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CLONING AND CHARACTERIZATION OF A HIGHLY CONSERVED HMG-LIKE PROTEIN (PF16) GENE FROM *PLASMODIUM FALCIPARUM*

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A novel gene encoding a protein of 147 amino acids (Pf16) has been cloned from *Plasmodium falciparum* and expressed in *E. coli*. The protein contains 19 methionines, all of which are localized in the NH_2-terminal 35 amino acid residues, and it is also rich in lysine. Pf16 is highly basic, contains a polyacidic domain consisting of aspartic acid and is related to the non-histone high mobility group proteins of higher eukaryotes. The gene is conserved among eight different species of *Plasmodium* so far examined, suggesting an important function for this gene product in the parasite's life cycle.

Malaria is one of the most important parasitic diseases in the world and abut 270 million people are threatened by this disease with an annual death toll exceeding 2 million (1, 2). It is caused by the protozoan parasite, *Plasmodium*. Of the four species that infect humans, *Plasmodium falciparum* causes the most severe form of the disease with an alarmingly high rate of morbidity and mortality. The global appearance of *Plasmodium* species that are resistent to chloroquine and other drugs poses a serious problem in combating malaria. In the last decade major efforts have been directed towards developing an effective vaccine but with only limited success (2).

The life cycle of the parasite occurs in two hosts, human and mosquito (3, 4). In humans, exo-erythrocytic and erythrocytic phases occur in the development of mature forms. Thus, unique gene products that are important for cell cycle control and developmental regulation could be identified and used as targets for antimalarial drugs. An enormous amount of work has been done on the isolation and characterization of stage-specific antigens (4, 5). In contrast, very little is known about the gene structure and regulation during various developmental stages in the life cycle of the parasite. For example, there is no information on the nuclear proteins that are involved in the structural organization of the chromatin, in DNA replication and in the regulation of gene expression. In eukaryotic cells histones and other nonhistone proteins together with DNA constitute chromatin. Some of these nonhistone proteins are involved in DNA replication and in gene expression (6). A class of proteins, commonly called the high mobility group (HMG) proteins, are ubiquitously distributed among all eukaroytic species (7, 8). Although their function is not known, it is likely they

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are involved in DNA replication and in transcriptional regulation (reviewed in 8). In this report, we describe the cloning and sequencing of an unusual gene (Pf16) from *P. falciparum*, which encodes a highly basic 147 amino acid protein that is rich in methionine, lysine, aspartic acid and asparagine. This gene is highly conserved among many different species of *Plasmodium* and appears to be related to the HMG and to the polyacidic proteins of higher eukaryotes.

MATERIALS AND METHODS

Screening of *Plasmodium falciparum* genomic library.

P. falciparum FCR/3 was grown, DNA was isolated and a *Hin*dIII library was established in λL47.1 as described (9). Approximately 5,000 plaques were screened by the immunoperoxidase method for antigen producing clones using human serum from a malaria patient. Seven positive plaques were obtained. One phage clone, λPL-Ag2, containing a 3.0 kb insert, was further characterized. The phage was amplified, DNA was prepared and the 3.0 kb insert was subcloned in pBR322.

Subcloning in pBR322.

The 3.0 kb *Hin*dIII insert in the λPL-Ag2 phage clone was gel purified, ligated to the *Hin*dIII linearized pBR322 and introduced into *E. coli* HB101 by transformation. Since there is a single *Eco*RI site in the pBR322 vector, digestion of the pPL-Ag2 plasmid DNA with *Eco*RI releases the 1.15 kb fragment. The large fragment containing the 1.85 kb fragment and pBR322 sequences were ligated by T_4 DNA ligase and used to transform *E. coli* HB101. From these transformants, the clone pPL-Ag2ΔR was selected. The 3.0 kb *Hin*dIII fragment was also digested with *Eco*RI and the resulting 1.15 and 1.85 kb fragments were independently cloned at the *Hin*dIII-*Eco*RI sites of pBR322. Two clones pPL-Ag2HR1 and pPL-Ag2HR3, containing the 1.15 kb and 1.85 kb inserts, respectively, were selected and used for further analysis.

Expression of Pf16 in *E. coli* minicells.

