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Amino acids in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum* involved in cycloguanil resistance differ from those involved in pyrimethamine resistance

(drug resistance/malaria/protozoan parasite/gene structure/polymerase chain reaction)

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ABSTRACT **Cycloguanil, the active metabolite of the antimalarial drug proguanil, is an inhibitor of dihydrofolate reductase as is another antimalarial, pyrimethamine. Its use has been limited by the rapid development of resistance by parasites around the world. We have determined the cycloguanil- and pyrimethamine-sensitivity status of 10 isolates of *Plasmodium falciparum* and have sequenced in all these isolates the dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) portion of the DHFR-thymidylate synthase (TS; 5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45) gene. Instead of the known serine-to-asparagine change at position 108 that is important in pyrimethamine resistance, a serine-to-threonine change at the same position is found in cycloguanil-resistant isolates along with an alanine-to-valine change at position 16. We conclude that pyrimethamine and cycloguanil resistance most commonly involve alternative mutations at the same site. However, we also have identified a parasite with a unique set of changes that results in resistance to both drugs.**

The dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) antagonists pyrimethamine and proguanil have played a major role in prophylaxis and treatment of *Plasmodium falciparum* infection; they are often used in conjunction with a sulphonamide drug (1) and, along with chloroquine, have been the most extensively used of all antimalarials over the past decade. However, there are increasing reports of resistance to both drugs, used alone or in combination with a sulphonamide (see ref. 2 for review). Resistance to these drugs is associated with heavy use of a drug in a particular area, and unlike resistance to chloroquine, the occurrence of resistance is sporadic and multifocal.

Proguanil is activated *in vivo* by the mixed function oxidase system of the hepatic microsomes to form the active compound cycloguanil (3). Both cycloguanil and pyrimethamine have been shown to strongly inhibit the DHFR of *Plasmodium berghei* (K_i of cycloguanil = 0.78×10^{-9} M) (4), an action that is selective for malarial enzymes. However, it is not known whether cycloguanil and pyrimethamine share the same binding site on the DHFR enzyme, nor is it clear whether the acquisition of resistance to one of the drugs necessitates concomitant resistance to the other.

That many parasites are resistant to both pyrimethamine and cycloguanil is well documented (5–7), but it is not clear whether this represents cross resistance or whether an accumulation of resistances has occurred. Sensitivities of parasites that break through various prophylactic regimens have shown both "doubly" resistant parasites (8) or parasites resistant to only pyrimethamine or cycloguanil. A study of acquisition of pyrimethamine resistance in *P. falciparum* (9) has shown that pyrimethamine resistance was associated with a slight increase in cycloguanil resistance.

Recent reports on the molecular basis of pyrimethamine resistance in *P. falciparum* have implicated an accumulation of point mutations being associated with increasing resistance to pyrimethamine (10–13). These studies also failed to demonstrate an increase in the copy number of the DHFR gene in pyrimethamine-resistant isolates. These observations accord with enzymological data showing that pyrimethamine-resistant isolates have no increase in enzymatic activity but have a change in the K_a of DHFR for pyrimethamine.

Plasmodium chabaudi has been made resistant to pyrimethamine by gradual increments in the concentration of pyrimethamine injected into the mice carrying the parasite, and the initial event in the development of resistance in this organism was a duplication of the chromosome carrying the DHFR-thymidylate synthase (TS; 5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) gene, with the end result being a doubling of the copy number of the DHFR-TS gene (14). Further selection of these parasites resulted in a greater increase in resistance towards pyrimethamine and the appearance of a point mutation in the DHFR gene in a similar position to the 108 change in *P. falciparum* (10, 11). A similar observation has been made on the *in vitro* selection of *P. falciparum* by pyrimethamine (15): the initial event in increased resistance was a duplication of the DHFR gene. From these observations it seems that initial events in the acquisition of pyrimethamine resistance may involve a low-level amplification of the DHFR gene, but higher levels of resistance, as are seen in field isolates, are caused by mutations within the DHFR gene.

