Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9109–9113, December 1988
Genetics

Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase–thymidylate synthase gene of *Plasmodium falciparum*

(malaria/protozoan parasite/gene structure/drug resistance)

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Communicated by Gerald M. Rubin, February 26, 1988

ABSTRACT **We describe the isolation and the sequence of the gene for the bifunctional enzyme dihydrofolate reductase–thymidylate synthase (DHFR–TS; EC 1.5.1.3 and EC 2.1.1.45, respectively) from two pyrimethamine-resistant clones of *Plasmodium falciparum*, HB3 and 7G8. We have also derived the sequence of the DHFR portion of the gene, by amplification using polymerase chain reaction, for the pyrimethamine-sensitive clone 3D7 and the pyrimethamine-resistant strains V-1, K-1, Csl-2, and Palo-alto. The deduced protein sequence of the resistant DHFR portion of the enzyme from HB3 contained a single amino acid difference from the pyrimethamine-sensitive clone 3D7. It is highly likely that this difference is involved in the mechanism of drug resistance in HB3. The sequence of the DHFR gene from other pyrimethamine-resistant strains contains the same amino acid difference from the sensitive clone 3D7. However, they all differ at one other site that may influence pyrimethamine resistance. The DHFR–TS gene is present as a single copy on chromosome 4 in all pyrimethamine-sensitive and pyrimethamine-resistant isolates tested. Therefore, the molecular basis of pyrimethamine resistance in the parasites tested is not amplification of the DHFR–TS gene.**

The antifolate drug pyrimethamine is used as an effective antimalarial agent. However, naturally occurring drug resistance has been described, and resistant mutants have been isolated by mutagenesis and drug selection (1–8). Pyrimethamine inhibits parasite growth by binding to the enzyme dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) (1), which exists in *Plasmodium falciparum* and a number of other protozoa (9, 10) as a bifunctional protein with thymidylate synthase (TS; 5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45). TS and DHFR catalyze sequential reactions in the synthesis of dTMP.

Resistance to antifolates such as pyrimethamine could result from a number of mechanisms, such as amplification of the dihydrofolate reductase–thymidylate synthase (DHFR–TS) gene, alteration of the DHFR–TS enzyme, or a reduction in the permeability of cells to the drug. Antifolate drug resistance in *Leishmania* has been described (11, 12) and can be a result of overproduction of the enzyme DHFR or a decrease in flux of the antifolate drug (13). In *P. falciparum*, one parasite clone has been reported to overproduce DHFR activity (8); however, the mechanism of this increase has yet to be elucidated. Inselburg *et al.* (14) have isolated a pyrimethamine-resistant strain of *P. falciparum* that produces above normal levels of DHFR activity and have shown that it produces an increased amount of a defective DHFR–TS enzyme.

Two pyrimethamine-resistant clones of *P. falciparum* (7G8 and HB3) have been studied in detail by *in vitro* analysis of the DHFR activity and have been compared to the drug-sensitive strain 3D7 (15). The three clones studied had approximately equal levels of DHFR activity, but 7G8 and HB3 were much more resistant to pyrimethamine than was 3D7. It was suggested that the mechanism of resistance was not due to overproduction of DHFR but to a decreased affinity for pyrimethamine by a structurally altered enzyme.

We describe the isolation and sequence of the DHFR–TS gene[§] for *P. falciparum* for the pyrimethamine-resistant clones HB3 and 7G8. Sequences of the DHFR portion of the gene were also derived by polymerase chain reaction (PCR) (16) for the pyrimethamine-sensitive clone 3D7 and the resistant strains Palo-alto, V-1, K-1, and Csl-2. Amino acid differences linked to pyrimethamine resistance were identified, but there is no evidence for amplification of the DHFR–TS gene.

MATERIALS AND METHODS

***In Vitro* Inhibition of *P. falciparum* by Pyrimethamine.** Mixed stages of *P. falciparum* at 1% parasitemia and packed cell volume were grown for 72 hr in the presence of freshly diluted pyrimethamine (in 0.1% acetic acid). Cultures of 100 μl were grown in 96-well culture dishes. At the end of the assay, 100 μl of fixative was added, and the parasitemia were assayed on the fluorescence-activated cell sorter as described previously (17).