E. coli χ984 cells harboring appropriate plasmid were grown overnight at 37°C and minicells were prepared and stored at -70°C as described (10). Minicells were labeled with 100 μCi ^{35}S-methionine (ICN, Irvine, CA) in T medium (10) for 1 hr at 37°C, centrifuged, resuspended in Laemmli's buffer (11) and electrophoresed on a 10 to 18% gradient polyacrylamide gel.

Expression of Pf16 from the T7 promoter.

The 1.1 kb *Xba*I fragment from the plasmid pPL-Ag2 (Fig. 1) was gel-purified, cloned into the *Xba*I linearized pGEM-3Z, and screened by colony hybridization using the *Xba*I fragment that was labeled by the random primer method (12). Positive clones were amplified and the plasmid DNAs from individual clones were tested for the orientation of the insert by restriction enzymes followed by agarose gel electrophoresis. The DNAs from two clones, Pf16-E2 and Pf16-C1, were then transferred into *E. coli* HB101 harbouring the plasmid pGP1-2 (13), which contains the coding sequences for T7 RNA polymerase.

^{35}S-labeling was done essentially as described (13; Tabor, personal communication). 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 polymerse 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 *trans*-label (sp. act. 1100 Ci/mmole; ICN, Irvine, CA) for 5 minutes. The cells were quick-frozen or fractionated into membranes and inclusion bodies essentially as described (14) and aliquots were boiled in Laemmli's buffer and analyzed on 10-18% gradient polyacrylamide gels. Gels were fixed, dried and exposed against x-ray film (XAR-5, Kodak) at -70°C.

Polymerase Chain Reaction (PCR).

DNA was isolated from 0.3 ml of infected blood samples with parasitaemia ranging from 0.2 to 0.5% by the phenol-chloroform method as described (9). DNAs were resuspended in 50 μl Tris-EDTA and 1 μl was used in each PCR reaction. As a positive control the 1.1 kb *Xba*I fragment (see Fig. 2) was used. The forward and reverse oligonucleotide primers correspond to the sequences 137 to 157 and 325 to 345 respectively (see Fig. 3). Reaction conditions include DNA from various species of *Plasmodium*, 1 μg of mammalian cell DNA or 1 to 10 pg of the 1.1 kb *Xba*I fragment in a volume of 100 μl containing dNTP at 0.1 mM, 10 μl 10X Taq polymerase buffer and 1 to 2 units of Taq DNA polymerse (Promega, Madison, WI). The conditions of amplification were 95°C for 2 minutes, 50°C for 2 minutes, and 65°C for 2 minutes and 35 cycles. Approximately 5 μl of the reaction products were analyzed as a 1% agarose gel in Tris-borate buffer.

DNA sequencing.

Appropriate restriction fragments were cloned in M13mp18 or mp19 and sequenced by the chain termination method (15) using sequenase (USB, Cleveland, OH). When necessary oligonucleotides spanning different regions of the restriction fragment were synthesized and used as primers for sequencing. Database searching for sequence similarity was done by using the Fast A program of Pearson and Lipman (16).

RESULTS

Cloning of Pf16 and expression in *E. coli*. As described in Materials and Methods, the λPL-Ag2 clone was identified from the genomic library by using antiserum from malaria patients. This suggests that the clone encoded a protein and that it could be expressed in *E. coli*. To further characterize this molecular clone, the 3.0 kb *Hin*dIII fragment was subcloned in pBR322 and the recombinant plasmid was transferred to *E. coli* χ984. Minicells were prepared and labeled with ^{35}S-methionine. An intense band of 14 kD could be readily detected (Fig. 1, lane B). A protein of 28 kD was also present (Fig. 1, upper small arrows). This was shown to be the ampicillin gene product, β-lactamase, of the pBR322 vector. In order to localize the coding sequences for the 14 kD product various deletion mutants were constructed as described in the Methods. The plasmid containing the deleted 1.15 kb *Eco*RI fragment (Ag2ΔR1) was introduced into the χ984 host and minicells were prepared and labeled with ^{35}S-methionine. The results indicate that a portion or all of the coding

