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Vaccine candidate MSP-1 from *Plasmodium falciparum*: a redesigned 4917 bp polynucleotide enables synthesis and isolation of full-length protein from *Escherichia coli* and mammalian cells

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ABSTRACT

The *Plasmodium falciparum* malaria parasite is the causative agent of malaria tropica. Merozoites, one of the extracellular developmental stages of this parasite, expose at their surface the merozoite surface protein-1 complex (MSP-1), which results from the proteolytic processing of a 190–200 kDa precursor. MSP-1 is highly immunogenic in humans and numerous studies suggest that this protein is an effective target for a protective immune response. Although its function is unknown, there are indications that it may play a role during invasion of erythrocytes by merozoites. The parasite-derived *msp-1* gene, which is ~5000 bp long, contains 74% AT. This high AT content has prevented stable cloning of the full-size gene in *Escherichia coli* and consequently its expression in heterologous systems. Here, we describe the synthesis of a 4917 bp gene encoding MSP-1 from the FCB-1 strain of *P.falciparum* adjusted for human codon preferences. The synthetic *msp-1* gene (55% AT) was cloned, maintained and expressed in its entirety in *E.coli* as well as in CHO and HeLa cells. The purified protein is soluble and appears to possess native conformation because it reacts with a panel of mAbs specific for conformational epitopes. The strategy we used for synthesizing the full-length *msp-1* gene was to assemble it from DNA fragments encoding all of the major proteolytic fragments normally generated at the parasite's surface. Thus, after subcloning we also obtained each of these MSP-1 processing products as hexahistidine fusion proteins in *E.coli* and isolated them by affinity chromatography on Ni^{2+} agarose. The availability of defined preparations of MSP-1 and its major processing products open up new possibilities for in-depth studies at the structural and functional level of this important protein, including the exploration of MSP-1-based experimental vaccines.

INTRODUCTION

Malaria caused by *Plasmodium falciparum* infections continues to be a serious health problem in major parts of the world. The identification of targets for immunologic interventions against this infectious disease remains, therefore, an important goal. Merozoites, which are the extracellular developmental form of the parasite that invade erythrocytes, expose on their surface a protein complex, which is the processing product of a 190–200 kDa precursor known as merozoite surface protein-1 (MSP-1; 1–5). Upon deposition in the merozoite membrane via a glycosyl-phosphatidylinositol (GPI) anchor, this precursor is proteolytically cleaved during late schizogony into fragments which remain associated with the parasite surface. Sequence analysis of *msp-1* genes of different *P.falciparum* strains has revealed that major regions of the protein are dimorphic belonging to either the K1 or the MAD20 prototype (Fig. 1A) while other parts are highly conserved (3). Two small regions near the N-terminus show higher variability. These features, as well as the presence of point mutations scattered throughout the molecule and evidence for intragenic recombination and/or gene conversion, confer a surprisingly limited polymorphism to these abundant surface proteins (3,5).

A number of experimental findings suggest that MSP-1 of *P.falciparum* may elicit a protective immune response against infections by the parasite. For example, in the rodent model, immunization of mice with the analogous protein of *Plasmodium yoelii* yielded protection (6), as did the transfer of monoclonal antibodies and immune serum against this protein (7–9). Sero-epidemiologic data (10,11) and results from several vaccination trials conducted with various *P.falciparum*-derived MSP-1 preparations in non-human primates also support the candidacy of this protein or parts thereof as promising components of a subunit vaccine against malaria tropica. In these trials, *Saimiri* or *Aotus* monkeys were immunized either with MSP-1 isolated from parasites (12–15) or with synthetic peptides or recombinant MSP-1 fragments (15–21). The recombinant fragments assessed most recently were primarily from the C-terminal region of MSP-1. Although the data from these trials support MSP-1 as a vaccine

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candidate, the MSP-1 protective effects measured in these trials barely satisfy requirements for statistical significance because group sizes were generally too small and since collected data were not confirmed in strictly comparable, subsequent trials. The two main reasons for this situation are the scarceness of suitable experimental animals and the difficulties associated with preparing sufficient amounts of well-defined MSP-1 from parasites. On the other hand, expression of full-length recombinant MSP-1 in heterologous systems has turned out to be most difficult if not impossible (22,23). This appeared primarily due to the high AT content of *P.falciparum* DNA which prevents the cloning and stable maintenance of large genes in *Escherichia coli*, thereby precluding crucial studies at the genetic and biochemical level which may have led to the elucidation of its function.

