trophozoites) of strains HB3 and DD2, size-fractionated on a 1.2% agarose-formaldehyde gel, transferred to nylon membrane and hybridized with the 1.1 kb *Xba*I fragment. The position of the 20S *P. falciparum* ribosomal RNA is indicated by an arrowhead and the transcript is indicated by an arrow. (B) Evidence that the 3′ end of Pf16 mRNA extends into the *Eco*RI-*Xba*I region. RT-PCR was done in two stages. First the RNA was reverse transcribed from primer **b** (see Fig. 1A). The cDNA products were used as templates in a 35 cycle PCR reaction using **a** and **b,** as the forward and reverse primers, respectively. RT-PCR using blood stage *P. falciparum* (DD2 strain) total RNA as template without reverse transcriptase (Lane 1) or with reverse transcriptase (Lanes 2 and 3). Lane 4 shows PCR using the same primers, but Pf16 genomic DNA (3 kb *Hin*dIII fragment, Fig. 1A) as template. Arrows on the left indicate size-marker positions and the arrow on the right indicates the expected 326 bp product. (C) Expression of Pf16 genomic and cDNA clones in *E. coli.* Cells were labeled and fractionated as described in Materials and Methods. Pf16-E (correct orientation) is Pf16 genomic clone [3] and PfHB3-E is the incomplete cDNA clone described here. Lanes 1, 2, and 3 correspond to Pf16-E; and, Lanes 4, 5, and 6 indicate PfHB3-E. Samples loaded are from crude cell lysate (Lanes 1 and 4), Triton X-100 supernatant (Lanes 2 and 5) or inclusion bodies fraction (Lanes 3 and 6). Dashes at the left indicate size-marker positions from top to bottom: 31, 20.4, 16.9, 14.4, 8.1, 6.2, and 2.5 kDa, respectively. The arrows indicate the full-length (~ 14 kDa) protein and the truncated (~ 5 kDa) protein. (D) *In vitro* translation of RNAs in reticulocyte lysate system. The ^{35}S-methionine-cysteine labeled translation products were analysed on 10-18% gradient SDS-polyacrylamide gel, followed by autoradiography. Lane 1: Negative control (without exogenous mRNA). Lane 2: Positive control (*xef-1* mRNA). Lane 3: *In vitro* transcribed mRNA from the partial cDNA clone, Pf HB3-2-4. Lane 4: *In vitro* transcribed mRNA from the full-length genomic clone, Pf16-E. The thin arrowhead on the right indicate the ~ 5 kDa (Lane 3) and the thick arrowhead indicates the ~ 14 kDa product (Lane 4).

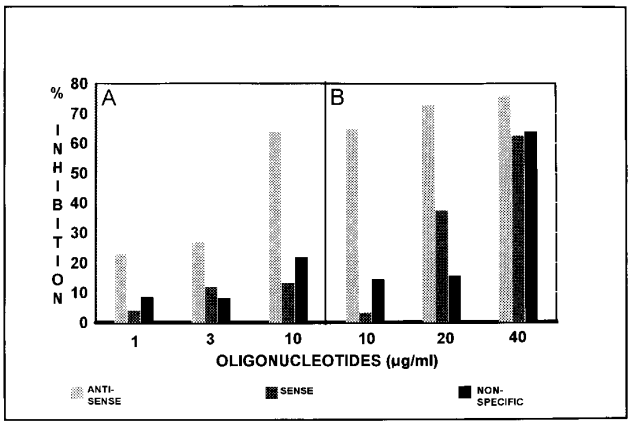

FIG. 4. Effect of antisense oligonucleotide on parasite. Sense and antisense oligonucleotides at the indicated concentrations were incubated with the parasite cultures. Smears were prepared and the number of rings were counted as described in Methods.

Pf16-E. The RNAs were translated in rabbit reticulocyte lysate system, optimized for translating uncapped transcripts. As shown in Fig. 3D, while the genomic clone transcript made the full size 14 kDa protein (Lane 4), the cDNA clone transcript made only the truncated protein of 5 kDa (Fig. 3D, Lane 3). Once again the upstream ORF does not appear to be translated.

