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Limited variation of the 5'cis-control region of the transmission blocking vaccine candidate *Pfs25* amid great genetic diversity of *Plasmodium falciparum* in Cameroon

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Genetic recombination during sexual reproduction within *Plasmodium* sp. contributes to parasite diversity and altered gene expression of certain surface markers. The *pfs25* gene involved in the upset of gametocytogenesis is a candidate antigen in transmission blocking vaccine. This study investigated the polymorphism of Pfs25 within its 5'cis-control region in field isolates from different ecotypes in Cameroon. Symptomatic patients and asymptomatic healthy school children with a positive smear and from different ecozones were included. Parasite DNA was extracted and polymorphisms within *pfs25, cg2-ω, msp-1, msp-2* and *glurp* genes were investigated by PCR-RFLP and DNA sequencing. Putative control elements of the 5'cis control regions of *Pfs25* were identified by PCGENE software and enzymes were selected whose sequences produced or abolished restriction sites by mutations. Malaria infection was mainly caused by *Plasmodium falciparum* with sporadic occurrence of *Plasmodium malariae* and *Plasmodium ovale.* Analysis of the *Pfs25* 5' cis-control region identified only one polymorphism (0.002%) that abolished an *RsaI* restriction site as part of the sequence TTTCTGTAC, located 40 bp downstream of the promoter and found at – 478 bp of the ATG. Analysis of the 5' cis-control sequence of *Pfs25* revealed minimal variation of the promoter region amid great zonal differences in parasite population. Altitudinal differences in parasite populations were not easily discernable.

Key words: *Plasmodium falciparum, Pfs25*, cis-control elements, genetic polymorphism*.

INTRODUCTION

The malaria parasite, *Plasmodium falciparum*, is responsible for the cases of malaria morbidity and mortality and remains a major public health problem in most tropical endemic countries in the world. Pregnant women and children are the most vulnerable. Control measures such as chemotherapy and protection against vectors have failed to reduce the burden of the disease. The genetic diversity of the malaria parasite is an obstacle to the control of the disease with many people harbouring multiple clones of parasites, genetically distinct in many characteristics (Ariey et al., 1999). Although polymorphic genes and microsatellite have been extensively used to characterize *P. falciparum* populations in various eco-

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zones, characteristics which identify individual *Plasmodium* within field isolates, have been difficult to define due to antigenic variation and genetic recombination during sexual reproduction. The diversity of the genotypes found and the frequencies at which they occur suggest that cross-mating between clones is frequent in nature (Babiker et al., 1997). In an attempt to assess this diversity, Wooden et al. (1992) and Ranford-Cartwright et al. (1991) used sequence information from merozoite surface proteins 1 and 2 as well as from genes which encode other surface antigens shown to be conserved in their N- and C-terminals but variant in their middle portions. Oligonucleotide primers to conserved sequences within block 1 and 2 and 1 and 4 respectively for *MSP1* and *MSP2* were made such that the variable regions of both genes are contained within the positions of the PCR primers. These primers were used later by Babiker et al. (1994) to confirm that the heterozygous forms occurring in oocyst were as a result of a mixture of gametocytes taken up by mosquitoes, of parasites belonging to different clones. The frequencies of alleles of the MSP1 and 2 were used to show that the frequencies of alleles found in an individual oocyst are more distinct than those found in blood stream forms with the interpretation that the high level of genetic diversity may be as a result of high intensity of transmission and sexual reproduction among the parasites.

The transmission blocking vaccine candidate *Pfs25* has been in focus for a long time and variations in its expression if they exist would be found in blood forms rather than in insect forms since this molecule is suggested to be important in transmission. Alterations of its expression through genetic recombination may result in the non-propagation of the parasites if these contain modified promoter regions. A single exon of 654 bp encodes the *Pfs25* molecule (Kaslow et al., 1993) and the constancy of the *Pfs25* sequence among the 8 laboratory isolates, even in parasites that do not produce gametes, suggests that DNA mismatch repairs and/or proof reading mechanisms are very active during DNA replication in malaria (Kaslow et al., 1993). Since the coding sequence has been demonstrated to be minimally variable, one might expect that any variation in gene expression, may, therefore reside within the 5' or 3' flanking regions which control gene expression. To study these flanking regions a chicken model for malaria was used to characterize control elements of the *Pgs28* surface antigen for subsequent comparison with *Pfs25* (Dechering et al., 1999; Mbacham et al., 1997, 2001). The *Pgs28* promoter was described to have a bipartite arrangement of its cis-control elements. Although other elements within the 5'region were A/T rich and looked similar to those of *Pfs25*, they did not affect expression considerably when mutated (Mbacham et al., 1997, 2001). The differential activation of the *Pfs16* and *Pfs25* promoters marks developmental switches during sexual

differentiation and demonstrated that the *Pfs16* is activated at the onset of gamecytogenesis while the *Pfs25* promoter is activated during the transition to the mosquito vector (Dechering et al., 1999).

Pfs25 having been suggested as a vaccine candidate for a transmission blocking vaccine required analysis and confirmation of the stability of its control sequences in field isolates to gain a better understanding of promoter functions and stable expression in *Plasmodium* sp. The identification of mutations within promoter region that affects essential gene expression may lead to the consideration of these changes in the design of intervention measures. The objective of this study was therefore to investigate the polymorphism of the 5' cis-çontrol regions of *pfs25* in genetically diverse field isolates from different ecotypes in Cameroon.