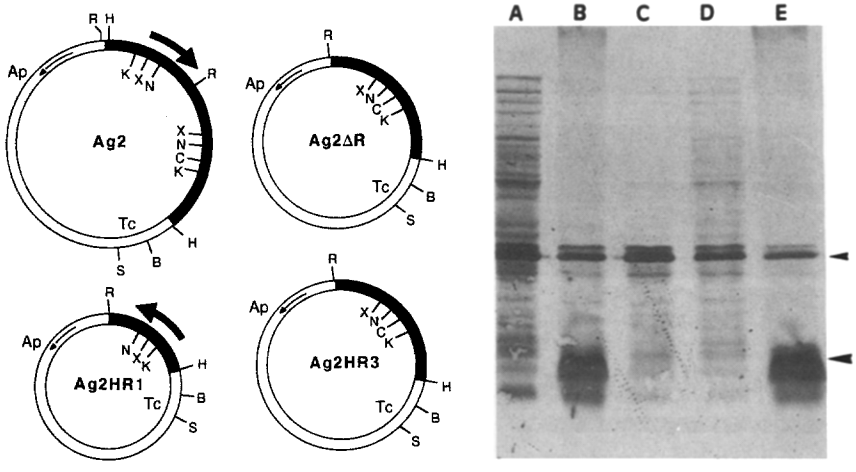

Fig. 1. Localization of the Pf16 coding sequences.

Left panel: Maps of deletion mutants. The 3.0 kb *Hin*dIII fragment with various restriction enzyme sites is shown in the circle marked Ag2. Solid line indicates the Ag2 sequences and open double line refers to the pBR322 vector. Thick arrow indicates the direction of transcription. In Ag2ΔR, the 1.15 kb *Eco*RI fragment (R to R) is deleted. In Ag2 HR1, the 1.15 kb *Eco*RI to *Hin*dIII fragment (R to H) was inserted in the pBR322 between the *Eco*RI and *Hin*dIII sites. Note the direction of the insert. Ag2HR3 is identical to the Ag2ΔR except that the 1.85 kb fragment of Ag2 (R to *Hin*dIII of the insert) was subcloned. K: *Kpn*I, X: *Xba*I, N:*Nde*I, C: *Cla*I, R: *Eco*RI, H: *Hin*dIII, B: *Bam*HI, S: *Sal*I. Ap: ampicillin gene and its direction of transcription is shown by a thin arrow in the open double line. Tc: Tetracycline sequences.

Right panel: Expression of Pf16 in *E. coli* χ984 minicells. The plasmids described in the left panel were introducted into the *E. coli* χ984 host. Minicells carrying the following plasmids were prepared and labeled with ^{35}S-methionine as described in Materials and Methods. Lane A: pBR322; B: Ag2; C: Ag2ΔR; D: Ag2HR3; and E: Ag2HR1. The 14 kD Pf16 protein is shown by a thick arrowhead and the 28 kD β-lactamase (ampicillin gene product) is shown by a thin arrowhead. Note the complete absence of the 14 kD band in Ag2ΔR and Ag2HR3 deletion mutants.

sequences reside in the 1.15 kb fragment because the deletion of the 1.15 kb fragment eliminated the 14 kD band (Fig. 1, circle marked Ag2ΔR and lane C).

Strong support for the presence of the coding region in the 1.15 kb *Eco*RI fragment came from the experiment in which the 1.15 kb *Hin*dIII-*Eco*RI (Fig. 1, Ag2HR1) was subcloned into pBR322 at the *Hin*dIII-*Eco*RI sites and the deletion mutant was introduced into *E. coli* X-984. Protein analysis indicated that the 1.15 kb fragment contained the corresponding coding sequence because the 14 kD band was completely restored (Fig. 1, circle Ag2HR1 and lane E). Parallel experiments with the 1.85 kb fragment, however, failed to restore the 14 kD band (Fig. 1, circle Ag2HR3 and lane D). These results suggest that the 1.15 kb but not the 1.85 kb fragment contains the coding sequences. When the proteins were resolved will, we found that the size of the protein, as revealed by its migration, was only about 13 kD (data not shown). This suggested to us that the coding sequence ends downstream of the *Eco*RI site. Since the protein is only about 140 to 160 amino acids and *Eco*RI reduced it by about 10 to 15 amino acids the amino end must lie about 400 bp upstream of the *Eco*RI site. That means the amino-end should be downstream of the *Xba*I or *Nde*I site (see Fig. 1). If this is true, then the coding sequences should also be in the 1.15 kb *Xba*I fragment. Therefore, the 1.15 kb *Xba*I fragment was inserted downstream of the T7 promoter in the pGEM-3Z vector in both orientations (Fig. 2). The recombinants were transferred into *E. coli* carrying the T7

