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Geographically Restricted Heterogeneity of the *Plasmodium falciparum* Circumsporozoite Protein: Relevance for Vaccine Development

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The design of a subunit vaccine against the malaria parasite relies on the epitopes recognized by T cells being identified and polymorphisms therein being defined. Here we present the analysis of a 354-bp fragment of the circumsporozoite (CS) protein encompassing defined proliferative and cytotoxic T-cell recognition regions. We reveal that the polymorphism of CS protein T-cell sites appears to be very limited among *Plasmodium falciparum* isolates prevalent in certain geographical regions, in particular Papua New Guinea. Furthermore, the more extensive polymorphism noted in other areas appears to be restricted. Although the extent of variation observed for the T-cell recognition domains suggests that any vaccine designed to stimulate this form of immunity will need to be polyvalent, this variation appears to be finite and the combination of peptides necessary for inclusion in a polyvalent vaccine may be small. If ways to increase immune responsiveness can be found, then a vaccine designed to stimulate CS protein-specific T-cell activity may prevent malaria.

The sporozoite coat protein, the circumsporozoite (CS) protein, is the most advanced candidate vaccine antigen for the sporozoite stage in the life cycle of the malaria parasite. Recent studies have suggested that the inability of humans to develop good immunity to *Plasmodium falciparum* sporozoites is related to polymorphisms within the T-cell antigenic sites of the CS protein (11, 12, 17). Although the *CS* gene is largely invariant, sequence variation has been noted to occur mainly in the immunodominant T-cell epitopic domains (9, 11, 14, 15, 17, 22, 26, 38). It has been observed that this variation is the result of nonsynonymous changes at the nucleotide level (2, 10, 24, 37). Thus, all the nucleotide variation within the *CS* gene results in coding changes. This observation has led to the suggestion that pressure at the protein level, possibly immunological pressure, has selected for variation. The implications of the extent to which sequence variations might affect the immune response to the CS protein and to sporozoites are not encouraging in terms of malaria vaccine development if T-cell recognition of the CS protein does prove to be restricted to variant epitopes.

Here we investigate further the occurrence of polymorphism in the T-cell epitope-encoding (3′ end) region of the *CS* gene and define the extent and degree of variation of described T-cell regions immunodominant for proliferative T cells (regions often referred to as Th2R [16] and Th3R [12] and occurring in the carboxy-terminal third of the protein, with Th2R occurring amino terminal to Th3R), as well as for murine and human cytotoxic T cells (15, 22, 26). We report the analysis of the sequence variation of a 354-bp fragment encompassing the immunodominant T-cell domains of the CS protein (nucleotides 886 to 1239) (amino acids 296 to 412 of the 7G8 clone [7]) of 38 previously unpublished clones, lines, or laboratory crosses of *P. falciparum* from geographically diverse regions. We establish that although the variation is restricted primarily to the immunodominant T-cell recognition regions, it appears to be limited. Furthermore, marked conservation of the *CS* gene in certain geographical areas is noted.

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MATERIALS AND METHODS

Parasite DNA. A total of 41 clones, cloned lines, or laboratory crosses of *P. falciparum* (38 previously unpublished) were studied, 22 of which came from widely separated areas in Papua New Guinea. The *P. falciparum* isolates used in this study are listed in Table 1. Parasite lines FCQ22 to FCQ41 were established from cells collected in 1980 from outpatients treated the Madang General Hospital, Madang, Papua New Guinea (6). FCQ49 to FCQ79 were established from cells collected over the period from 1981 to 1989 from patients in Brisbane, Australia, who were infected during travels in Papua New Guinea and the Solomon Islands. MAD20 and MAD71 were kindly supplied by David Walliker from isolates established in Madang in 1984 (27). Parasite lines 005 to 051 were established from cells collected from the village of Buksak, Madang Province, in August 1990. MS2, AE7, and AE28 were kindly provided by Savanat Tharavanij. All isolates were maintained in continuous in vitro culture, as described by Limpaiboon et al. (23), for a period of at least 1 month. Parasite DNA was kindly provided by Temduang Limpaiboon.