MATERIALS AND METHODS

Collection sites

Samples from symptomatic malaria cases were collected from various locations where the human population density in Cameroon is high, in order to provide the greatest variety. Samples were therefore collected from two ecologically distinct sites: Forest (Yaounde, Limbe) and from the savannah regions, (Nkambe and Dschang) of Cameroon. Samples were further collected from upland forest areas (Kumba and Fontem) or from asymptomatic healthy school children below the age of 10 years in the Buea health district schooling and living at different altitudes (400, 700 and 1000 m as above sea level). Samples (n = 659) collected from all age groups between 1999 - 2003 were used for DNA extraction and stored at -80°C prior to analysis.

Sample handling

Informed consent was obtained from patients or parents before blood were collected. All individuals found positive for malaria were treated by the attending physician with quinine. Patients who had been diagnosed as having malaria and selected for the study and who later refused to donate blood were still treated for malaria. Control samples from parasite-free individuals were obtained from individuals attending the hospital for reasons other than malaria such as; broken bones, regular pregnancy visits, eye problems or as companion to patients in the hospital. Asymptomatic cases were collected from school children in the Buea health district following informed consent from their parents. Cases of malaria were diagnosed on the field using the rapid stain (Diffquick®). A second set of slides was stained with giemsa and further confirmed microscopically. Microscopic speciation was performed at the anatomo-pathology unit of the Faculty of Medicine and Biomedical Sciences and the Biotechnology Center of the University of Yaounde I. Parasites and white blood cells were counted per 50 fields, and the parasite density was calculated based on the estimate that 8000 leukocytes are contained in 1 μl of whole blood. Slides were considered negative if no parasites were seen within 100 fields.

Between 2 and 3 ml of blood were drawn by veninpuncture into tubes containing Acid-Citrate-Dextrose buffer ACD (38 mM citric acid, 74 mM sodium citrate, 14 mM dextrose) (Barker et al., 1992; Laserson et al., 1994) modified by the replacement of dextrose

Table 1. Primers used in polymorphism studies.

WFM27	(5'GTTGCTTGTGTAGAACATACGTTCC)
WFM29CM	(5'GATAACGAAAAAAATGAATAAATTGAAG)
WFM42R	(5'GGAAAAGAAACAAACTGTAAAGTTTATTCAT)
WFM43	(5'GTATGTATATCTCACAGAGCTTATAAATGTTGC)
W1 5'cg2-ω	(AAAAAAGATGATAATGATAATCAAAATGACG)
W7 3'cg2-ω	(CAATATACAAAAAAAAAACATCTACATC)
W4 5'cg2-ω	(AGAGGTTGATGAGGATGCCTGGTTTTACTG)
W6 3'cg2-ω	(CTACATCTTGTAACTTTAATCTTAATAACC)

with NaCl 5.6% and the addition of EDTA to a final concentration of 3 mM and renamed ACSE. Blood samples were stored at 4°C for up to a maximum of 2 h while being transported to the laboratory for processing for the extraction of DNA by the Phenol/Chloroform or by saponin lysis method (Laserson et al., 1994). Alternatively blood samples from finger pricks (samples collected from 2002 onward) were placed on Whatman 3 MM filter paper and stored in the presence of silica gel as desiccant. These were extracted by the Chelex method as described by the manufacturers (Biorad).

Primers for expression polymorphism

Oligonucleotides primers were designed for the *pfs25* coding and 5' flanking sequences: WFM42R contained sequences which matched 31 bp downstream of the ATG of *pfs25* coding sequence. WFM29CM, WFM43 and WFM27 were complementary to sequences located respectively at 160, 661 and 633 bp upstream of the ATG. Primers for *MSP1* (O1/O2 and N1/N2) were designed from conserved regions of block 1 and 2, whereas *MSP2* (S2/S3 and S1/S4) primers contained sequences from within the central variable region of block 2 of the merozoite surface antigen gene, *MSP2*. Primers (G4/G5 and G1/G3) were those that enclose variable repeats of the glutamine rich protein gene (*glurp*) (Wooden et al., 1992; Randford-Catwright et al., 1992). Primers for *Pfs25* and *Cg2* polymorphisms were designed by Dr. Mbacham (Table 1).

PCR amplification

PCR buffers contained in final concentrations, 70 mM Tris pH8.8, 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, 100 g/ml of BSA. Deoxynucleotides (dNTPs) were from the Sigma Company and reconstituted in nuclease free water (Gibco). DNA extracts (3 µl) were used to perform amplification reactions using Amplitaq (Perkin-Elmer Cetus, Norwalk, CT). Reaction conditions for the *pfs25* gene were set at 94°C for 4 min and 40 cycles of 94°C for 1 min, 60°C for 2 minutes and 72°C for 90 s. At the end of 40 cycles, partially extended products were subjected to one round of 94°C for 1 min, 60°C for 3 min and extended at 72°C for 5 min. Products were stored at -20°C until used in experiments. Conditions for the *cg2*-ω outer primers (W1 and W7) were 30 cycles of 94°C for 25 s 50°C for 45 s; 68°C for 150 s. The primary amplicon was nested with the primers (W4 and W6) for 30 cycles at 94°C for 25 s; 55°C for 35 s; 72°C for 150 s and the elongation step for 10 min. The genes *msp1, msp 2* and *glurp* had amplifications performed as described by Randford-Catwright et al. (1991). Control reactions included, one positive tube with 6 ng of 3 D7 or W2 DNA from *P. falciparum*, negative

controls had no *Taq* I polymerase or target DNA. Amplified products were visualized over UV by staining with ethidium bromide.