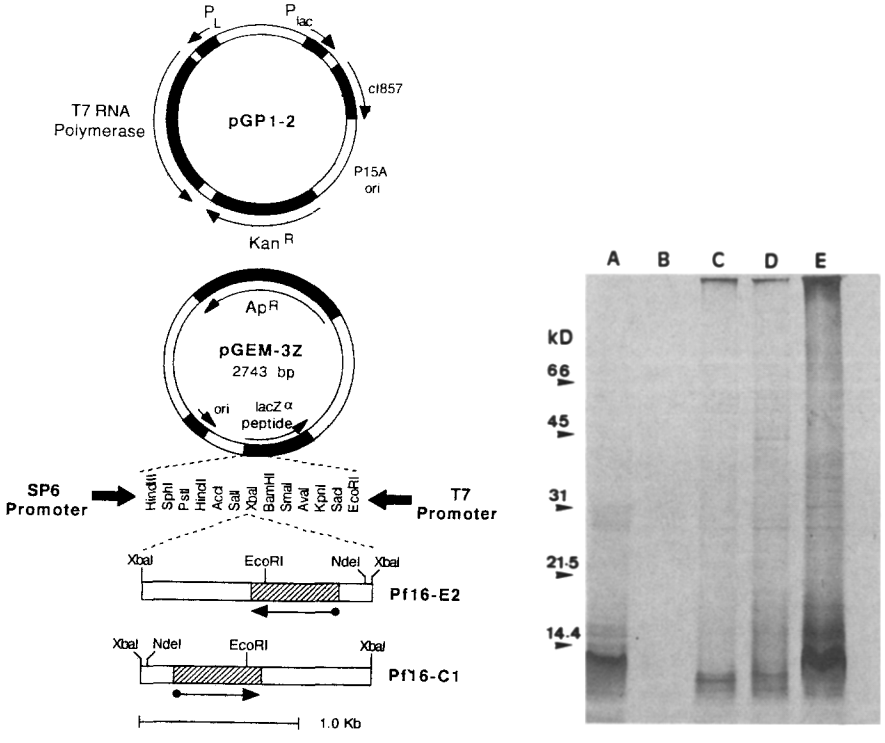

Fig. 2. Further localization of the coding sequences of Pf16.
The 1.1 kb *Xba*I fragment (see Fig. 1, X in Ag2 for the *Xba*I sites) was subcloned in pGEM-3Z in both orientations as shown in the figure. The cells were labeled with ^{35}S-methionine-cysteine as described in the Materials and Methods and the proteins were fractionated on a 10-18% polyacrylamide gel. Lane A: Pf16-E2; B: Pf16-C1; C: cytoplasm; D: membranes; E: inclusion bodies. Note that almost all the 14 kD protein is present in the inclusion bodies. The numbers at the left indicate the size of the markers in kD.

RNA polymerase-containing plasmid, pGP-1-2. Induction of T7 RNA polymerase by raising the temperature to 42°C followed by metabolic labeling with ^{35}S-methionine and SDS-PAGE analysis revealed an intense ^{35}S-labeled band of about 14 kD. This protein was detected when the fragment was inserted in the right orientation i.e., in the direction of transcription from the T7 promoter (Fig. 2, Pf16-E2 and lane A) but not in the opposite orientation (Fig. 2, Pf16-C1 and lane B). These results strongly suggest that the coding sequence reside downstream of the upstream *Xba*I site and extend beyond the *Eco*RI site. It should also be noted that almost all the methionine incorporated was present in the 14 kD band. This experiment also enabled us to identify the coding strand. Further analysis to localize the 14 kD protein in *E. coli* indicated the presence of this protein exclusively in the inclusion bodies (Fig. 2, lane E) but not in the cytoplasm (lane C) or membranes (lane D).