As yet there is little information on the mechanism of resistance to cycloguanil and its relationship to pyrimethamine resistance. To investigate this, we have taken parasites from the field that are resistant to either drug or to both drugs, sequenced their DHFR genes, and assessed the copy number of the gene in each isolate. We reason that any change in the sequence of this gene will most likely have been selected for by pressure from either pyrimethamine or cycloguanil as these have been the only two DHFR inhibitors that have been widely used against malaria. We show that there are separate changes in the DHFR gene that are different in cycloguanil-resistant parasites than in pyrimethamine-resistant parasites and that no amplification of the DHFR gene occurs.

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Abbreviations: DHFR, dihydrofolate reductase; TS, thymidylate synthase; IC_{50pyr} and IC_{50cyc}, the 50% inhibitory concentration of pyrimethamine and cycloguanil; strain PA2, strain Palo-alto 2.

MATERIALS AND METHODS

Parasites and DNA. *P. falciparum* clones HB3 (Honduras) and 3D7 (the parent was isolated from an airport worker in Amsterdam) were obtained from D. Walliker (Department of Genetics, University of Edinburgh, EH9 3JN); clones 7G8 (Brazil), Palo-alto (Uganda), and V1 (Vietnam), from R. Howard (DNAX); FCR3/A2, from W. Trager (The Rockefeller University, New York); K1 (Thailand), from G. Knowles (Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea); isolate Csl-2 (Thailand), from M. Malone (Commonwealth Serum Laboratories, Melbourne, Australia); and FAC8 (a clone of ITG2.F6, Brazil), from B. Biggs (Walter and Eliza Hall Institute, Melbourne, Australia). *P. falciparum* isolate FC27 was isolated from Madang, Papua New Guinea. DNA was produced after guanidine hydrochloride lysis of parasites (16).

It will be noted that the value for the sensitivity for Palo-alto is different than previously published (10). We have found that our "stabilates" of Palo-alto parasites contain two different parasite lines, one sensitive to and the other resistant to pyrimethamine, and it is the data from the sensitive isolate that was presented in the previous paper; the data in this paper come from a different stable isolate of the parasite. The sequences of the DHFR gene for both isolates are consistant with their pyrimethamine resistance status (see below). To prevent confusion, the Palo-alto isolate discussed in this paper will be called "Palo-alto 2" (PA2).

Sensitivity Assays. Nonsynchronized parasites at an initial parasitemia of 1% were grown at 2% hematocrit in fresh erythrocytes. The medium contained folate at 0.01 mg/liter and *p*-aminobenzoic acid at 0.0005 mg/ml, which are at lower concentrations than previously used (10). Freshly diluted drug was added to the parasites with each daily medium change, and the assay was terminated after 72 hr with the addition of formaldehyde fixation solution. The final parasitemia was assayed on the FACSCAN as described (17). The concentration of the drug that inhibits growth to 50% of the control growth has been termed the IC_{50}. The IC_{50} of pyrimethamine is referred to as IC_{50pyr} and that of cycloguanil is IC_{50cyc}.

DNA Sequencing. Oligonucleotide primers from both the 3′ (GTATCTTTGTCATCATTCTTTAAAGGCATATC) and 5′ (AAGCTTTTCTCCTTTTTATGATGGAACAAGTCTGCGAC) ends of the DHFR portion of the DHFR-TS gene were used in a polymerase chain reaction as described (18). The products of the polymerase chain reaction were cloned into M13mp18 and M13mp19 and sequenced by the dideoxy chain-termination method (19).

Southern Blot Analysis. *Xba* I-digested DNA from each isolate was separated on an agarose gel, blotted to nitrocellulose, and probed with a radiolabeled *Eco*RI fragment from the 3′ end of the DHFR-TS gene.