Relative distances migrated were extrapolated from a standard curve using the migrated distances of the molecular markers and their known molecular sizes as reference. Statistical calculations were performed using Epi info software and the chi-square was used to compare differences in means or proportions between groups.

Mapped restriction site polymorphism

The 5'flanking region of *Pfs25* sequence was used to perform an electronic cleavage analysis for identification of restriction enzyme sites. The putative control elements were identified by PCGENE. The putative promoter TATATAAG, 402 bp upstream of the ATG with putative initiation sequence TTATTTA, 114 bp downstream of its promoter. The second TATATAAT (327 bp upstream of the ATG, with 4 putative initiation start points with sequences TTATTTA at 39 bp, TATTTTC at 46 bp, CTATTTT at 52 bp and TTTTATT at 57 bp each down stream of the promoter. Two other control elements identified by deletion-replacement were also incorporated in the analysis YAAGGAATA and a putative initiation site at -435 bp from the ATG (CTGTACT). Restriction endonucleases were chosen based on the possibility of these sequences to acquire single mutation creating restriction sites (Table 2). In addition restriction enzymes with sites within the *pfs25* 5'flanking region obtained from an electronic restriction cleavage were used. The enzymes used were those of *VspI, SspI, Dra I* and *Rsa I*. Following this analysis, PCR products (20 µl) were precipitated with ethanol in the presence of 0.3 M sodium acetate pH5.2 and re-suspended in 10 µl of nuclease free water. Some 625 samples from different geographic locations were analysed by PCR-RFLP essentially as described by the manufacturers (New England Biolabs). Digested products were run in a 2% submarine agarose gel contained in Tris-Borate-EDTA buffer (Sambrook et al., 1989).

Sequencing of PCR amplified products

The PCR products were purified by electroelution, phenol and chloroform washes and precipitated with ethanol in the presence of 0.3 M sodium acetate. Gel purified products were then used in cycle sequencing as described by the manufacturers (Amersham). Essentially, 500 pmole of DNA and 1 pmole of primer were used in reactions with 8 units of Thermo Sequenase polymerase. Reactions were chain-terminated in the presence of 225nCi of [α-^{32}P] ddNTPs by subjecting these to 30 cycles of 90°C for 30 s, 60°C for 30 s and at 72°C for 90 s. Reactions were cooled to room temperature (25°C) and then 3.5 µl of stop solution was added.

Table 2. Enzyme choices for restriction fragment length polymorphism of the 5'flanking sequence *pfs25.*

Putative Control Element	Probable mutation	Suggested enz before mutation	Suggested enz post mutation	Restriction site of enz
CTATTTT	CTAATTT		*Tsp5091*	AATT
TTTTATT	TTTAATT		*Tsp5091*	AATT
TTATTTAA	TTAATTAA	/	*Tsp5091, PacI*	AATT, TTAATTAA
TATTTTC	TAATTTC		*Tsp5091*	AATT
TATATAAT	TATTTAAT	/	*MseI*	TTAA
TATATAAG	TATTTAAG	/	*MseI*	TTAA
TAAGGAATAA	TAAGGAAGAA	/	*MboI*	GAAGA(N)$_8$
CAAGGAATAA	CAAGGAAGAA	/	*MboI*	GAAGA(N)$_8$
CTGTACT	CAGTACT	*RsaI*	*ScaI, RsaI*	AGTACT, GTAC
	CTNTACT	*RsaI*	/	GTAC
	CTGNACT	*RsaI*	/	GTAC
	CTNTNCT	*RsaI*	/	GTAC
	CTGTANT	*RsaI*	/	GTAC

Table 3. Study population characteristics.

Site	Limbe	Y'de	Kumba	Dschang	Nkambe	Buea Health District (0 – 1000 m)
1 - 5	107	26	33	8	41	27
6-10	29	8	21	8	18	80
11-30	41	33	27	34	0	0
>30	36	38	24	20	0	0
Total	213	105	105	70	59	107
Geo mean parasites/ul	32450	32422	3660	8900	9424	2130

Table shows the number of participants per age group in the different sites. The geometric mean parasitaemia has equally been shown for the different sites.

Samples were heated at 72°C for 5 min prior to separation on 8% polyacrylamide gels in the presence of 7 M urea, 40% formamide and glycerol tolerant buffer containing (10.8 g Tris base, 3.6 g Taurine and 200 mg Na$_2$ EDTA.2H$_2$0 per litter). Gels were fixed in 10% methanol and 10% acetic acid and dried under negative suction and exposed to Hyperfilm-MP for 24-36 h before being developed.

RESULTS

Study population characteristics

Women and men were equally represented and all age groups were represented in the study. Parasite densities were calculated and ranged from 40–200,000 parasites/µl of blood. Asymptomatic individuals had significantly lower parasitemia (p <0.005) (Table 3) except for an abnormally high individual with 110000 parasites/µl of whole blood. Of the 249 asymptomatic children examined at the Buea health district, 43% (107) of them had parasites and their mean body temperatures was normal (36.7^{o}C). These were distributed as follows: Soppo, Mutengene and Molyko, had 36.48, 52 and 36% of children infected with the malaria parasites. The mean parasites density were 2960 parasites/µl Soppo, 1080 parasites/µl in Molyko and 2360 parasites/µl in Mutengene. Microscopic speciation was done on positive slides and the result showed that children in Soppo and Mutengene were having a mono-infection with *P. falciparum.* However, at Molyko, 92.10% of infection was caused by *P. falciparum.* A mixed infection of *P. falciparum* + *P. malariae* (5.26%) and *P. falciparum* + *P. ovale* (2.64%) was observed in Molyko, whereas 3.0% of the parasites in Dschang were *P. malariae*. No other species were seen in the other sites.