Parasites and DNA. *P. falciparum* clones HB3, 3D7, XP2, and X5 were obtained from D. Walliker (Department of Genetics, University of Edinburgh; Edinburgh EH9 3JN) (18); clones 7G8 (19), Palo-alto (Uganda), and V-1 (Vietnam) were from R. J. Howard (National Institutes of Health, Bethesda, MD); and clone FCR-3/A_2 (20) was obtained from W. Trager (The Rockefeller University, New York). K-1 (Thailand) was a gift from G. Knowles (Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea). Csl-2 was obtained from Margaret Maloney (Commonwealth Serum Laboratory, Melbourne, Australia) and was isolated from a tourist returning from Thailand. The derivation of clones D10 and E12 have been described elsewhere (21). Parasites were maintained in culture, and DNA was produced as previously described (22).

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Abbreviations: DHFR, dihydrofolate reductase; TS, thymidylate synthase; PCR, polymerase chain reaction.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03772).

Oligonucleotides. Radioactively end-labeled oligonucleotides corresponding to the thymidylate synthase protein consensus sequences Met-Ala-Leu-Pro-Pro-Cys-His (ATGGCWTTRCCWCCWTGYCAT) and Pro-Phe-Asn-Ile-Ala (CCWTTYAAYATHGCT) (where W = A or T, R = A or G, Y = C or T, and H = A or C or T) were used to isolate a 3.3-kilobase (kb) *Eco*RI fragment from DNA of the *P. falciparum* clone FCR-3/A_2. This DNA fragment contained the 3′ end of the TS gene and was then used to isolate the same *Eco*RI fragments from clones 7G8 and HB3.

DNA Sequencing. Appropriate restriction fragments of the DHFR–TS gene were made and subcloned into M13mp18 and M13mp19 and were sequenced by the dideoxy chain termination method (23). When suitable restriction sites were not available, oligonucleotide primers were synthesized to complete the sequence. The DHFR–TS gene was sequenced on both strands for HB3, 7G8, and V-1.

Polymerase Chain Reaction. Oligonucleotide primers from the 5′ (AAGCTTTTCTCCTTTTTATGATGGAACAAGTCTGCGAC) and the 3′ (GTATCTTTGTCATCATTCTTTAAAGGCATATC) end of the DHFR gene were used to amplify this region with Taq polymerase by using PCR as described (16). The amplified fragment was cloned into M13mp18 and was sequenced. At least four independent clones were sequenced for HB3, 3D7, V-1, Csl-2, K-1, and Palo-alto from two different PCR reactions.

Pulsed-Field Gradient Gel Electrophoresis and Southern Blot Analysis. Chromosomes from *P. falciparum* clones D10, E12, 3D7, HB3, and 7G8 were fractionated in a contour-clamped homogeneous electric field (24) apparatus by pulsed-field gradient electrophoresis for 34 hr by using 150-sec pulses at 160 V and then were transferred to Hybond-N membranes (Amersham). Genomic DNA from each strain was digested with either *Eco*RI or *Xba* I, fractionated on a 0.7% agarose gel, and transferred to Hybond-N membranes. All filters were hybridized in 5× SSC (1× = 0.015 M sodium chloride/0.15 M sodium citrate, pH 7)/1× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% $NaDodSO_4$/500 μg of salmon sperm DNA at 65°C.

RESULTS

Pyrimethamine Resistance of *P. falciparum* Strains. As a step toward understanding the molecular basis of pyrimethamine resistance in *P. falciparum*, we chose to isolate the gene for DHFR–TS, the target of action for this antifolate drug. We chose the pyrimethamine-sensitive clone 3D7 and the resistant clones HB3 and 7G8, as their levels of DHFR activity and pyrimethamine sensitivity have been extensively studied (5, 15). We also examined the strains V-1 (Vietnam), K-1 (Thailand), Csl-2 (Thailand), and Palo-alto (Uganda). To confirm the response of these isolates to pyrimethamine, we cultured them in the presence and absence of the drug (Fig. 1). The pyrimethamine-resistant clone 7G8 and strains V-1, K-1, and Csl-2 are ≈100 times more resistant to the drug than the sensitive clone 3D7. The strain Palo-alto and clone HB3 show an intermediate level of resistance.