Having established the approximate coordinates of the coding region, we subcloned the 0.5 kb *Xba*I-*Eco*RI and 0.65 kb *Eco*RI-*Xba*I fragments in M13mp18 or 19 and determined their sequence. The 973 nucleotide sequence is shown in Fig. 3. Examination of the sequence of both strands revealed a single open reading frame (ORF) of 147 amino acids (aa) in only one strand. As observed

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   1  TCTAGAGAAAAATAAATTTGCTATTTGGATTAGTGATACCATATGTATTGATGATAATGA   60

  61  AGAGCTAATTGTATTAACAGATGCAATTAGTAAGGAAATTAATACCATATCATACGAATT  120

                                                        r1
 121  AGAAGATACCAAAAGTGATGATGAAGAGGGGGACGATGATGAAAGAGATGATGATGATGA  180
                      MetMetLysArgGlyThrMetMetLysGluMetMetMetMetM
                                  r2
 181  TGAAAAAGGTGATGATGAAAGAGGTGATGATGATGATGAAAAAGGTGATGATGAAGATGA  240
      etLysLysValMetMetLysGluValMetMetMetMetLysLysValMetMetLysMetM

 241  TGAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTGAAAAAGTGAAAAAGGGGGTAAGTGTAA  300
      etArgLysLysLysLysLysLysLysSerGluLysValLysLysGlyValSerValL

 301  AAAAAGAAAAGAAAAATAAACATAATAATAGTCATAAATATAGTGATAATGATGATGAAG  360
      ysLysGluLysLysAsnLysHisAsnAsnSerHisLysTyrSerAspAsnAspAspGluG

 361  AAGATGATGATGAAGATGACGATGATGATAATGATGATGACGATGATGATAATAATAATA  420
      luAspAspAspGluAspAspAspAspAsnAspAspAspAspAspAspAsnAsnAsnA

 421  ATAATAATAATAATGGTTTACACAAAGATAAGAAAAAAACAGGCATTTCTGCAAGTATAT  480
      snAsnAsnAsnAsnGlyLeuHisLysAspLysLysLysThrGlyIleSerAlaSerIleL

 481  TAAACAATGCTGCTAGTGTTATTGTTTCTGATCGTTTAAGAAGAAGAAATAAGAATTCAT  540
      euAsnAsnAlaAlaSerValIleValSerAspArgLeuArgArgArgAsnLysAsnSerL

 541  TAGCACATAATAACGGAACAAGAAATGCAAGCATTAAATAAAAGACAACATGAATTAAAA  600
      euAlaHisAsnAsnGlyThrArgAsnAlaSerIleLysEnd

 601  GCGCAAAAGATTAATGATATTAAAATAAGATTTTCCAAAGGAACAAATGATTATAAAGAT  660

 661  TTAAATAAAAAGAATATTAAAAAATTAGAAGATTTAAAAACATATAATGATCCAGATTTA  720

 721  TTACCTAAAGATTAAAGACCAAATATAATATGTGTAGATAATAAACATGAAGATATTATT  780

 781  ACCTATAAATGGATTACAGATCCATTCCATGTATCTACTATAAAAAATTTAAGTTCAAAT  840

 841  TATGAAGATAATAATGATATTTTTGTTTTACGTAAAAACTTTTTAGTACCAGGAAATCAA  900

 901  GGAGTAGTGAAAGGAGAATTAAATAGATTTCCGACATTACAACAAAATCAAATGTATATT  960

 961  AGAGAATTGATAT  973
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Fig. 3. Sequence of the Pf16 gene.

The sequence of the *Xba*I fragment is shown with the single open reading frame of 147 amino acids. Two twelve-amino acid repeats (r1 and r2) are underlined by a thin line and the polyacidic aspartic acid and glutamic acid-rich sequence is shown by a double underline. The basic region resembling trans-activation domains of other proteins is indicated by a solid line. Other basic regions containing lysines that resemble HMG-1/-2 are shown in Fig. 5.