Oligonucleotides. The oligonucleotide primers used for polymerase chain reaction amplification of the *P. falciparum* *CS* gene were based on the sequence of the 7G8 clone (7) and synthesized on an Applied Biosystems nucleotide synthesizer. The sequence of the sense oligonucleotide (26-mer: 5′-GAA TTC CCT AAT AAA AAC AAT CAA GG-3′) corresponded exactly to the region immediately 3′ to the central NANP repeats (nucleotides 871 to 885). The sequence of the antisense oligonucleotide (27-mer: 5′-A GAA TTC ACT CAA ACT AAG ATG TGT TC-3′) corresponded exactly to the region five bases 3′ to the end of the coding region (nucleotides 1245 to 1264).

Phosphorylation of oligonucleotides. Sense and antisense oligonucleotide primers (1 to 15 μg) were phosphorylated by

TABLE 1. Origins of *P. falciparum* DNA

DNA Source	Country	Details	Reference
D10	Papua New Guinea	Cloned from FCQ27	1
FCQ22	Papua New Guinea	Line	6
FCQ27	Papua New Guinea	Line	6
FCQ30	Papua New Guinea	Line	6
FCQ31	Papua New Guinea	Line	6
FCQ33	Papua New Guinea	Line	6
FCQ41	Papua New Guinea	Line	6
FCQ46	Papua New Guinea	Line	
FCQ49	Papua New Guinea	Line	
FCQ50	Papua New Guinea	Line	
FCQ64	Papua New Guinea	Line	
FCQ65	Papua New Guinea	Line	
FCQ79	Papua New Guinea	Line	
MAD20	Papua New Guinea	Line	27
MAD71	Papua New Guinea	Line	27
005	Papua New Guinea	Line	
008	Papua New Guinea	Line	
009	Papua New Guinea	Line	
014	Papua New Guinea	Line	
018	Papua New Guinea	Line	
021	Papua New Guinea	Line	
051	Papua New Guinea	Line	
MS2	Thailand	Line	
AE7	Thailand	Line	
AE28	Thailand	Line	
T9-94	Thailand	Cloned from T9	34
MCK$^+$	Malaysia	Monkey-adapted line	8
3D7	The Netherlands	Cloned from NF54 line	35
NF54	The Netherlands		30
W/L	Nigeria	Line	21
PALO ALTO	Uganda	Monkey-adapted line	5, 20
HB3	Honduras	Cloned from H1	35
7G8	Brazil	Cloned from IMTM22	3
ItG2	Brazil	Cloned from Ituxi 084	18
X5	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35
X10	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35
X11	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35
XP8	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35
XP9	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35
XP12	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35
XP13	Honduras and The Netherlands	Progeny of cross of HB3 and 3D7	35

using T4 polynucleotide kinase (20 U) (New England Biolabs) at 37°C for 30 min, extracted once with phenol-chloroform, precipitated with sodium acetate-ethanol, and resuspended in distilled water.

Polymerase chain reaction. Two polymerase chain reactions per template were carried out, one with the sense strand phosphorylated (kF/oligo R) and the other with the antisense strand phosphorylated (oligo F/kR). Amplifications consisted of 28 cycles with 50 to 100 ng of DNA, 20 pmol of each primer, 0.2 mM each deoxynucleotide (Pharmacia), and 1 U of *Taq* polymerase (Perkin-Elmer Cetus). Reaction buffer was as described by Perkin-Elmer Cetus, and reaction conditions were as follows: 94°C, 30 s; 55°C, 30 s; and 72°C, 30 s, all with a Hybaid thermal cycler. To prevent contamination, DNA template was always added by using a positive displacement pipette. Also, stock mixes of the primers were made, and DNA was added only at the last moment. Negative controls were included, some without template, some without enzyme, some with mismatched primers, and some with other templates. These did not amplify DNA.