Pf16 is essential for parasite growth. We have presented evidence for two ORFs in the cDNA (Fig. 2) and also showed that only the downstream ORF, which is the same as Pf16, could be translated into protein in both *E. coli* and in reticulocytic lysates. If Pf16 function is vital for the parasite, blocking the expression of Pf16 should inhibit parasite growth. In order to examine the biological role of Pf16, antisense phosphorothioate oligonucleotides spanning the AUG codon of Pf16 nucleotides 1418 to 1438 (Fig. 2) was incubated with red blood cells infected by the parasite. As a control, the sense and non-specific oligonucleotides were also used. The results (Fig. 4) clearly indicate that the antisense oligonucleotide inhibited parasitemia in a concentration-dependent manner. For example, at 10 μg/ml, antisense oligonucleotide reduced parasitemia by more than 65% (two different experiments) whereas the sense oligonucleotide had no effect. Indeed the nonspecific oligonucleotide is slightly more inhibitory than the sense oligonucleotide. However, both the sense and nonsense oligonucleotides exerted non-specific inhibitory effect at higher concentrations. These results strongly suggest that the Pf16 protein is essential for parasite development and that the 2.2 kb mRNA encodes Pf16 but not the upstream ORF. Even if the upstream ORF is translated, the results presented here suggest that Pf16, which is the same as downstream ORF, is important for parasite's growth.

DISCUSSION

The study of mRNA processing in malarial parasites is an important new area of research, with potential implications for the design of new chemotherapeutic strategies, especially anti-sense blockade. We have reported earlier [3] the cloning and characterization of an HMG-like protein (Pf16) gene from *P. falciparum*. High mobility group (HMG) proteins have recently gained prominence with the demonstration by several investigators of the presence of highly conserved sequences (HMG boxes) in several eukaryotic transcription factors [6-8]. These domains appear to bind and bend DNA and activate transcription. Pf16 is a highly basic protein with clusters of positively and negatively charged domains similar to those present in other DNA-binding proteins [3]. This feature considered together with the fact that this gene is highly conserved in different species of plasmodium [3], suggest an important role for this protein in plasmodial gene regulation.

Pf16 is an intronless gene. In the present study, we have isolated a partial complementary DNA clone of $\sim$ 1.5 kb that encodes the Pf16 protein. As assessed by the nucleotide sequence this clone is incomplete at its 3′ end, representing only up to the N-terminal 41st amino acid residue of this 147 a.a. protein. However results of Northern blot analysis and RT-PCR indicate that the Pf16 mRNA is $\sim$ 2.2 kb long, co-linear with its genomic DNA and that transcription possibly extends 0.6 kb downstream of the coding sequences.

The 5′ untranslated region contains a putative ORF. The sequence of this putative, 279 amino acid protein was compared with the protein sequences in the data bank. Except for a 34% identity over a 38 amino acid region, with the serine-repeat antigen precursor protein of *P. falciparum*, no significant homology with any published protein sequences was found. Also, results of our bacterial expression studies and *in vitro* translation experiments suggest that this ORF is not translated. Hence, it is reasonable to conclude that the entire 1420 nucleotide region upstream of the Pf16 start codon may constitute the 5′ UTR or that this mRNA may encode two proteins. At this stage, it is premature to dismiss the existence of a novel protein corresponding to this ORF. Further experiments with antibodies raised against a polypeptide synthesized from the upstream ORF followed by immunoprecipitation with *Plasmodium* extracts would definitively demonstrate whether this ORF codes for a protein.

RT-PCR reveals that the 3′ UTR is at least 230 bases long. These results are consistent with data presently available on plasmodial mRNA, which show all of them to possess unusually long 5′ and 3′ UTRs [1,9,10]. Malarial parasite mRNAs are capped and lack trans-spliced leader sequences [19]. The sequence 5′AAAA3′ is a signature sequence preceding the initiation codon in over twenty *P. falciparum* genes [20]. This motif is also present in the Pf16 mRNA, although separated by three bases from the start codon. A unique feature of the 5′ end of Pf16 mRNA is the presence of an uninter-

rupted stretch of 59 U residues. The functional significance, if any of this poly(U) region is not known.

Rapaport *et al.* [21] showed that antisense oligonucleotide phosphorothioates are readily taken up by the parasitized human erythrocytes. Using oligonucleotides specific for dihydrofolate reductase-thymidylate synthase gene as a target, they have demonstrated inhibitory effect by anti-sense oligonucleotides. If Pf16 is essential for parasite growth and survival, inhibition of its expression should inhibit parasitemia. The results from three independent experiments strongly suggest that Pf16 is essential as the anti-sense oligonucleotide inhibited the development of the parasites, especially the step from schizonts to rings (Fig. 4). If the cDNA clone codes for the protein corresponding to the upstream ORF, the antisense oligonucleotide used here would not have any effect because it is specific only for the downstream ORF. These results suggest that Pf16 serves a vital function for the parasite and that the antisense oligonucleotide strategy can be potentially used to control malaria. Further experiments to understand the function of this HMG-like Pf16 and the mechanism of inhibition by antisense oligonucleotide are in progress.

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