Thus, we decided to synthesize a 4917 bp polynucleotide encoding MSP-1 of the Colombia FCB-1 strain (24,25) and change the AT content such that it could be maintained and expressed in a variety of hosts. Herein, we describe the design and synthesis of this gene based on human codon frequencies. We also report the cloning of this synthetic gene and its controlled expression in *E.coli* and in mammalian cells. Purification of full-length protein from both expression systems yielded material that is recognized by several monoclonal antibodies known to interact with conformational epitopes. Moreover, the strategy employed for the synthesis of the gene allowed for subcloning, production and purification of all major processing products of MSP-1. The availability of large amounts of MSP-1 and its fragments opens up new possibilities for the thorough investigation of this prominent malaria antigen.

MATERIALS AND METHODS

Sequence design

The amino acid sequence of MSP-1 of the Colombia FCB-1 strain (25) was translated into a DNA sequence with an average codon composition similar to that found in human coding sequences (26).

This was achieved by using a random number generator to make each codon assignment, a process that proved to be truly random, because each run of the program yielded a different synthetic allele of MSP-1. One sequence was chosen as the master sequence and modified in a number of ways to eliminate sequences that might be detrimental to efficient transcription and translation of the synthetic gene. All analysis programs mentioned below were from the Genetics Computer Group program collection (27). Positions where the introduction of additional endonuclease cleavage sites appeared feasible without changing the amino acid sequence were identified with the 'Map' program using the option 'silent'. 'Find Patterns' was used to search for consensus sequences that are indicative of prokaryotic promoters, poly(A) signals and exon–intron boundaries. Prokaryotic factor-independent RNA polymerase terminator structures were identified with the 'Terminator' program. Inverted repeats which might lead to the formation of undesirable secondary structures were identified with the 'Stemloop' program. All these structures, when encountered, were eliminated by using alternative codons. Moreover, long runs of purines (>7 nt) that may cause transcriptional termination in some viral systems were disrupted. Finally, the stability of the resulting RNA molecule was assessed with 'Fold RNA'. This analysis was performed on overlapping fragments because the software restricted the length of the input sequence to 1200 bases. Any structures more stable than the mRNA of the human glyceraldehyde-3-phosphate dehydrogenase were eliminated.

Design and synthesis of oligonucleotides

The oligonucleotide primers used for the PCR-based synthesis of high molecular weight, double-stranded DNA were restricted in size to maximally 120 nt in order to ensure that at least 50% of the PCR products were error free. The primers were designed using the Oligo 4.0 program and the following parameters were considered. The overlapping region between two oligonucleotides used in one PCR reaction was on average 18 nt long. The internal stability of each overlapping oligo pair was $-\Delta G \geq 9$ kcal/mol. Potential hairpin and duplex structures with $-\Delta G \geq 8$ kcal/mol were eliminated as were false priming sites. The oligonucleotides were synthesized on a 1000 Å pore size glass support using an Applied Biosystems 394 synthesizer following standard protocols. They were purified by electrophoresis in 10% polyacrylamide (PAA) containing 7 M urea. Upon electroelution from gels, they were precipitated with ethanol.

Asymmetric PCR-based synthesis of double-stranded DNA fragments

The overall strategy of fragment synthesis is outlined in Figure 2. In general, four assays were performed in parallel. The ratio of the oligonucleotide pair used in each reaction was 5:1 in order to yield the appropriate asymmetrically amplified product (28). Products A, B, C and D, respectively, resulted from four PCR assays that contained the oligonucleotide pairs O1/O2 and O5/O6 in ratios of 25:5, O3/O4 and O7/O8 in ratios of 5:25 pmol, respectively. Five PCR cycles were performed in 50 µl of 10 mM Tris–HCl, 1.5 mM $MgCl_2$, pH 8.3, containing 2.5 U Taq polymerase (Boehringer, Mannheim) and the four deoxynucleotide triphosphates (200 µM each). The optimized cycling conditions (Omnigene TR3 CM220 thermocycler) were 10 s at 94°C (denaturation), 30 s at 55°C (annealing) and 60 s at 72°C (polymerization). Product E was prepared by combining the products from assays A and B, and F from assays C and D. After amplifying for five cycles, we added 25 pmol of O1 and O8 to 5 pmol of E and F, respectively, to re-establish asymmetric oligonucleotide compositions and amplified for an additional eight cycles. Product G was prepared by combining these latter assays and amplifying for another 12 cycles. PAGE was used to follow the various synthetic steps. The final product G was separated from other reaction products by electrophoresis in 1% agarose gels, eluted from the gel slice according to the Qiaex II procedure (Qiagen, Düsseldorf), digested with *Bam*HI and *Cla*I and ligated into an appropriately cleaved pBSK* plasmid. This plasmid is identical with pBSK (Stratagene, Heidelberg) except that the *Xba*I and *Spe*I sites within the multiple cloning site were replaced with *Nhe*I, *Mlu*I, *Nco*I and *Sty*I sites. The resulting vectors were transferred into *E.coli* strain SG13009. DNA fragments of the expected size liberated from plasmids of bacterial clones after *Bam*HI/*Cla*I cleavage were further analysed. Usually, 10–20 clones containing such inserts were sufficient to identify either error-free 600–800 bp DNA fragments or fragments containing small numbers of errors that could be eliminated by combining the error-free portions of two fragments via an internal cleavage site. All fragments were finally combined via their compatible unique cleavage sites positioned at either end (Fig. 1B). After assembly was complete, the *msp-1*^{S1} gene was sequenced in its entirety with a standard set of sequencing primers.