Lambda exonuclease digestion. Single-stranded templates for sequencing were obtained by digesting amplified polymerase chain reaction products (1 to 2 μg of DNA) with Lambda exonuclease (2 U) (Bethesda Research Laboratories, Inc.) at 37°C for 30 min.

Purification of single-stranded DNA templates for sequencing. Single-stranded DNA templates were prepared for sequencing by either phenol-chloroform extraction (four times) followed by ammonium acetate-ethanol precipitation or glass-milk purification (Prep-A-Gene DNA purification kit; Bio-Rad). DNA templates were reconstituted in approximately 7 μl of distilled water and used in sequencing reactions.

DNA sequencing. DNA templates were sequenced by using the Sequenase version 2.0 kit (U.S. Biochemicals) under conditions described by the manufacturer.

S1 nuclease digestion. Sequences of specific lines shown to be identical by direct sequencing were verified by digestion with S1 nuclease. Equal quantities of each of two DNA templates were mixed, denatured at 94°C for 10 min, annealed at 50°C for 2 h, cooled to room temperature, and digested with S1 nuclease (1 U) (Pharmacia) at room temperature for 60 min. The digestion was stopped by incubation for 10 min at 65°C. A control sample of each template alone was similarly digested. Digested templates were elec-

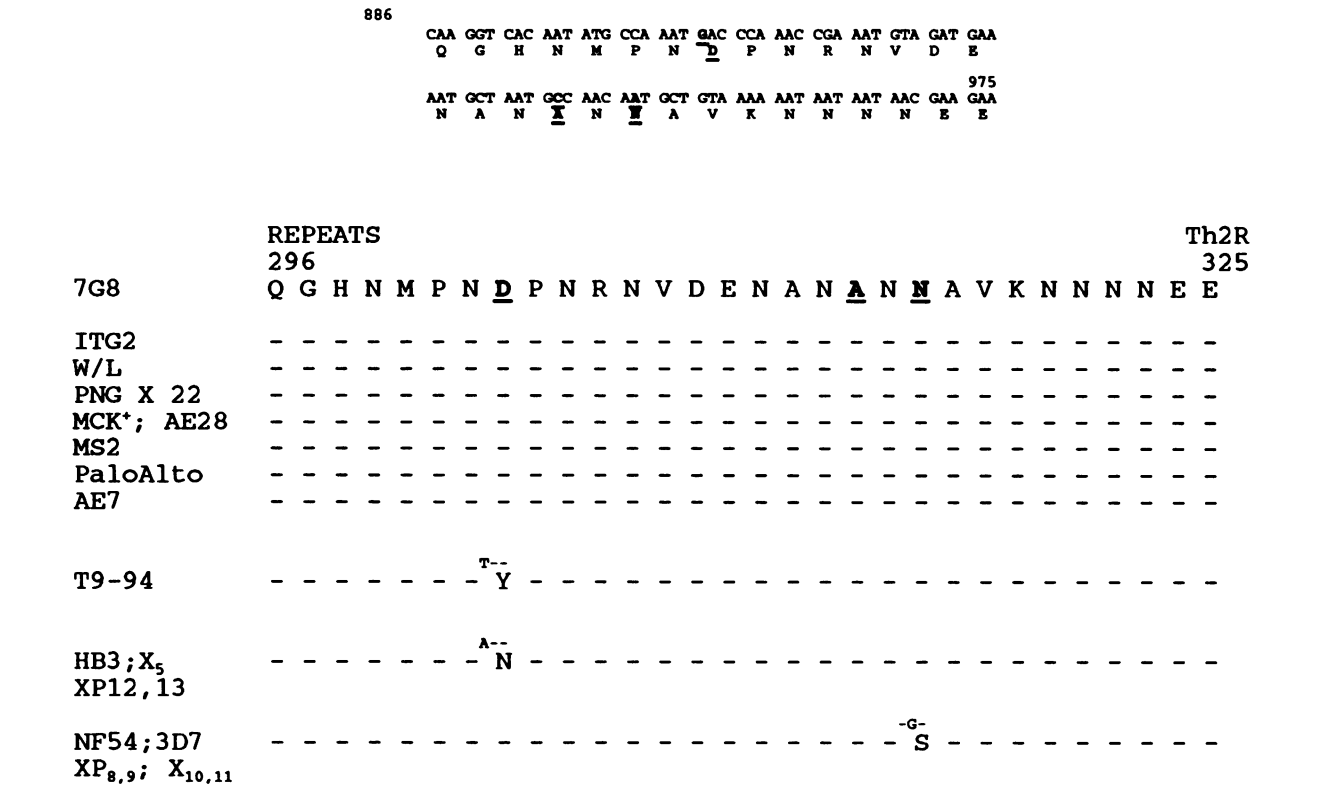

FIG. 1. Known variants within the 3′ non-Th2R-Th3 domain. Nucleotide and deduced amino acid numbering is according to that of the 7G8 sequence (7). Identity with the 7G8 sequence is indicated by dashes. Nucleotide mutations are shown above the position at which the corresponding amino acid substitution is first listed. Underlined, boldface residues are those shown previously and here to be polymorphic. Residue A (314) was not shown to be variant in any of the clones or lines studied here. Geographical origins and sources of the isolates are as listed in Table 1.

trophoresed on a 4% NuSieve gel and stained with ethidium bromide. Any nucleotide mismatches were indicated by the presence of additional faster-migrating DNA fragments along with a concomitant decrease in the intensity of the band of the double-stranded DNA.

Nucleotide sequence accession numbers. Nucleotide sequences not previously published have been submitted to the GenBank data base, with accession numbers M77202, M77203, M77204, and M77205.