Altitudinal polymorphisms in the Buea health district

Asymptomatic samples were analysed for polymorphisms of the *msp-1, msp-2* and *glurp* genes. Based on size polymorphism of nested-PCR products for *msp-1*, the most represented alleles in Mutengene, Molyko and

Soppo were respectively, the 540, 490 and 570 bp alleles (Figure 1). The 460 bp allele was only present in Molyko. The 570 bp allele seemed to increase in frequency as the altitude rose. This difference was statistically significant (p<0.05). For the *msp2*, no pattern was easily discernable. However, the 500 bp allele seemed to decrease significantly with altitude (p <0.05). For the *msp2* polymorphism, there was a general bimodal distribution around the 560 bp allele although in Molyko there was an outlier population of parasites with the 680 bp allele (40%). The 560 allele for the *glurp* gene seems to define a bimodal distribution about it again except for the 680 bp gene which seems to predominate in Mutengene. Overall there were plethora of alleles for all three genes and taken together, combinations of these genes may define different populations of parasites with distinct phenotypes.

cg2-ω as a marker for genetic diversity

The genes that have traditionally been used for low technology population genetics have been those of *msp1*, *msp2* and *glurp*. We used primers designed to the borders of the repeat regions of *cg2-ω* and were able to demonstrate that there were zonal differences in the band size allelic frequency (Figure 2). Following amplifi-cation some samples did amplify with *msp1* primers but not with *cg2-ω* primers (sample L49 from Limbe). Similarly, samples OM from Kumba and L79 from Limbe demonstrated more clonal populations with the cg2-ω primers and not with *msp1* primers. L09, L21 and L36 for *msp1* showed multiple bands and single bands each with cg2 primers. In Dschang and Kumba the dominant allele was 530 bp, different from the 655 bp allele dominant in Yaounde (Figure 3). Along the same lines, the 560 bp allele was most represented in Limbe and Nkambe. However, none of these allelic distributions followed any zonal pattern. Although *cg2-ω* looked similar in Limbe and Nkambe, the *msp1* of the two sites were different with the most represented allele in Yaounde being the 490 bp (cf - Limbe, and Kumba), whereas it was the 540 bp in Nkambe. Taken together the cg2- ω could add to the definition of different parasite populations for each of the zones.

Multiple infections in clonal populations of *P. falciparum* were seen in Limbe and with an equal representation of single (37%) and mixed infections (37%) (Figure 4). Equally present were triple and quadruple infections at 16 and 10% of the samples. In the other sites only a low proportion of double clones were observed probably reflecting the intensity of transmission.

Minimal variation of the promoter region of pfs25 in field isolates

Different PCR parameters were tested to establish condi-

tions for the amplification of *pfs25* specific products. To increase recovery for single gene products from PCR amplification of *pfs25,* a nested pair of oligonucleotide primers was used at an annealing temperature of 60°C (see Materials and Methods). Samples after nested amplification were incubated with restriction endonucleases and only *RsaI* was able to digest sample JA93K from Kumba, with the disappearance of a 50 bp piece. Samples were selected at random and sequences to determined identification of any heterogeneity which might otherwise be missed by restriction analysis of individual amplicons. Upon direct sequencing of 12 PCR products the polymorphism of the *Rsa I* site at position -435 bp upstream of the ATG of *pfs25* site was confirmed (Figure 5). Incidentally this region is found within the 489 bp region that gave a minimal 5% of parental activity (Mbacham, 1997; Dechering et al., 1999) suggesting that it might not have a significant effect on transcription.