Isolation and Sequence Analysis of the DHFR–TS Gene. Oligonucleotides corresponding to a consensus sequence of the TS gene were used to screen an *Eco*RI genomic library of the *P. falciparum* clone FCR-3/A_2, and a 3.3-kb fragment was isolated. Sequencing of one end of this fragment revealed homology to the TS gene of other organisms (25). This probe was used to isolate the same 3.3-kb *Eco*RI fragment from HB3 and 7G8 and an overlapping 4.8-kb *Xba* I fragment extending the DHFR–TS sequence an additional 1 kb toward the 5′ end of the gene, which was isolated as a 5-kb *Eco*RI fragment (Fig. 2). Although a number of clones had extensive deletions, the longest clone was a 4.4-kb fragment that contained only a short deletion. Two independent 2.0-kb clones were also isolated and fully sequenced.

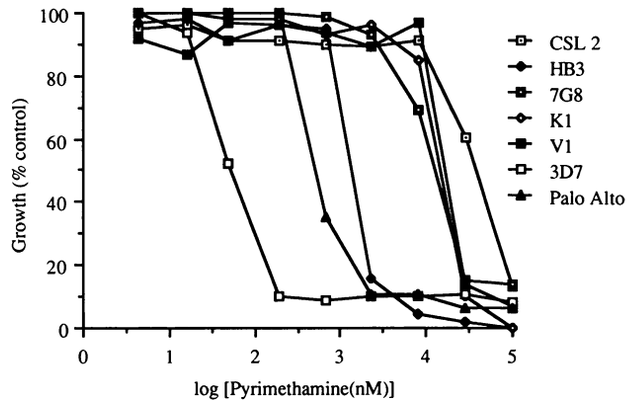

FIG. 1. *In vitro* inhibition of *P. falciparum* by pyrimethamine. The effect of the antifolate inhibitor pyrimethamine was assayed in duplicate after cultivating asynchronous parasites in 96-well dishes for 72 hr in the presence of various concentrations of the drug. The parasitemias were calculated after fixation and staining with Hoechst dye 33258 in a fluorescence-activated cell sorter.

The sequence of the DHFR–TS gene of the pyrimethamine-resistant clone HB3 is shown in Fig. 3. Translation of the sequence gave a single long open reading frame of 608 amino acids with no apparent introns. The putative protein has a deduced molecular mass of 72 kDa, which is in agreement with that measured by polyacrylamide gel electrophoresis (14). The proposed open reading frame contains homology to the DHFR and TS genes of other species throughout the sequence and differs by two amino acids from the DHFR sequence of FCR-3/A_2 (26, 27). The region 400 base pairs upstream of the first methionine codon shown (Fig. 3) has been fully sequenced (data not shown) in an undeleted area of the DNA clone, and it is greater than 90% A+T-rich and has many stop codons. We conclude from these sequence data and the homology with other species that this open reading frame encodes the DHFR–TS gene of *P. falciparum*.

Structure of the DHFR–TS Protein. Comparisons of the *P. falciparum* DHFR–TS protein sequence with other species suggest that the DHFR domain consists of ≈198 amino acids located at the amino terminus and that TS consists of 285 residues located at the carboxyl terminus. The enzyme domains are separated by a hinge region that, unlike the enzymically active regions, is not homologous to the same region of the *Leishmania tropica* DHFR–TS bifunctional protein (28). The secondary structure of the DHFR domain appears to be conserved in that the eight β-helices can be

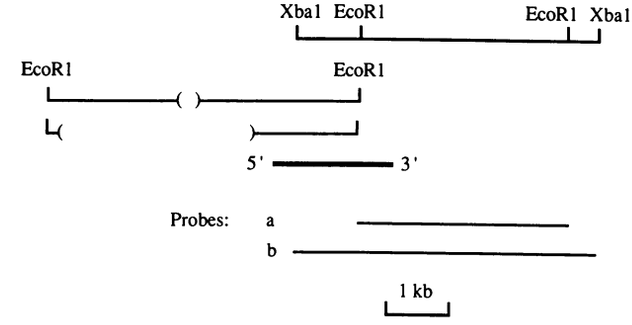

FIG. 2. Structure of genomic DNA encompassing the DHFR–TS gene of HB3. The open reading frame encoding the DHFR–TS protein is shown (▬). The *Eco*RI fragment containing the 5′ end of the DHFR gene contained deletions, which are shown by ( ). Two independent DNA fragments are shown, one of which has an extensive deletion.