RESULTS

Prior to this study, a number of *P. falciparum CS* genes had been sequenced commencing with the original sequence, that for 7G8 (4, 7, 10, 13, 24, 25, 37). We have sequenced an additional 38 clones and lines representing parasites from various countries, in particular Papua New Guinea, from which no previous epidemiological data were available, as well as three previously sequenced clones and lines.

We observed that nucleotide substitutions occurred predominantly in the first codon position and less frequently in the second codon position. Deduced amino acid sequence comparisons revealed that polymorphisms were always nonsynonymous. This had previously been shown in the sequences of other *CS* genes (2, 4, 10, 24, 37).

Two variant amino acid positions were noted in the 30-amino-acid region between the invariant repeats (NANP) and the immunodominant T-cell domain Th2R (Fig. 1). Variation at amino acid position 303 D (7G8) has not previously been reported. Two novel substitutions were noted at this position, Y (T9-94) and N (HB3 and progeny). No variation was observed in the 15-amino-acid region between the Th2R and Th3R domains, known as region II (7). Furthermore, no variation was observed in the 31-amino-acid region between Th3R and the carboxyl-terminal end of the protein (amino acids 381 to 412 of the 7G8 sequence).

Seven variant amino acid positions were observed in the Th2R region relative to the 7G8 sequence, and four variant amino acid positions were observed in the Th3R region. All these positions have been previously noted to vary in other lines (Fig. 2 and 3). Furthermore, variation at positions 367, 369, 370, 372, 374, 376, and 377 (within the Th3R domain) represent polymorphism within the human (positions 351 to 395) (15, 26) and murine (positions 368 to 390) (22) cytotoxic T-lymphocyte (CTL) epitopes.

A striking feature revealed by our analysis was that the sequences of all 22 Papua New Guinea isolates (PNG isolates) were identical. This was confirmed by S1 nuclease digestion experiments. The apparent conservation contrasts markedly with the extensive polymorphism of *P. falciparum* isolates from other countries. DNA fingerprinting (with a non-CS probe) could show that most of the PNG isolates were distinct (see below).