Synthesis of full size MSP-1 and of MSP-1 fragments in *E.coli*

The DNA encoding MSP-1^{S2}, p83 (without signal peptide, Fig. 1B), p30, p38, p42 and p19 (the latter without GPI anchor signal, Fig. 1B), respectively, were transferred from pBSK* vectors to the expression vector pDS56 (29) via their *Bam*HI and *Cla*I cleavage sites. By utilizing the *Bam*HI insertion site of this vector, six histidines were attached to the N-terminus of the respective protein. This allows the purification of the resulting protein via Ni^{2+} chelate chromatography (29).

The various vector constructs were transferred into the Lac repressor providing *E.coli* SG13009. Cultures derived from individual clones containing the proper plasmids were grown to early log phase (OD_{600} 0.2) and induced with IPTG (1 mM) for 3 h. With the exception of p38, all fragments as well as the full-size p190 were produced in high yields (between 2 and 10% of the total protein), but even p38 was readily purified in sufficient quantity to allow characterization. For isolation of the various expression products, cell pellets were dissolved in 6 M guanidinium hydrochloride and applied directly to Ni^{2+} chelate columns. Before the adsorbed material was eluted, the column was developed with a reverse gradient running from 6 to 1 M urea in 0.5 M NaCl, 0.05 M Tris–HCl, 20% glycerol, pH 7.4. Elution with an imidazole gradient (0–500 mM imidazole hydrochloride in 0.05 M Tris–HCl, 10% glycerol, pH 7) yielded MSP-1^{S2} as well as all the fragments in highly purified and soluble form.

Expression of MSP-1-encoding DNA in mammalian cells

The *msp-1*^{S1} gene was inserted as a *Mlu*I–*Cla*I fragment into plasmid pBi-5 (30) where it is co-regulated with the luciferase gene by the bidirectional promoter P_{bi-1}. The activity of P_{bi-1} is entirely dependent on tTA, the tetracycline controlled transcriptional activator (31). The resulting plasmid pBi-5/MSP-1^{S1} was used to transiently express the gene in HeLa and CHO cells that constitutively produce tTA. Thus, HtTA-1 (31) and CtTA-1 cells were co-transfected with pBi-5/MSP-1^{S1} and pUHD16-1 following a modified (30) calcium phosphate method. The latter plasmid gives rise to β-galactosidase which serves as a standard for determining transfection efficiencies. MSP-1^{S1} expression was induced by removal of doxycycline (Dox) from the culture and cells were harvested after 30 h to determine luciferase activities in cell extracts as described previously (31). The production of MSP-1 was visualized by western blot analysis using monoclonal antibody mAb 5.2 (32) and compared with lysates prepared from uninduced cells.

HtTA-1 and CtTA-1 cell lines that control the synthesis of MSP-1^{S1}

To integrate the *msp-1*^{S1} gene controlled by P_{bi-1} into the genome of HtTA-1 cells, the cells were grown in 35 mm dishes to 40–50% confluency and co-transfected with 2.9 µg of linearized plasmid pBi-5/MSP-1^{S1} and 0.1 µg of linearized plasmid pHMR272 (30), which confers resistance to hygromycin B. After 24 h, the cells were transferred to 10 cm dishes and maintained in medium containing 300 µg/ml hygromycin B. Resistant clones were isolated, expanded separately and analysed for luciferase activity (31) in the absence and presence of Dox (100 ng/ml). Several clones which exhibited efficient regulation of luciferase activity in a Dox-restricted manner were then analysed for tTA-dependent production of MSP-1 by western blot analysis. Further subcloning produced the cell line HtTA-9319 which efficiently expressed MSP-1 as well as the *luc* gene. The MSP-1-expressing CHO cell line CtTA-27/29 was generated in an analogous way.