RESULTS

Cycloguanil and Pyrimethamine Sensitivities of *P. falciparum* Isolates. Ten parasite isolates and clones from different continents were tested for both cycloguanil and pyrimethamine resistance. The sensitivity curves thus generated are depicted in Fig. 1. As the *in vitro* level of sensitivity of the parasite towards cycloguanil has not been linked to any clinical correlate of drug effectiveness, the terms sensitive and resistant are only used relatively. The IC_{50} of the most pyrimethamine-resistant isolate (Csl-2) is nearly 10,000 times greater than the sensitive clone (3D7), whereas Csl-2, also the most cycloguanil-resistant isolate, has an IC_{50} for cycloguanil that is <1000 times that of 3D7. A comparison of each IC_{50} for both drugs is presented in Fig. 2. This shows that 3D7 has the lowest IC_{50} to both drugs and that Csl-2 has the greatest IC_{50} to both drugs, FAC8 and FCR3 have both high IC_{50cyc} values and low IC_{50pyr} values, whereas K1, V1, PA2, 7G8, and HB3 have low-to-moderate IC_{50cyc} values, and yet all have increased IC_{50pyr} values. Csl-2 is the only isolate studied that is highly resistant to both cycloguanil and pyrimethamine.

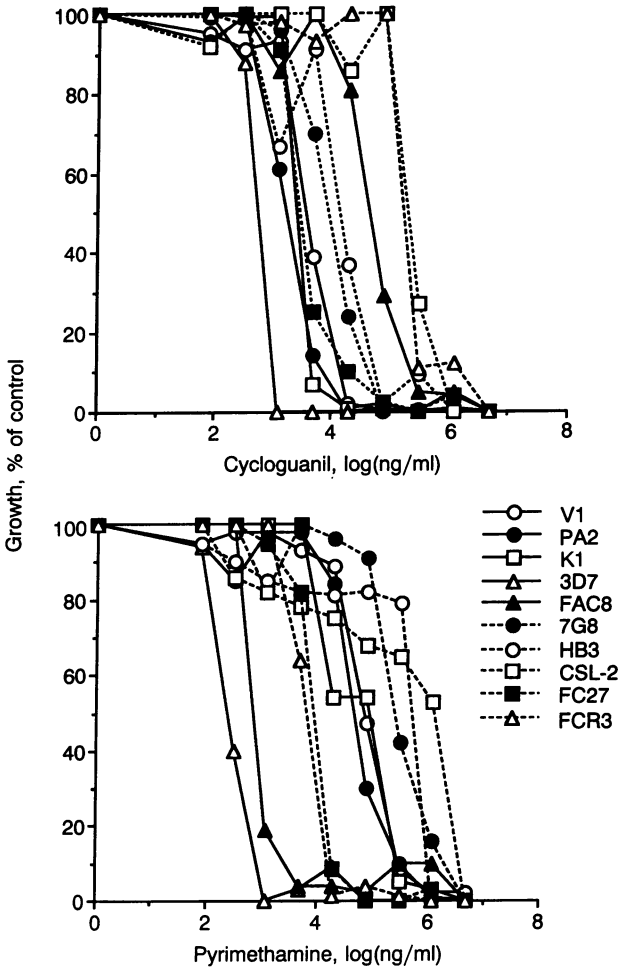

FIG. 1. *In vitro* inhibition of isolates and clones of *P. falciparum* by cycloguanil (*Upper*) and pyrimethamine (*Lower*). The effect of the various concentrations of the drugs on the growth of the parasites was determined by assaying the percent parasitemia after 4 days of culture in the presence of the drug.

Sequence of the DHFR Portion of DHFR-TS. Products from the polymerase chain reactions of the DHFR portion of the

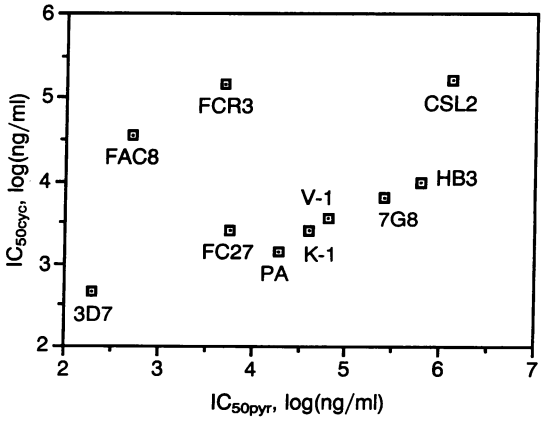

FIG. 2. Comparison of the IC_{50} of isolates to pyrimethamine and cycloguanil. The IC_{50} values were calculated as the concentration of drug that resulted in 50% growth as compared with parasite growth in the absence of drug.