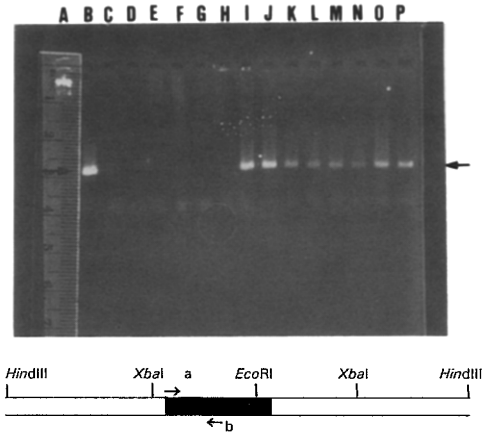

Fig. 4. Evidence that the Pf16 gene is conserved among *Plasmodium* species.
DNA isolated from various species of *Plasmodium* was amplified by the PCR and analyzed on an agarose gel. Lane A: *Hin*dIII digest of the λ phage DNA; B: PCR product generated from the 1.1 kb *Xba*I fragment (Fig. 2) was used as a positive control; C: no DNA; D: HeLa cell DNA; E: rat DNA; F: mouse DNA; G: hamster DNA; H: Salmon sperm DNA. DNAs from different species of *Plasmodium* were shown in lanes I to P. Lane I: *P. falciparum*; J: *P. malariae*; K: *P. brasilianum*; L: *P. simium*; M: *P. coatneyi*; N: *P. vivax*; O: *P. inui* and P: *P. fragile*. Arrow at right indicates the 210 bp PCR product.

above by deletion analysis, 15 aa were found downstream of the *Eco*RI site, confirming the results of protein analysis. The molecular weight calculated from the amino acid sequence is more than 16 kD, whereas the size detemined by the PAGE is approximately 14 kD. This anomalous migration might be due to the basic nature of the protein. This protein is referred to as Pf16, reflecting the calculated molecular weight.

Conservation of the Pf16 gene in *Plasmodium* species. Most of the *Plasmodium* genes encoding surface proteins have diverged remarkably in various species of *Plasmodium* and also within individual isolates (5). To test whether Pf16 is conserved in other species of *Plasmodium*, we carried out amplification by the polymerase chain reaction using oligonucleotides covering about 69 amino acids at the 5′ end. Eight different *Plasmodium* species (given in the legend to Fig. 4) were examined. DNA was isolated from parasitized blood samples (kindly supplied by W. Collins, CDC, Atlanta, GA) and subjected to the PCR. All the species tested positive as evidenced by the presence of the same 210 bp fragment (Fig. 4, lanes I to P). Human, salmon sperm, mouse, and rat DNAs gave negative results (Fig. 4, lanes C to H). PCR amplification of DNA isolated from *P. knowlesi* λgt11 cDNA library also yielded the same size fragment (data not shown) suggesting that this sequence, as expected, is transcribed into mRNA, because the library was derived from the cDNA (17). These results suggest that the Pf16 gene sequence is highly conserved among various *Plasmodium* species. Northern analysis also indicated that the 1.15 kb *Xba*I fragment probe detected a mRNA of 1.4 kb and the size of the cDNA clone from *P. knowlesi* was also 1.4 kb (data not shown).

DISCUSSION

Unusual features of Pf16. There are several unusual features about this protein. It is highly basic (pI 10.4) and rich in methionine, lysine, aspartic acid and asparagine. At the amino end, a 12 aa sequence is repeated twice with only a single nucleotide change in the second repeat. There are 19 methionines in the first 35 amino acids. This region is followed by a string of eight lysines. Overall