DISCUSSION

Genetic diversity plays a major role in the natural acquisition of immunity to malaria infection giving to the individual who is exposed to many more parasites, a comparative advantage over how one is limited in exposure. This immune pressure also propagates the generation of variants (Snounou et al., 1999). The PCR typing methodology provides the advantage of a great versatility in rapidly determining the genetic structure of a parasite population by size polymorphism. The single copy genes *msp-1*, *msp-2* and *glurp* offer such a large allelic polymorphism pool from which to draw conclusions (Wooden et al., 1992; Zwetyenga et al., 1998). This plethora of alleles number highlights the intensity of malaria transmission (Ariey et al., 1999). Most invest-tigations have employed the subtypes of the *msp1* and *msp2* to distinguish between subtypes of the same population. Microsatelite markers have been used to investigate *Plasmodium* diversity except that this is not convenient for African settings. Most of these genes used for polymorphism studies do not define a measurable phenotype and we therefore present the possibility of using *cg2-ω* as a marker of diversity. The *cg2* gene segregated with the resistance phenomenon when a resistance strain and a sensitive strain of *P. falciparum* were crossed (Wellems et al., 1991; Su and Wellems, 1994; Su et al., 1997). It was evaluated as a marker for resistance to chloroqiune and abandoned in favour of the *Pfcrt* gene because it did not correlate 100% with the resistance phenotype. This current study demonstrates that the diversity of *Plasmodium* could be further assessed using such a marker because it gives it the added advantage that the diversity phenotype associated with it is measurable. From this study, zonal and altitu-dinal differences that exist may define particular clades of *Plasmodium.* However these clades would have to be
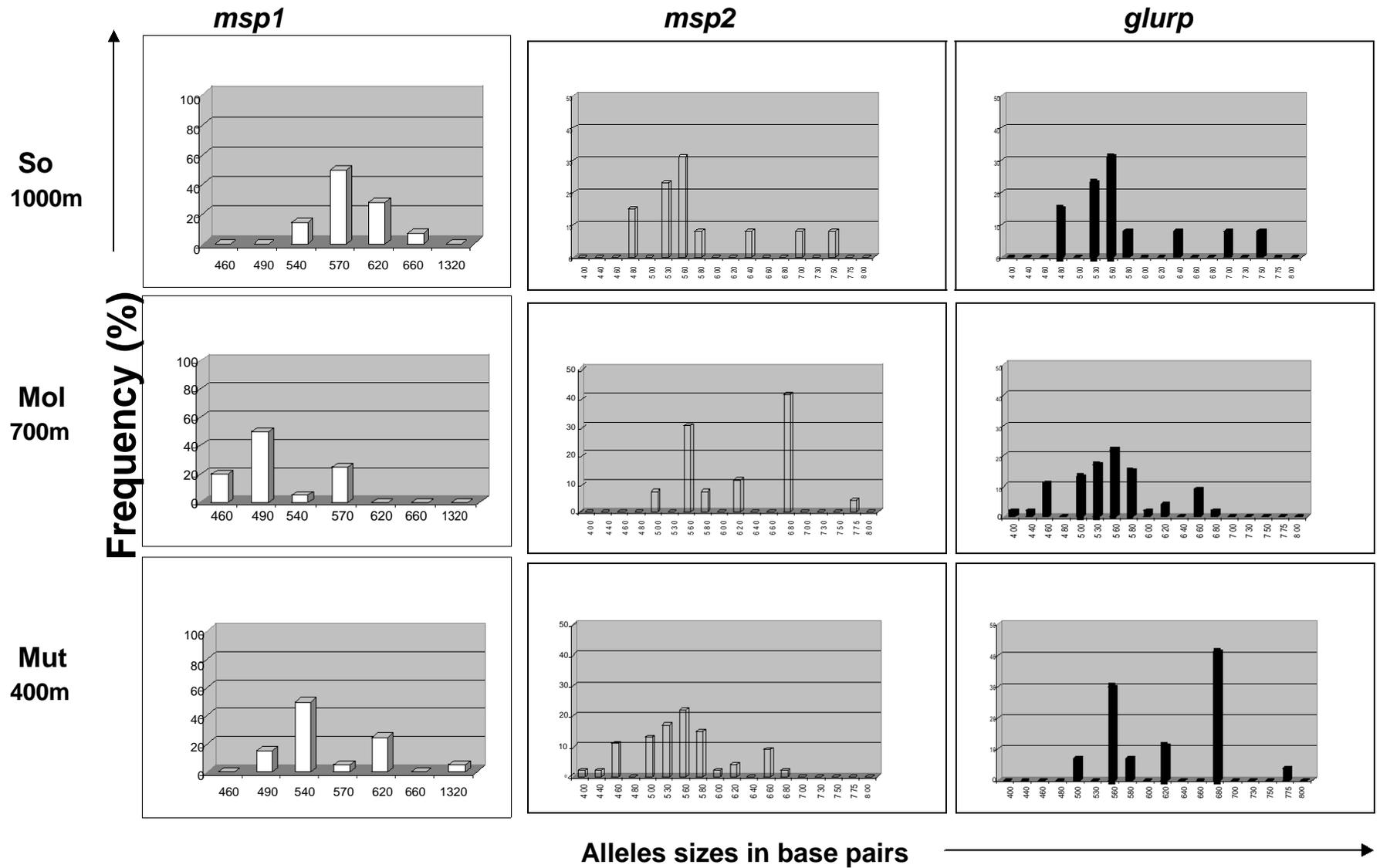
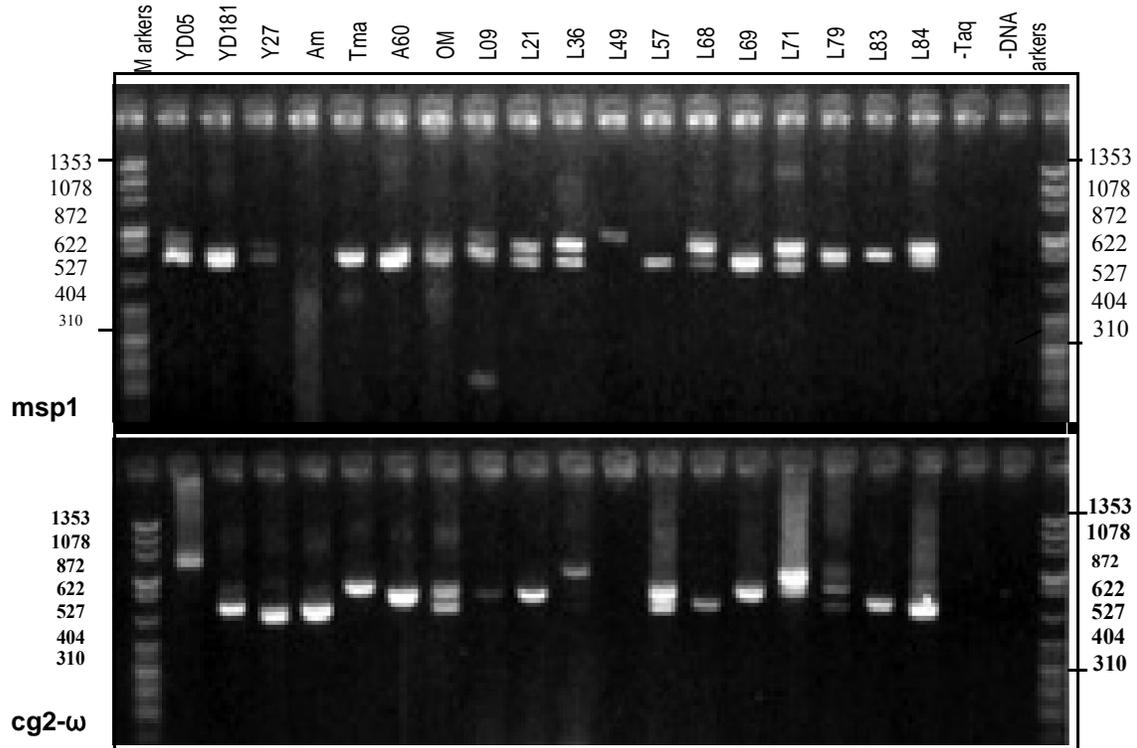**Figure 1.** Altitudinal variation of population structure genes *P. falciparum.*