Table 1. Summary of the positions that are different in each isolate and the IC_{50cyc} and IC_{50pyr} of isolates

Isolate	IC_{50pyr}, μg/ml	IC_{50cyc}, μg/ml	Residues*				
			16	51	59	108	164
3D7	0.12	0.45	Ala	Asn	Cys	Ser	Ile
FC27	5.6	2.8	Ala	Asn	Cys	Ser	Ile
FAC8	0.5	35	Val	Asn	Cys	Thr	Ile
FCR3	5.0	160	Val	Asn	Cys	Thr	Ile
HB3	631	10	Ala	Asn	Cys	Asn	Ile
K1	39	2.8	Ala	Asn	Arg	Asn	Ile
V1	63	3.5	Ala	Asn	Arg	Asn	Ile
PA2	20	1.6	Ala	Asn	Cys	Asn	Ile
7G8	251	6.3	Ala	Ile	Cys	Asn	Ile
Csl-2	1400	180	Ala	Asn	Arg	Asn	Leu

*The amino acid positions refer to those in Cowman *et al.* (10).

bifunctional DHFR-TS gene were cloned into M13, and for each isolate four clones were sequenced in both orientations. The amino acid changes that were identified are presented in Table 1 along with the sensitivity status of the isolates. The TS portion of the gene remains unchanged in all isolates in which it has been sequenced.

Interestingly, all of the differences are in the third codon and result in changed amino acids. There are differences in five positions in the gene, and there is a correlation between the position of mutation and the IC_{50} of that isolate to either pyrimethamine or cycloguanil. The two isolates with low IC_{50pyr} and high IC_{50cyc} (FCR3 and FAC8) have a valine at position 16 instead of alanine and a threonine at position 108 in place of serine. FAC8 is a subclone of ITG2.F6, and the DHFR gene of this isolate had been sequenced by Peterson *et al.* (11), who reported an alanine at position 16; however, they have resequenced the DHFR gene from this isolate and confirmed the presence of a valine at this position (24), in agreement with our results. Position 108 is also changed in all pyrimethamine-resistant isolates, but to an asparagine. Other pyrimethamine-resistant isolates have in addition the cysteine at position 59 changed to an arginine. Isolate 7G8 has a unique change—namely, residue 51 is changed from an asparagine to an isoleucine. In addition 7G8 has the position 108 change that is usually associated with pyrimethamine resistance. Csl-2 is the only parasite that we have found (with the possible exception of HB3) that is highly resistant to both cycloguanil and pyrimethamine. As well as the usual mutations at position 59 and 108, it also has an isoleucine at position 164 changed to a leucine. As can be seen by comparing the number of mutations in a given isolate and its IC_{50pyr}, there seems to be a correlation between degree of resistance and extent of mutations in the DHFR gene.

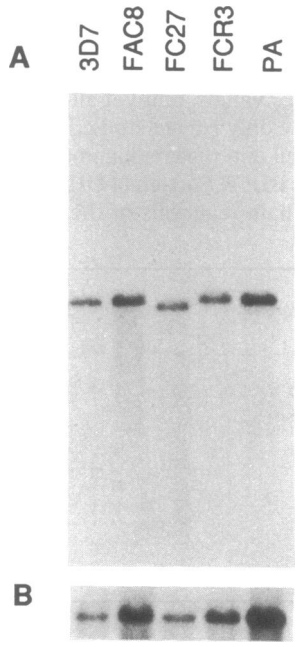

FIG. 3. Each isolate has only one copy of the DHFR-TS gene. *Xba* I-digested genomic DNA from isolates 3D7, FAC8, FC27, FCR3, and PA2 was hybridized with an *Eco*RI fragment covering the DHFR portion of the DHFR-TS gene (*A*). Loading was checked by hybridizing the same filter to the known single-copy gene *Hsp-70* (*B*).