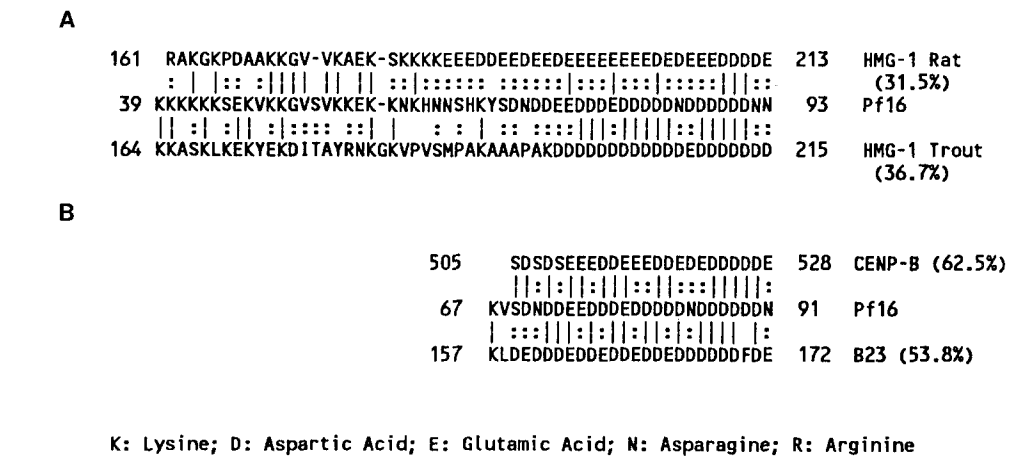

Fig. 5. Pf16 is homologous to HMG proteins.
Two HMG proteins from rat and trout and two nuclear proteins are compared. The numbers on the left and right indicate the amino acid positions in each protein. It appears that the CENβ-B and B23 are more related to Pf16 than are HMG-1 to Pf16. However, if we compare only the polyacidic domain (glutamic acid or aspartic acid), the extent of homology in the 20 to 24 amino acid polyacidic region of the HMG-1 and Pf16 is more than 70 to 80%. |, exact match; : conserved charged amino acid.

the methionine content is 13% and lysine is 26%. At the COOH end, there exists a domain that is rich in arginine and other basic amino acids. Similar positively-charged domains of several proteins have been implicated in DNA binding (18).

Protein homology search in the data base revealed partial identity to the non-histone high mobility group (HMG-1) proteins of Chinese hamster, rat and pig as well as to the nuclear proteins, CENP-B and B23 (Fig. 5). This suggests that the Pf16 is probably a nuclear protein. Like the other HMG-1 proteins, a polyacidic region of 19 aspartic acid and glutamic acid residues is present in Pf16. However, unlike the HMG-1 of higher eukaryotes where this acidic sequence is at the COOH end (8), in Pf16 it is present in the middle of the protein. In addition, the polyacidic region is highly related to two other proteins, the CENP-B and a nucleolus protein B23 (19), with a 52 to 62% similarity in a string of 20 to 24 amino acids. Since aspartic acid and glutamic acids have similar charges, this stretch of polyacidic region is virtually identical to the HMG-1 of trout and rat (Fig. 5). Like HMGs, Pf16 is also rich in basic amino acids, especially lysine. These features strongly suggest, but does not prove, that Pf16 belongs to the HMG family of proteins. Further experiments are required to establish its relationship with HMG proteins.

Conservation of Pf16 sequences. The evidence presented above clearly indicated that the gene is highly conserved among *Plasmodium* species but is quite distinct from higher eukaryotes. Although we have not tested other species of evolutionarily distant phyla, these results never the less document the relative conservation of this sequence. Sequence analysis of the PCR products further attests to the strong homology among different species. For example, in *P. simium* and *P. inui* only 6 and 8 nucleotide changes were detected in a total of 210 nucleotides so far sequenced (Guntaka and Kandala, unpublished data). These nucleotide changes have resulted in a few amino acid changes. This strong conservation of the gene may indicate a vital role for this gene product in the growth cycle of the parasite.

Possible function of Pf16. Several nuclear proteins, including HMG-1/-2, a centromeric protein CENP-B, nucleoplasmin and DNA topoisomerase I contain polyanionic regions (19). The strong

conservation of the polyacidic domain in Pf16 suggests that Pf16 might also interact with other nuclear proteins (19). It is also possible, because of the conserved basic domains, that the Pf16 might serve as a transcription factor. The synthesis of HMG1 was shown to be cell cycle regulated (20) suggesting an important role for these proteins in the cell cycle. Thus, in all likelihood, Pf16, is a good candidate to be involved in gene regulation as it is highly conserved among *Plasmodium* species. Further experiments are required to determine the structure and function of Pf16. If it indeed serves a crucial function in the cell cycle of the parasite, then antimalarial chemicals that are specific for this protein could be designed and developed.

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