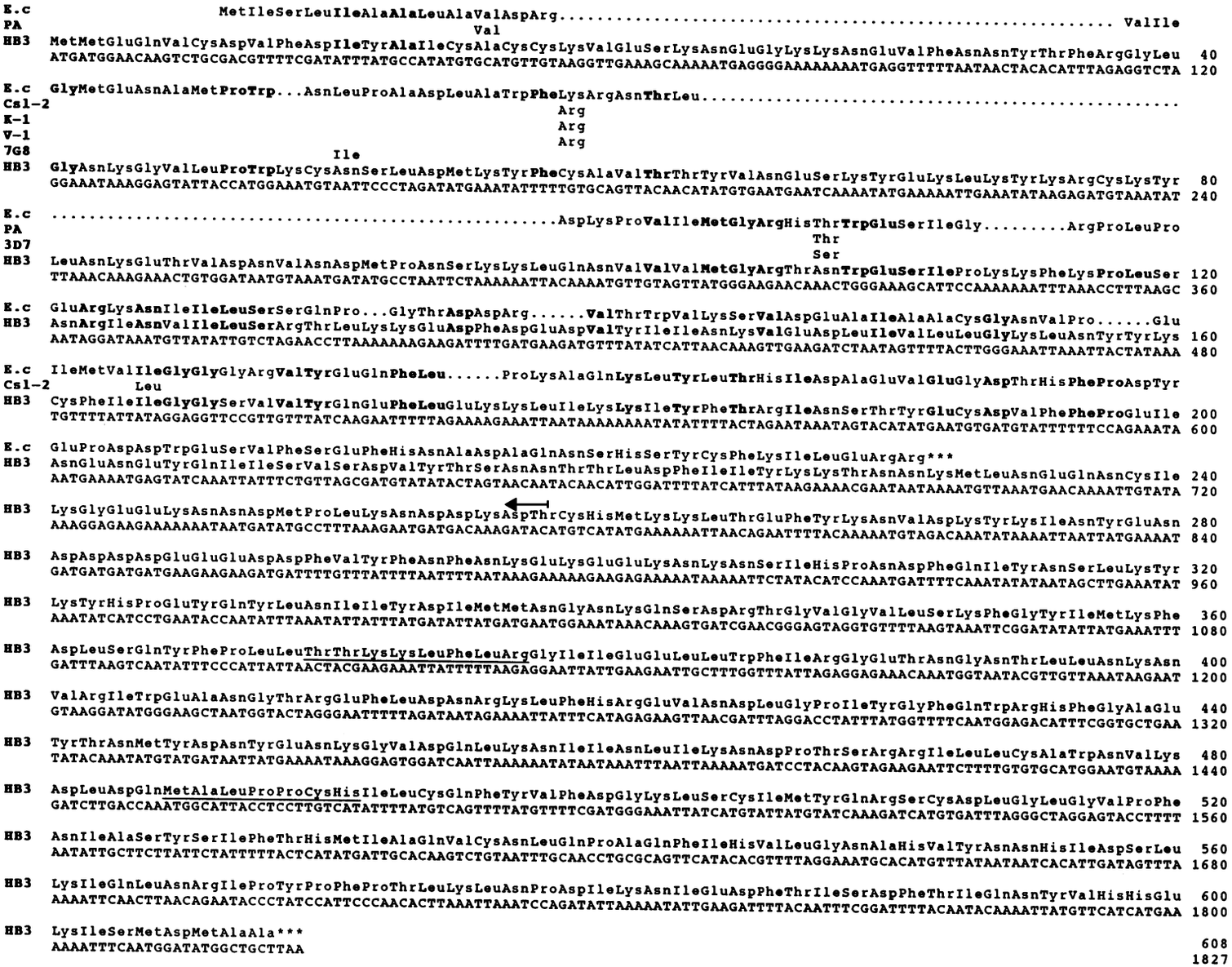

FIG. 3. DNA sequence and deduced protein sequence of the DHFR–TS gene of parasite clones HB3, 7G8, and V-1 and comparison with *Escherichia coli* DHFR. The nucleotide sequence of the pyrimethamine-resistant clone HB3 is shown in full with the deduced protein sequence. Only the amino acid differences are shown for the drug-resistant clone 7G8 and strain V-1. Nucleotides and amino acids are numbered at the right. The *E. coli* (E.c) DHFR protein sequence is shown aligned with the *P. falciparum* DHFR sequence. Bold-face residues show locations of invariant amino acids. Underlined amino acids in the TS domain represent conserved residues present in the active site of the enzyme (see text). Also shown are the positions of amino acid differences derived by PCR and sequencing of the pyrimethamine-sensitive clone 3D7 and the resistant strains Csl-2, K-1, and Palo-alto (PA). The arrow at nucleotide 773 shows the 3′ boundary where the DHFR region was amplified. All amino acid residues are identical to HB3 except where shown.

identified, two of which are important in substrate binding (29). X-ray crystallographic analysis of the *Lactobacillus* DHFR sequence has identified 32 amino acids that have interactions with NADPH and methotrexate in the active site (30). In the *P. falciparum* DHFR protein sequence, 11 of these residues are identical to the *Lactobacillus* sequence.

The TS domain commences at amino acid 323 and continues to the carboxyl terminus of the protein and is much more highly conserved than the DHFR portion of the molecule. Amino acid residues 370–377 (Thr-Thr-Lys-Lys-Leu-Phe-Leu-Arg) of the TS domain (Fig. 3) correspond to a conserved consensus sequence of Thr-Thr-Lys-Arg(Lys)-Xaa$_{2-3}$-Arg-(Lys) that is involved in binding