Purification of MSP-1^{S1} from HeLa cells by immunoaffinity chromatography

HtTA-93/9 cells were grown to confluency in 10 cm dishes containing EMEM medium supplemented with 10% FCS. Cells from 20 such cultures were washed twice with PBS and suspended in 4 ml TNET lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, 1% Triton X-100, pH 7.4) containing a cocktail of protease inhibitors (PMSF, aprotinin, antipain, bestatin, pepstatin and leupeptin, each at 5 µg/ml, and 50 µg/ml TLCK). The suspension was kept in ice for 30 min before it was centrifuged at 300 000 *g* and 4°C for 30 min. The supernatant was 'cleared' by passing it through a 1.5 ml column packed with protein A–Sepharose 4 fast flow (Pharmacia Biotech) and the flow-through was collected. For immunoaffinity chromatography the flow-through was then applied to a 1.5 ml mAb 5.2 (ATCC, HB9148)/protein A–Sepharose 4 fast flow column (33) equilibrated with TNET buffer. The column was washed with 5 bed vol of TNET, pH 7.4, followed by 5 bed vol of TNET, 0.65 M NaCl, pH 8.0, and by 2 bed vol of 150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 6.8. Adsorbed protein was eluted with 0.1 M glycine buffer, pH 2.5, and fractions were collected into a previously titrated volume of 1 M Tris–HCl, pH 8.0. The protein was stored in 20% glycerol at –20°C.

Analysis of MSP-1 isolated from *E.coli* and CHO cells by western blot

MSP-1^{S} purified from *E.coli* via Ni^{2+} chelate chromatography and MSP-1^{S1} isolated from CHTA-27/29 cells by immunoaffinity chromatography was subjected to electrophoresis in 8% PAA in the presence of SDS (2%) but under non-reducing conditions. Transfer of the protein onto ImmobilonP membranes (Millipore) was carried out in transfer buffer (0.01% SDS, 25 mM Tris, 192 mM glycine, 20% methanol) for 90 min at 350 mA.

Approximately 0.1 µg of protein was applied per lane. The membrane-bound proteins were exposed to the various monoclonal antibodies and visualized via anti-mouse IgG AP-conjugate (Sigma, A2179) following standard procedures (33).

RESULTS

Design of a polydeoxyribonucleotide encoding an MSP-1 sequence

The MSP-1 coding sequence chosen for redesign is from *P.falciparum* strain FCB-1. Table 1 shows the bias towards A and T in the codons of the parasite gene in comparison with codon frequencies found in human coding sequences. By back-translating the amino acid sequence of the *msp-1* gene into DNA with human codon frequencies, the AT content was reduced from 74 to 55%. The redesigned gene was further modified to exclude sequences that may be problematic during synthesis or cloning and expression of the polynucleotide in various heterologous systems (Materials and Methods). These included a perfect *E.coli* promoter sequence lying upstream of a consensus-type translational start signal which gave rise to efficient expression from an internal start site. By making use of the degeneracy of the genetic code, we eliminated all the potentially problematic sequences by changing individual base pairs without affecting the encoded amino acid sequence.

Table 1. Comparing the codon frequencies of the *msp-1* gene of *P.falciparum* with human coding frequencies reveals an extreme bias towards A/T-containing codons in the parasite DNA