Analysis of the progeny of a laboratory cross between the HB3 clone and the 3D7 clone (35) revealed that the XP12, XP13, and X5 progeny inherited the *CS* gene sequence of the

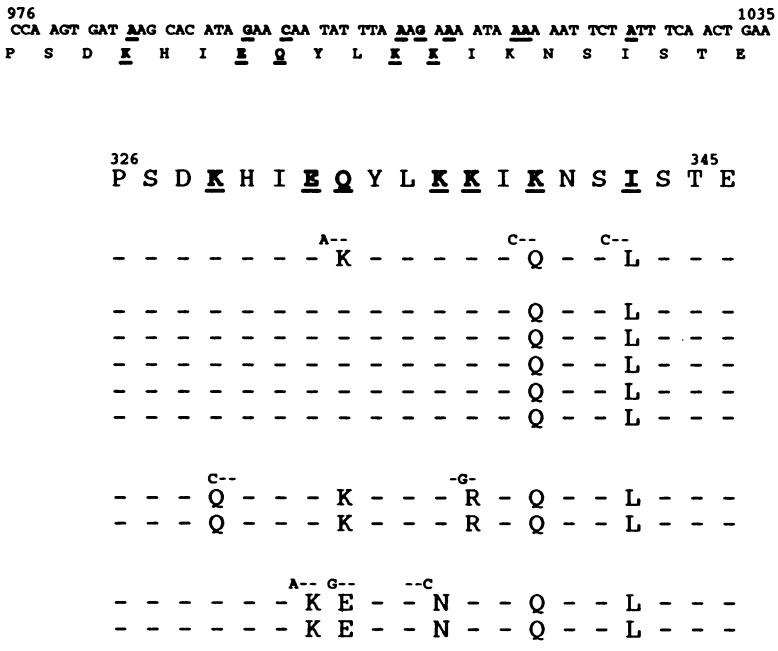

FIG. 2. Th2R variants in CS proteins of laboratory and field isolates of *P. falciparum*. Nucleotide and deduced amino acid numbering and origins and sources of the isolates are as described in the legend to Fig. 1.

HB3 parent, while the XP8, XP9, X10, and X11 progeny inherited the sequence of the 3D7 parent.

Reanalyzed sequences of previously published clones and lines (7G8 [7], NF54 [4], and HB3 [37]) were identical to the published sequences. The sequence of the D10 clone reported by Yoshida et al. (37) was, however, noted to differ at three nucleotide positions (positions 1100, 1105, and 1114) from the sequence of all PNG clones and lines, including D10, analyzed in this study. D10 has been amplified and sequenced repeatedly by us, with the same resultant sequence.

For each line and clone analyzed, deduced amino acid sequences constituting each of the immunodominant T-cell domains were evaluated. Five sequence patterns were found in the Th2R domain (Fig. 2), all of which have been previously described. Six sequence patterns were noted in the