folate cofactors (30). Also, amino acids 485–491 (Met-Ala-Leu-Pro-Pro-Cys-His) (Fig. 3) are homologous to the active site of the TS enzyme of other species (25, 31), and the cysteine residue at 490 can be assigned as the nucleophile essential for the conversion of dUMP to dTMP (31). The TS domain ends in a conserved region with a penultimate alanine, a characteristic of the carboxyl terminus of all TS sequences.

DHFR–TS Is Encoded by a Single-Copy Gene on Chromosome 4. To test if the mechanism of pyrimethamine resistance in *P. falciparum* is due to DHFR–TS gene amplification, we used Southern blot analysis to probe genomic DNA of resistant and sensitive clones (Fig. 4). The *Eco*RI fragment containing the 3′ end of the TS gene (probe a; see Fig. 2) was hybridized to *Xba* I digests of genomic DNA from the pyrimethamine-sensitive clone 3D7 and resistant clones HB3 and 7G8 as well as the strains K-1, V-1, Palo-alto, and Csl-2. The DHFR–TS probe hybridized to a 4.8-kb *Xba* I fragment to an equal extent in all isolates. The same filter was hybridized to a known single-copy gene, Ag 44 (32), and this probe hybridized equally to all isolates (data not shown). Therefore, the DHFR–TS gene is present as a single-copy gene in all of the isolates tested.

Pulsed-field gradient electrophoresis was used to determine the chromosomal location of the DHFR–TS gene. *P. falciparum* contains 14 chromosomes, some of which show length polymorphisms between different clones (33). Hybridization of the *Xba* I fragment containing most of the DHFR–TS gene (probe b) to chromosomes of *P. falciparum* isolates D10, E12, 3D7, XP2, X5, HB3, and 7G8 identified a single hybridizing chromosome (Fig. 5). This was unambiguously identified as chromosome 4 by its very characteristic length polymorphisms (33).