Figure 2. cg2-ω as a marker for genetically diverse p. falciparum populations. An garose gel (2.5%) analysis of PCR amplified blood samples obtained from the different ecoozones of Cameroon, using primers shown in Table 1. (A) *msp-1* alleles and (B) *cg2-ω* alleles. Markers used were *pBR322* DNA digested with *msp1* restriction enzyme and Φx 174 DNA digested with *HaeIII* restriction enzyme, sizes (in base pairs) are indicated on both sides of the gel. YD05, YD181 are samples from Kumba, L09, L21, L36, L49, L68, L69, L71, L79, L83, L84 are samples from Limbe. –*Taq*: PCR mixture without *Taq* Polymerase. –DNA: PCR mixture without exogenous DNA Template. For each sample 4 µl of the PCR reaction was applied. The gels were stained in 0.5 µg/ml of ethidium bromide and observed by u.v. transilluminator.

associated with a phenotype; either virulence or pathogenic traits for it to have meaning. It however demonstrates the possibility of using both the *cg2*-ω repeats along side the traditional *glurp* and *msp* genes. The significant variation of some molecules with altitude - 500 bp allele of the *msp2* and the 680 bp allele of *glurp* gene which reduced in frequency with altitude or the 570 bp allele for *msp1* which increased with altitude is discernable. If it is further validated it can be used as a tool for the rapid assessment or measure in parasite evolution and control.

Gene expression is a multi-step process that involves a plethora of factors and sequences. Although consensus sequences have been defined for broad based eukaryotic and prokaryotic systems, most of the factors and the sequences they bind are highly specific. In previous studies we demonstrated that the promoters of *Plasmodium* differ from other eukaryotic systems and from each other even for genes of similar molecular compart-