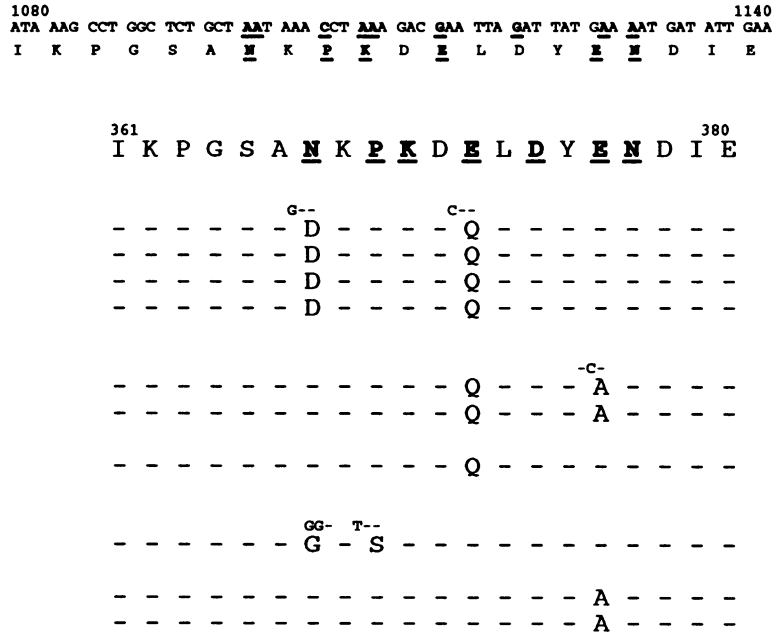

FIG. 3. Th3R variants in CS proteins of laboratory and field isolates of *P. falciparum*. Nucleotide and deduced amino acid numbering and origins and sources of the isolates are as described in the legend to Fig. 1. Residues K (370), D (374), and N (377) were not shown to be variant in any of the clones or lines studied here.

Th3R and CTL domains (Fig. 3), of which two had not previously been described (sequence patterns 3 and 4). In several instances, the *CS* genes of isolates which encoded the same Th2R sequence differed in their corresponding Th3R and CTL sequences, and vice versa, giving a larger repertoire of Th2R-Th3R variant combinations. For the 41 genes sequenced in this study, eight sequence patterns were found for the region encompassing the Th2R, Th3R, and CTL domains, three of which had not previously been described. Thus, although the individual amino acid positions shown to be variant here have been previously reported to vary, we have observed novel sequences within the Th3R region.

DISCUSSION

In this study, we have analyzed partial sequences of 41 independent *P. falciparum CS* genes. The parasites came from different countries, in particular Papua New Guinea, from which there have been no previous epidemiological data. The region of the gene analyzed was chosen because it represents immunodominant T-cell regions for both $CD4^+$ (9, 11, 14, 17, 38) and $CD8^+$ (15, 22, 26) T cells, both of which are strongly implicated in immunity to sporozoites and vaccine development (Fig. 4). It has been established that the majority of the sequence variation in the *CS* gene is restricted to these immunodominant T-cell domains. Also, the region of the *Plasmodium berghei* and *Plasmodium yoelii* CS proteins recognized by CS-specific CTL (31, 32, 36) is homologous to this region of the *P. falciparum* CS protein (Fig. 4). Variation of the CS protein has been shown to preclude cross-reactivity in the case of murine CD4 T cells (11, 12). Although it makes intuitive sense that variation will also preclude cross-reactivity for human T cells, this has not yet been addressed experimentally.

There appear, however, to be constraints in the ability of the parasite to change. Examination of the actual variation shows that it preferentially occurred at certain positions and that certain residue substitutions were much more common than others (Fig. 2 and 3). In any given sequence pattern, substitutions occurred at no more than five positions in Th2R and two positions in Th3R (relative to the 7G8 sequence) and there were no more than three different residues at each of the varying positions.

Assimilation of observed variation with that reported in the literature revealed 119 sequences in the immunodominant Th2R domain and 98 sequences in the immunodominant Th3R domain. Geographical regions represented included Thailand, Malaysia, Brazil, Honduras, The Netherlands, Africa, and Papua New Guinea. Twelve patterns were noted among the 119 sequences in the Th2R domain, and nine patterns were noted among the 98 sequences in the Th3R domain and in the human and murine CTL domains. In total, 21 different sequence patterns were representative of the entire spectrum of variation of the immunodominant T-cell region of the *P. falciparum* CS protein (Fig. 4). The sequences illustrated in Fig. 4 can be divided into three apparent groupings: one predominantly Asian, one predominantly African, and one other.