DHFR Gene Is Present as a Single Copy Gene in All Isolates. It has been shown that a number of the isolates discussed have only one copy of the DHFR-TS gene (10, 11). To complete this analysis for the isolates discussed in this paper, DNA from the parasites were digested with *Xba* I, separated by agarose gel electrophoresis, and blotted onto nitrocellulose. This was hybridized to an *Eco*RI fragment from the 5′ end of the cloned DHFR-TS gene. As can be seen in Fig. 3, all tracks hybridize to the same degree as compared to a control probe (Hsp70), indicating that there are the same number of copies per genome in each of the isolates. As 3D7 has been shown to contain only one copy of the gene, then the same must also be true of the others. Therefore, gene amplification is not the explanation for the differing drug sensitivities of the isolates.

DISCUSSION

Dihydrofolate antagonists play a major part in the pharmocopeia and are used against an array of organisms ranging from tumor cells to parasites and bacteria. They are effective because the DHFR of many organisms differ from that of their host and therefore are selective targets for certain drugs. However, as is the case with many drugs, resistance to these DHFR inhibitors develops in the target organism. Possible mechanisms of resistance to these compounds include decreased permeability of the organism to the drug, increased expulsion of the drug from the cell, and modification of the drug leading to its inactivation. Changes in the DHFR enzyme and increased expression of the enzyme also serve to overcome the effect of the drug.

We present here the IC_{50} values of pyrimethamine and cycloguanil for 10 different isolates of *P. falciparum* and the sequence and copy number of the DHFR gene in each isolate. We have shown that all combinations of resistances between these two drugs are possible—i.e., increased IC_{50pyr} but low IC_{50cyc} and vice versa as well as an increased IC_{50} for both drugs.

Sequence analysis of the DHFR gene from these 10 isolates shows no silent mutations. The explanation for this may lie in the codon usage employed by *P. falciparum*, which is heavily biased towards A+T-containing codons (20); hence, the possibilities of silent mutations are more limited. A similar observation was made after a study of the sequences of DHFR genes from a further six isolates of *P. falciparum* (11).

The mutations that lead to pyrimethamine resistance have already been well documented, and our data on several more isolates strengthen the importance of the asparagine at position 108 being responsible for pyrimethamine resistance and that extra changes at positions 51 and 59 further increase the IC_{50pyr}.

The two isolates that have elevated IC_{50cyc} values only (FAC8 and FCR3) have a difference at residue 108 as well. This change results in a threonine at this position, distinguishing it from the change leading to pyrimethamine resistance. Both FAC8 and FCR3 also have a change at position 16 but lack the change at position 59. No other DHFR gene from any other parasite sequenced (10, 11) has these changes,

which suggests therefore that these changes in FAC8 and FCR3 may be important in the development of cycloguanil resistance. In a similar study, Peterson *et al.* (24) have reached identical conclusions to those presented here.

Csl-2, which is highly resistant to both drugs, has the usual mutations associated with pyrimethamine resistance and a change at position 164. Therefore, it is possible that this accumulation of changes results in its also being cycloguanil resistant. Therefore, accumulation of changes in the DHFR gene may lead to either increasing IC_{50pyr} values and/or cross resistance to cycloguanil.

It is also of interest that it is residue 108 of the DHFR gene that is altered in both pyrimethamine- and cycloguanil-resistant parasites. This residue is an important residue in the active site of *Lactobacillus casei*, as structural studies have shown that it is part of an α-helix that interacts with methotrexate (21). As any change to the enzyme is likely to result from a tradeoff between the ability of the enzyme to avoid inhibition by these drugs and the continued efficiency of its enzymatic function, there are likely to be few changes that would lead to a selective growth advantage in the presence of drug pressure but not cause too much of a disadvantage under normal growth conditions. Enzymatic studies on the DHFR enzyme from isolate 7G8 and the parent of FAC8 (ITG2F6) have demonstrated that the K_m values for DHFR from both isolates were virtually identical, and yet there was a large difference in the enzymatic inhibition constant for pyrimethamine, as would be expected from their *in vitro* sensitivities to pyrimethamine (22). A similar result was reported with clones 3D7, HB3, and 7G8 (23): the enzymatic activity of DHFR among the isolates was similar, but the concentration of pyrimethamine required to inhibit the enzymatic activity was much higher for HB3 and 7G8. This implies that the change at position 108 has had little effect on the enzymatic activity of the enzyme while greatly affecting the ability of pyrimethamine to bind the enzyme.