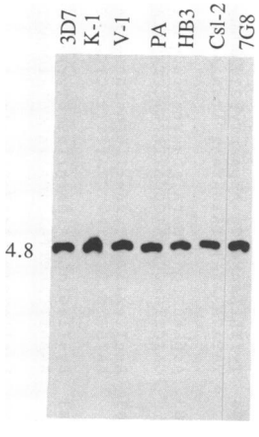

FIG. 4. The DHFR–TS gene is not amplified in resistant parasite clones. *Xba* I-digested genomic DNA from parasites 3D7, K-1, V-1, Palo-alto (PA), HB3, Csl-2, and 7G8 was hybridized with the radiolabeled *Eco*RI fragment containing the 3′ end of the DHFR–TS gene (probe a). The molecular size of the hybridizing fragments is shown in kilobase pairs.

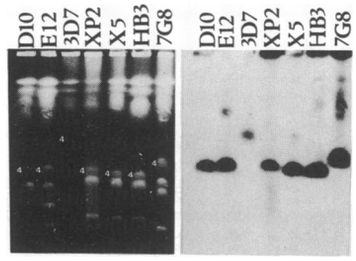

FIG. 5. Chromosomal localization of the *P. falciparum* DHFR–TS gene. (*Left*) Ethidium bromide-stained pulsed-field gradient electrophoresis gel of *P. falciparum* chromosomes. 3D7 chromosomes were used as the reference clone for numbering chromosomes (33). (*Right*) Blot of the same gel hybridized with radiolabeled probe b, the *Xba* I fragment containing most of the DHFR–TS gene. Note that the quantity of 3D7 DNA is lower than the other DNAs, and consequently the hybridization signal is also lower in that lane.

DHFR–TS Gene Sequence of Pyrimethamine-Resistant Isolates. Chen *et al.* (15) have analyzed DHFR activity and pyrimethamine resistance of the *P. falciparum* clones 7G8 and HB3 and have compared them to the sensitive clone 3D7. They concluded that the mechanism of resistance in the pyrimethamine-resistant clones HB3 and 7G8 is due to a structurally altered enzyme in both cases. To analyze this at the DNA level, we isolated the DHFR–TS gene of 7G8 and sequenced it in full (Fig. 3). The DHFR region of 3D7 was amplified by PCR, and the sequence was compared to HB3 and 7G8 (Fig. 3). The pyrimethamine-resistant clone HB3 contains only one amino acid difference from that of the sensitive clone 3D7 in the DHFR domain: residue 108 is serine in 3D7, but it is asparagine in HB3. The clone 7G8, which is also resistant to pyrimethamine, has asparagine at residue 108 and isoleucine instead of the asparagine at residue 51 in 3D7 and HB3.

We obtained the sequence of the DHFR region of the DHFR–TS gene by PCR from a number of other pyrimethamine-resistant strains of *P. falciparum* to ascertain if particular amino acid differences were linked to resistance as suggested by the 7G8 and HB3 DHFR sequences. The pyrimethamine-resistant strains Csl-2, K-1, and V-1 all have an asparagine at residue 108 (Fig. 3). However, they also have arginine at position 59 instead of cysteine as found in 3D7, HB3, and 7G8. Csl-2 DHFR also encodes a conservative difference, leucine at residue 164. This residue is isoleucine in all other DHFR sequences analyzed. The Palo-alto strain of *P. falciparum* has a threonine at residue 108 and a valine at position 16, the same amino acids reported for the pyrimethamine-sensitive clone FCR3 (26).

DISCUSSION

We have isolated and sequenced the DHFR–TS gene of *P. falciparum* from two pyrimethamine-resistant clones, HB3 and 7G8, and have obtained the sequence of the DHFR portion of the pyrimethamine-sensitive clone 3D7 and several resistant strains. Hybridization of the DHFR gene to genomic DNA of the pyrimethamine-sensitive and -resistant parasites revealed that the molecular basis of drug resistance, in those parasites tested, is not due to amplification of the DHFR–TS gene as both resistant and sensitive clones have a single gene on chromosome 4. The DHFR–TS gene behaved in a normal Mendelian manner in a cross between 3D7 and HB3 (18), as would be expected for a single chromosomally located gene. This is in agreement with previous analysis of the level of DHFR enzyme activity in the resistant clones HB3 and 7G8 (15) and the strain K-1 (7). Previously it has been reported that the Palo-alto strain had a 30- to 80-fold increase in DHFR activity (8), but we have no evidence for amplification at the

gene level. It is of course possible that the Palo-alto strain in our laboratory has changed as it has not been maintained under drug-selective pressure.