Lockyer et al. (24), in their analysis of the sequence variation of the *P. falciparum CS* gene, found considerable polymorphism in the Th2R and Th3R domains of isolates from a single small endemic area (5-mile radius) of The Gambia. They concluded that the degree of sequence variation in parasites present at the same time and place was so extensive as to preclude the use of all variants in a polyvalent subunit vaccine: the total numbers of potential variants in Th2R and Th3R were estimated to be 432 and 128, respectively, assuming that any combination of substitutions could occur. This conclusion is not, however, supported by the analysis presented here. Our data suggest that the polymorphism of CS protein T-cell sites is limited among *P. falciparum* isolates prevalent in certain geographical regions, notably Papua New Guinea. Furthermore, although comparison of *P. falciparum* isolates from widely separated geographical regions (Asia and Africa) reveals more extensive polymorphism, this variation appears to be limited. Characteristic DNA fingerprint patterns of many of the parasites sequenced in this study have been previously reported by Limpaiboon et al. (23), who used the 7H8/6 probe. With the exception of some of the PNG isolate DNA, distinct patterns were noted for all parasites. FCQ31 and FCQ49 were similar to FCQ22; and FCQ30, FCQ50, FCQ64, and MAD20 were similar to FCQ27. Four of the PNG parasites (FCQ46, FCQ65, FCQ79, and MAD71) have not been fingerprinted. Importantly, however, there were at least 12 distinct fingerprints among 18 of the PNG isolates sequenced (23, 32a).

A situation similar to that reported here for Papua New Guinea has been noted in Brazil. Data presented by Yoshida et al. (37) revealed that of 24 field isolates of *P. falciparum* which came from widely separated areas from the Amazon region in Brazil, 19 displayed a sequence identical to that of the Brazilian sequence 7G8. A second sequence was represented by four of the isolates and differed at five nucleotide positions in the Th2R domain. A third Brazilian sequence was noted for one clone (ItG2) analyzed in this study and was the same as that reported by Lockyer et al. (24) for a similar clone (ItG1 G2). The Th3R and CTL domains were both found to be invariant in the Brazilian isolates, as noted for the PNG isolates. Furthermore, the sequence of the PNG isolates was not the same as any of the three Brazilian sequences but was identical to the sequence of Wellcome/Liverpool (Africa) and T9-94 (Thailand).

The proposed mechanism of genetic variation in the *CS* gene is that by which any organism accumulates point mutation (28). Recombination, as the origin of variation, is thought to be unlikely because generalized recombination extensive enough to produce consistent point mutations in a single position is undocumented and would be thought to produce highly deleterious genetic effects. That genetic recombination is not the origin of variation in the *CS* gene is supported to a small degree by data presented in this study. Analysis of the seven progeny of a laboratory cross of the HB3 isolate and the 3D7 clone revealed that the *CS* gene sequence of each of the progeny was identical to that of one or the other of the parent sequences.

Like the CS protein, the *P. falciparum* major merozoite surface antigen, MSA1 (a potential candidate for an asexual-blood-stage malaria vaccine), also shows extensive antigenic diversity (27). The variation in the *MSA1* gene appears, however, to be principally dimorphic rather than polymorphic at the genetic level (5, 29). Genetic recombination, restricted to the 5′ end of the *MSA1* gene, is thought to be the mechanism responsible for creating new genotypes (29, 33).

The extent of variation observed for the CS protein CTL and helper T-cell recognition domains suggests that any vaccine designed to stimulate this form of immunity will need to be polyvalent. The ideal vaccine would contain such CTL epitopes combined with $CD4^+$ T-helper epitopes from the same protein, as well as the B-cell epitope, $(NANP)_n$, for