mentalization (Dechering et al., 1999; Mbacham et al., 2001). This complexity is further accentuated by stage or organ specificity within *Plasmodium*. On evolutionary grounds more energy is conserved by organisms in regulating expression at transcription initiation, so we investigated the possibility that transcription could be regulated and so foster our understanding of *pfs25* gene regulation. Against the background of diverse parasitism, the possibility of variation of the cis-control sequences of the candidate anti-transmission blocking vaccine gene, *pfs25* demonstrated long stretches of poly dT and poly dA upstream of the *pfs25* gene. Such stretches were also identified and described in *Plasmodium berghei* and *Plasmodium chabaudi* DHFR-TS and calmodulin 5'flanking regions. While no such repeat elements were found in *P. falciparum pfs25*, such repeats were found in *Toxoplasma gondii* to regulate expression (Soldati and Boothroyd, 1993). It is also possible that secondary structures found in these regions could contribute to ex-
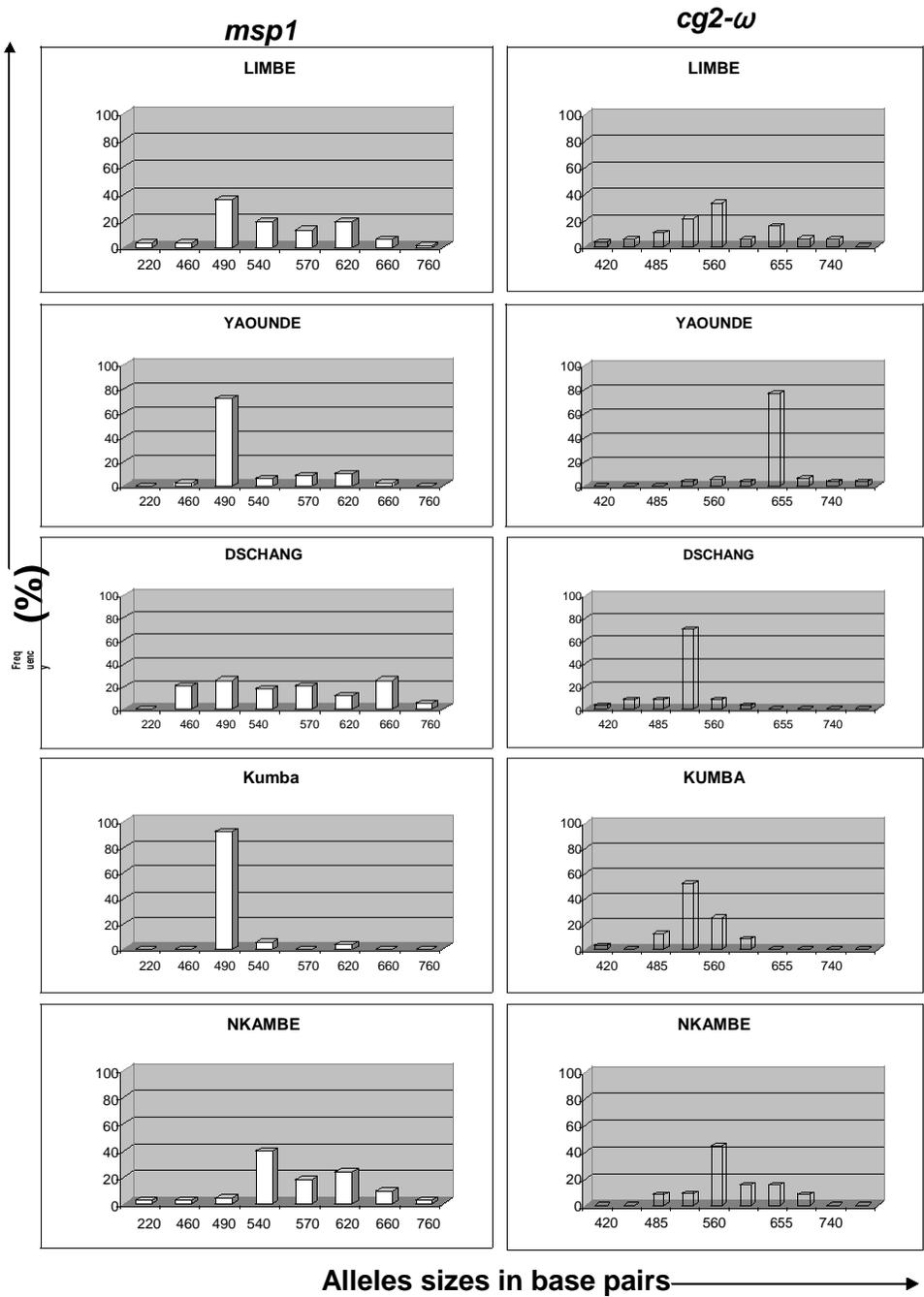
Figure 3. Zonal variation of population structure genes *P. falciparum*.

pression in *Plasmodium*. The features of the 5'Flanking Sequence (5'FS) of *pgs28* and *pfs25* make them interesting given that the kind of sequences that bind architectural proteins are abundant within the 5'FS of these genes.The architectural protein, lymphoid enhancer factor, LEF-1 binds to the minor groove of DNA molecules and is capable of causing bends of up to 120

degrees (Giese et al., 1995). Such molecules greatly facilitate interactions since elements which would otherwise be far apart are brought into close proximity by such folding. Since the poly dA or dT tracts are rigid structures, they frequently cause bends and are inflexible making them difficult to wind around the nucleo-protein complex at enhancer sequences. Similar DNA binding proteins
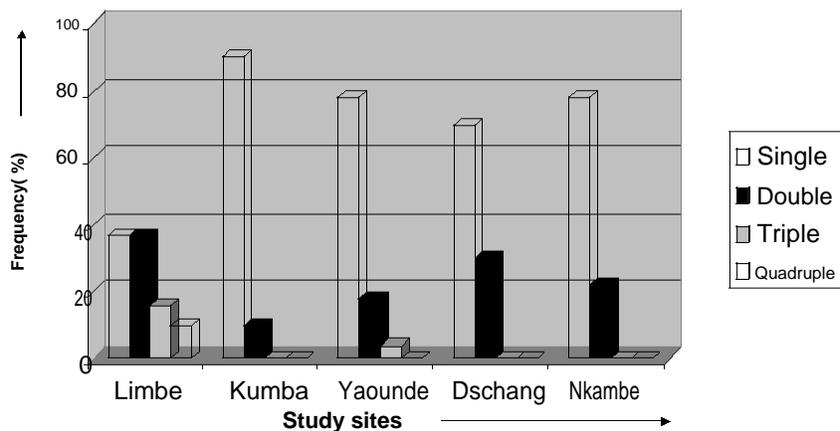
Figure 4. Eco zone differences in extent of multi-clonal infections of *Plasmodium falciparum*.
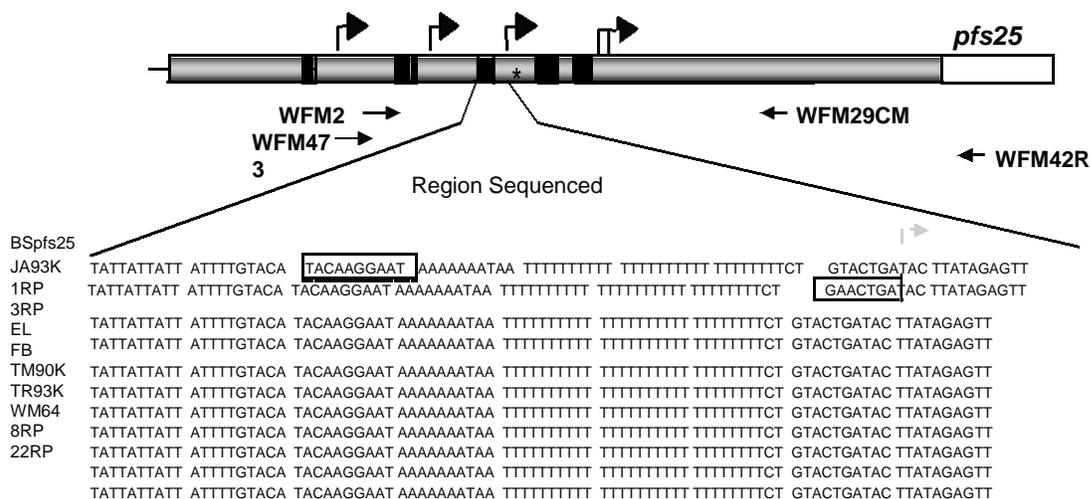