DHFR activity has previously been isolated from the pyrimethamine-resistant clones 7G8 and HB3 and has been compared to the drug-sensitive clone 3D7. These results suggested that the drug resistance in these resistant *P. falciparum* clones is a result of structural changes in the DHFR–TS protein. Comparison of the 3D7 DHFR sequence to that of HB3 revealed only one amino acid difference, at residue 108, that can account for the drug resistance of this clone. In the *Lactobacillus casei* three-dimensional DHFR sequence, this residue is located in an α-helix that contains important residues that interact with methotrexate (29), another antifolate drug. The DHFR sequence of 7G8 also has the asparagine at residue 108, but it also has a difference at residue 51 (isoleucine). 7G8 is 50-fold more resistant to pyrimethamine than HB3 and, therefore, this second amino acid change is required to obtain high-level drug resistance.

Comparison of the DHFR sequence of the pyrimethamine-resistant strains Csl-2, K-1, and V-1 to HB3 and 7G8 showed that an asparagine at amino acid 108 is linked to resistance, in contrast to pyrimethamine-sensitive clones 3D7 and FCR3 (26), which either have a serine or a threonine. This conclusion is in agreement with the data of Peterson *et al.* (26), who have sequenced the DHFR gene of 3D7, HB3, and 7G8 as well as a number of other pyrimethamine-resistant and -sensitive strains. Csl-2, V-1, and K-1 are as resistant to pyrimethamine as 7G8 and are at least 20-fold more resistant than HB3; however, they all have the same amino acid at position 108 (asparagine). The three southeast Asian strains (Csl-2, V-1, and K-1) all have an arginine at position 59, and it is likely that this amino acid combined with the asparagine at residue 108 results in the high-level pyrimethamine resistance observed. Csl-2 has a third amino acid difference compared to the drug-sensitive DHFR sequences; however, this is a very conservative change (Ile-164 to Leu) and is unlikely to have a significant effect on binding of pyrimethamine. The Palo-alto sequence of the DHFR gene region is identical to that derived for the pyrimethamine-sensitive clone FCR3 (26), suggesting that either the low-level resistance seen in Palo-alto is the same as that for FCR3 or that other factors are involved in the drug resistance of this strain. The Palo-alto DHFR sequence differs from 3D7 in that it has threonine at residue 108 in place of serine, and this may explain the increased resistance of this strain compared to 3D7.

Isolation of the DHFR–TS gene from a pyrimethamine-resistant clone of *P. falciparum* will enable analysis of the protein products of these genes by the expression of large amounts of enzyme in heterologous systems. This should facilitate identification of those amino acids involved in the pyrimethamine resistance of these *P. falciparum* clones. Furthermore, development of a system for genetic transformation in this parasite should be aided by the use of pyrimethamine-resistant DHFR–TS genes as dominant selectable markers. The cloning and sequencing of the 7G8 DHFR–TS gene is a step closer to this aim.

We thank Jenny Thompson and Denise Galatis for expert technical assistance and Margaret Moloney of the Commonwealth Serum Laboratories for parasites. We are grateful to Peter Coleman of Commonwealth Scientific and Industrial Research Organization Protein Structure Laboratory for helping compare the *E. coli* and *L. casei* three-dimensional structure with the primary sequence of the *P. falciparum* DHFR. We thank D. J. Kemp, R. F. Anders, and G. F. Mitchell for critical comments on the manuscript. We also thank R. L. Coppel for help with the computer graphics. We thank David Peterson (National Institutes of Health) for communication of results prior to publication (ref. 26). This work was supported by the John D. and Catherine T. MacArthur Foundation, the Australian National Health and Medical Research Council, the Alcoa Foundation, and the United Nations Development Programme/World Bank/World Health Organization Special Programme for Training in Tropical Diseases. A.F.C. was supported by a C. J. Martin fellowship from the Australian National Health and Medical Research Council for part of this work.

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