It is also evident that there is some other factor(s) operating, as there is a correlation between resistance to the two drugs. Excluding isolates FAC8, FCR3, and Csl-2, the increase in IC_{50pyr} is mirrored by a smaller but nonetheless real increase in IC_{50cyc}. As these increases in IC_{50} are not related to changes in the DHFR gene, there are likely to be other factors outside the DHFR gene that exert some influence on the susceptibility of the parasite to the DHFR antagonists. These factors may also play a part in the different IC_{50pyr} values of parasite lines having identical DHFR gene sequences, for example HB3 and PA2. This agrees with the observation and conclusion reached by Watkins *et al.* (8). Therefore, this work has implicated specific changes in the DHFR gene for both cycloguanil and pyrimethamine resistance.

The identification of mutually exclusive point mutations in the DHFR gene encoding resistance to the two most widely used DHFR inhibitors and the observation that multiple mutations are required for resistance to both drugs suggests an innovative approach to the employment of these agents. The combined use of proguanil and pyrimethamine, especially in areas where resistance is low to either drug would decrease the frequency of resistance to either drug and therefore prolong the effective lifetime of both drugs.

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1. Scott, H. V., Rieckmann, K. H. & O'Sullivan, W. J. (1987) *Trans Roy. Soc. Trop. Med. Hyg.* **81,** 715–721.
2. Peters, W. (1984) in *Antimalarial Drugs II*, eds. Peters, W. & Richards, W. H. G. (Springer, Berlin), pp. 225–236.
3. Armstrong, V. L. & Smith, C. C. (1974) *Toxicol. Appl. Pharmacol.* **29,** 90–98.
4. Ferone, R., Burchall, J. J. & Hitchings, G. H. (1969) *Mol. Pharmacol.* **5,** 49–59.
5. Charles, L. (1961) *Bull. W.H.O.* **24,** 457–463.
6. Schapira, A. (1984) *Trans. Roy. Soc. Trop. Med. Hyg.* **84,** 359–362.
7. Watkins, W. M., Sixsmith, D. G. & Chulay, J. D. (1984) *Ann. Trop. Med. Parasitol.* **78,** 273–278.
8. Watkins, W. M., Howells, R. E., Brandling-Bennett, A. D. & Koech, D. K. (1987) *Am. J. Trop. Med. Hyg.* **37,** 445–451.
9. Sixsmith, D. G., Watkins, W. M., Chulay, J. D. & Spencer, H. C. (1984) *Am. J. Trop. Med. Hyg.* **33,** 772–776.
10. Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. M. & Foote, S. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9109–9113.
11. Peterson, D., Walliker, D. & Wellems, T. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9114–9118.
12. Zolg, J. W., Plitt, J. R., Chen, G.-X. & Palmer, S. (1989) *Mol. Biochem. Parasitol.* **36,** 253–262.
13. Snewin, V. A., England, S. M., Sims, P. F. G. & Hyde, J. E. (1989) *Gene* **76,** 41–52.
14. Cowman, A. F. & Lew, A. F. (1989) *Mol. Cell Biol.* **9,** 5182–5188.
15. Inselburg, J., Bzik, D. J. & Horii T. (1987) *Mol. Biochem. Parasitol.* **26,** 121–134.
16. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
17. Foote, S. J., Thompson, J., Cowman, A. F. & Kemp, D. J. (1989) *Cell* **57,** 921–930.
18. Saiki, R. K., Gelfand, D. H., Stoffel, S. S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Weber, J. L. (1987) *Gene* **52,** 103–109.
21. Blakly, R. L. (1984) in *Folates and Pteridines*, eds. Blakly, R. L. & Benkovic, S. J. (Wiley, New York), Vol. 1, pp. 346–396.
22. McCutchan, T. F., Welsh, J. A., Dame, J. B., Quakyi, I. A., Graves, P. M., Drake, J. C. & Allegra, C. J. (1984) *Antimicrob. Agents Chemother.* **26,** 656–659.
23. Chen, G., Mueller, C., Wendlinger, M. & Zolg, J. L. (1987) *Mol. Pharmacol.* **31,** 430–437.
24. Peterson, D. S., Milhous, W. K. & Wellems, T. E. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3018–3022.