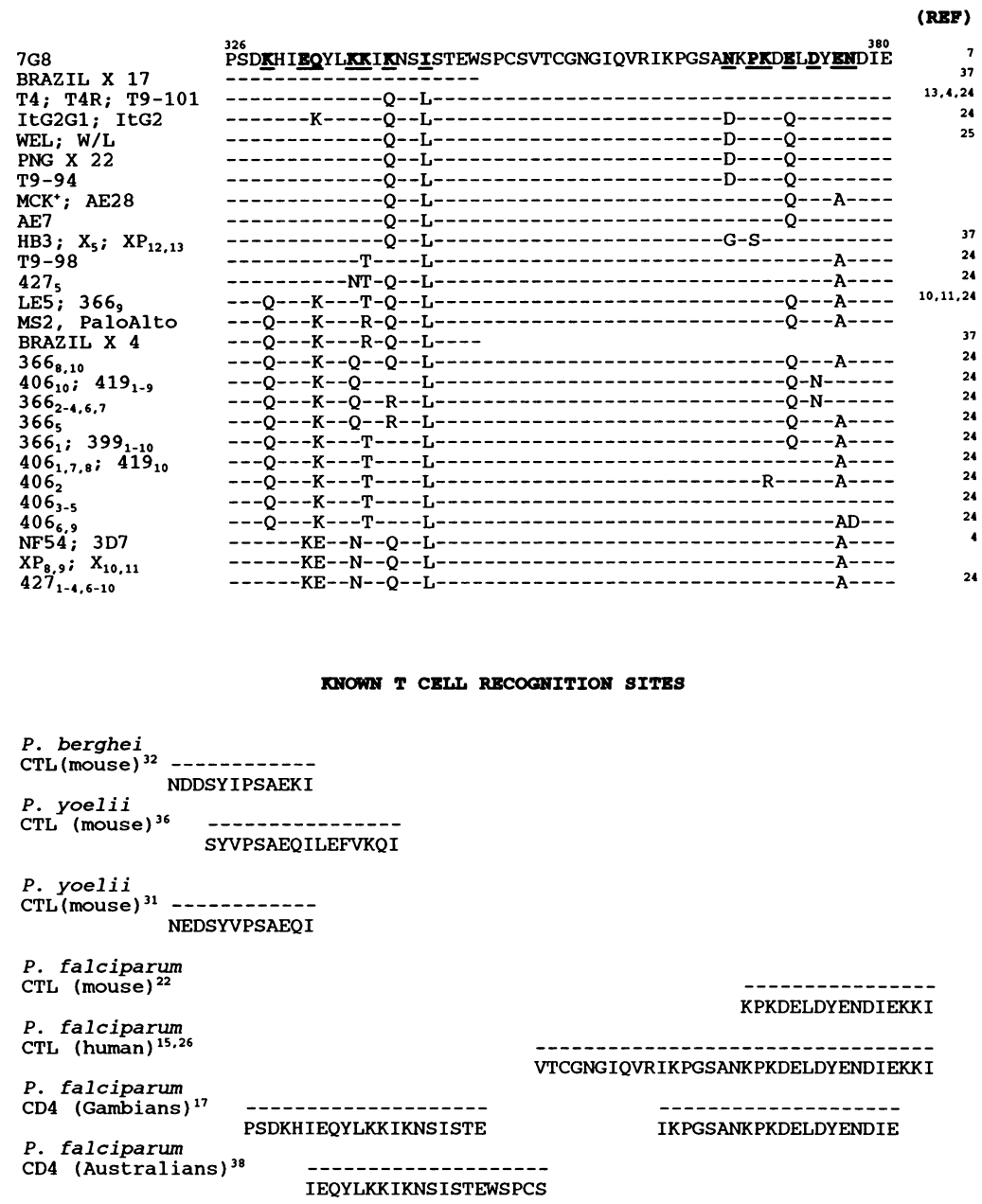

FIG. 4. Polymorphic variants in CS proteins of laboratory and field isolates of *P. falciparum*, including published sequences. Published sequences are referenced (REF) in the right margin. Nucleotide and deduced amino acid numbering and origins and sources of the isolates are as described in the legend to Fig. 1. Locations of known T-cell recognition sites are depicted with respect to the variant residues.

natural boosting following exposure to the parasite. The feasibility of such a vaccine would, however, depend on the degree of polymorphism in these epitopes exhibited by parasite populations: it seems likely that many of the defined sequences within the T-cell domains may not cross-react (11, 12, 19). We have, however, demonstrated that the extent of variation observed for the CTL as well as the $CD4^+$ recognition regions is finite and that the combination of peptides necessary for inclusion in a polyvalent vaccine may be limited. These peptides between them could be expected to be immunogenic for most people and may elicit cross-reactive responses to those parasite strains not represented (9). If ways to increase immune responsiveness can be found, a vaccine designed to stimulate CS protein-specific T-cell activity may prevent malaria.

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