Figure 5. Genetic polymorphism of *pfs25* promoter region.

Legend: The black boxes represent the putative promoters with their corresponding initiation site (arrows) ,29CM, 42R and 43 are primers used in the simplification either for sequencing or for PCR-RFLP. The strip box is the position of the pfs25 gene coding region. The left open box represents the putative promoter whose putative initiator (right open box) acquired a mutation (sample JA93K).

have been described which either reverse kinks or create bends in DNA (Thanos and Maniatis, 1995). These molecules effect subtle structural changes capable of rendering the DNA flexible to wrap around the nucleo-protein complex during the formation of the enhance-osome. An example of such a protein is the HMG 1(Y) (Falvo et al., 1995), which facilitates nucleo-protein complex formation through protein-protein interaction involving nuclear factor NF-kappaB, the activating trans-cription factor-2 (ATF-2), the high mobility group HMGI(Y) proteins and the positive regulatory domains, PRDI-IV,

at the human Interferon-gamma gene enhancer element.

In testing the null hypothesis that there are no variants in field isolates, PCR combined with mapped restriction analysis and sequencing identified only one polymor-phism at the *RsaI* restriction site part of which is located within the sequence TTTCTGT, located 40 bp down-stream of the regulatory element at -478 bp from the ATG of *pfs25*. The mutant had this transcription initiation sequence TTTCTGT was identified by the PCGENE software as a putative transcription start point. So far a limited number of twelve isolates have been sequenced

and demonstrated to have no variation in their sequences. It is uncertain if this mutation will affect expression since the identified site of transcription initiation by RACE-PCR is 190 bp downstream of the putative promoter and warrants further investigation. Variation of promoter elements have been described and correlated with virulence. McGuire et al. (1994) described mutations of the TNF promoter and correlated this with severity of malaria. Mutations were identified 308 nucleotides upstream of the initiation point where a guanine to adenine change resulted in increased TNF- production. Although this finding is being contradicted by carefully planned experiments in which this mutation was not associated with survival in severe sepsis or responsible for lipopolysaccharide inducibility. Other demonstrations of promoter mutations associated with severity of disease have been described in HIV/AIDS infection such that clades HIV-1C and HIV-1E which are the faster spreading subtypes in Africa and Asia respectively have mutations within the promoter and binding sites for enhancer elements NK-kappa B. These mutations have been correlated with divergent activation of transcription and may explain some of the differences observed in transmission and pathogenesis. So far minimal variation is observed in the promoter regions of *pfs25*, similar to findings within the protein itself (Kaslow et al., 1993). These findings are suggestive of the fact that mutations within the flanking control regions or coding sequences that affect survival become deleterious to the parasite and are selectively eliminated from being transmitted. If such mutations that affect expression would be found then they could offer good molecular targets for intervention. However no drug or immune pressure exist on this molecule and there may not be a reason for the parasite to alter its structure to accommodate such stress.

The nature of its 5'FS is such that rigidity is frequently encountered given its richness in homopolymeric tracts. DNA binding proteins have been described in literature that specifically recognizes homopolymeric tracts of more than 10 bp in length. One such molecule is Datin (Winter and Varshavsky, 1989) which binds and acts as a repressor. Others in this category include netropsin, distamycin and Hoechst 33258 which are minor grove binding drugs (Juo et al., 1996). Their structure lacks the C_2-NH_2 protruding group from the floor of the minor groove from adenine as opposed to guanine which permits a tight drug/DNA contact at A-T regions. Evidence cited in Juo et al. (1996) demonstrates that the variability of the minor groove allows for a range of deformabilities for binding of more than one drug molecule in such regions. These properties and studies of such structures should help in the design of new therapies for malaria. The electrophoretic mobility shift assay studies indicated that one could obtain substantial shifts because the factors bound with high affinity and avidity. It is possible to identify these factors through the use of antibodies made to conserved epitopes. Once identified, it is possible to isolate these factors and establish *in vitro* cell free systems that can be used to study the influence of environmental fac-tors on expression.

There exist cryptic promoters within the studied sequence and *Plasmodium* could use this as a survival strategy and capable of using these elements to ensure transmission, in situations in which its preferred sequences have been altered. Such a strategy may be important for molecules such as the *pfs25* whose blocking, using antibodies have shown how imperative it is for transmission from man to mosquito. It is the conclusion that there is minimal variation within the 5' FS of the *pfs25* gene even amid great genetic diversity. The control elements within this region can therefore be used to drive stable expression of *pfs25* in vaccine studies.

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