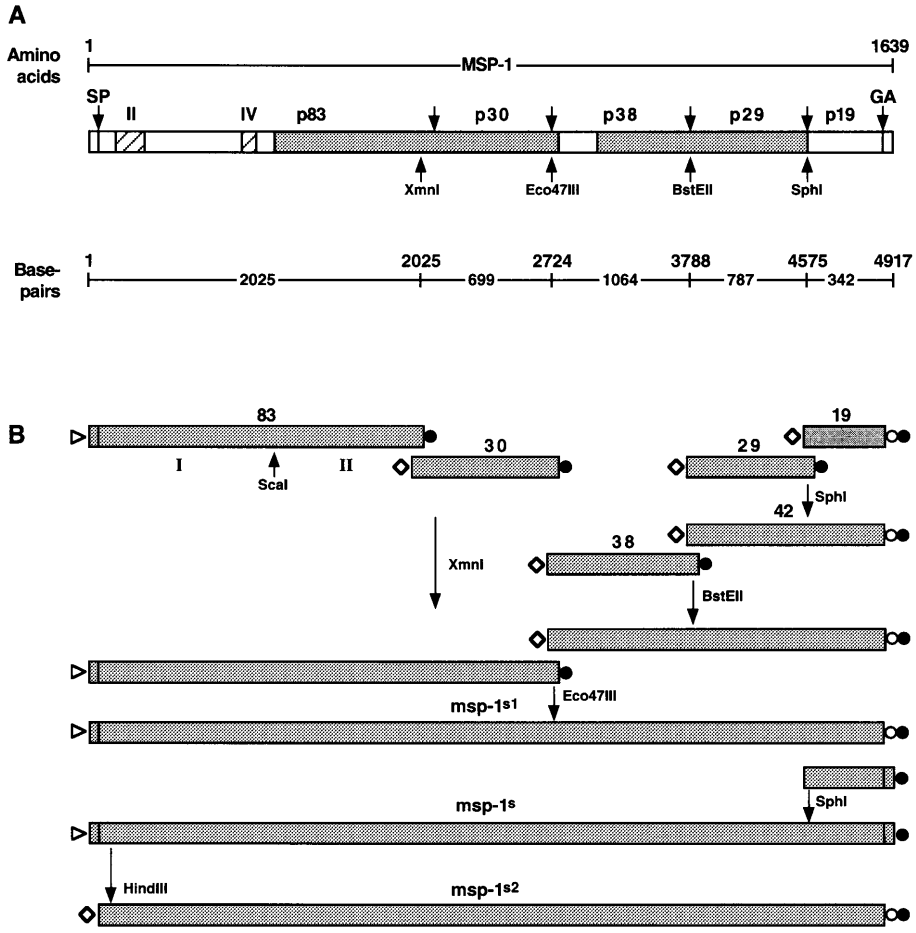
		Codon frequencies (%)							
Amino acid	Codon	MSP-1 native	MSP-1 synthetic	human coding seq.	Amino acid	Codon	MSP-1 native	MSP-1 synthetic	human coding seq.
Ala	GCA	53	22	23	Leu	CTA	2	2	7
	GCC	8	49	40		CTC	3	30	20
	GCG	2	0	10		CTG	0	51	41
	GCT	37	29	27		CTT	17	8	13
						TTA	69	1	7
Arg	AGA	62	30	20		TTG	9	8	13
	AGG	10	17	20					
	CGA	10	9	11	Lys	AAA	86	35	42
	CGC	0	26	19		AAG	14	65	58
	CGG	0	9	21					
	CGT	19	9	9	Met	ATG	100	100	100
Asn	AAC	22	58	54	Phe	TTC	33	79	55
	AAT	78	42	46		TTT	67	21	45
Asp	GAC	13	64	53	Pro	CCA	60	33	28
	GAT	87	36	47		CCC	10	27	32
						CCG	2	6	11
Cys	TGC	10	60	55		CCT	29	35	28
	TGT	90	40	45					
					Ser	AGC	5	27	24
Gln	CAA	94	36	26		AGT	24	7	15
	CAG	6	64	74		TCA	42	7	15
						TCC	6	24	22
Glu	GAA	94	38	42		TCG	2	2	6
	GAG	6	62	58		TCT	21	33	18
Gly	GGA	48	25	25	Thr	ACA	57	28	28
	GGC	7	43	34		ACC	6	41	36
	GGG	0	15	24		ACG	2	8	12
	GGT	46	18	17		ACT	35	22	24
His	CAC	26	57	59	Trp	TGG	0	0	100
	CAT	74	43	41					
					Tyr	TAC	19	54	56
Ile	ATA	41	12	15		TAT	81	46	44
	ATC	6	55	49					
	ATT	53	34	36	Val	GTA	45	6	12
						GTC	5	33	24
						GTG	4	48	46
						GTT	46	13	18

The synthetic MSP-1 sequence was adjusted to the human codon frequencies using a random number generator. Multiple additional adjustments, e.g. for generating unique cleavage sites, eliminating splice donor and acceptor signals, etc., were made thereafter. The codon frequencies shown for the synthetic gene represent the final sequence synthesized which, when compared with the native gene, contains base pair changes at 1317 positions.

Searching for hidden recognition sequences for restriction endonucleases permitted us, again by incorporating single base pair exchanges, to position unique cleavage sites at or near the major processing sites where the MSP-1 precursor of FCB-1 is proteolytically cleaved during schizogony (Fig. 1A). Thus, the endonucleases *Sph*I, *Bst*EII and *Eco*47III cleave the synthetic MSP-1 coding sequence within one, four and one amino acid, respectively, of the processing sites which separate p19 from p29, p29 from p38 and p38 from p30 (Fig. 1A). Moreover, the *Xmn*I site at position 2025 is within 28 amino acids of the putative processing site that separates p83 from p30 (34). Finally, unique cleavage sites were placed at either end of the full-size gene: a *Mlu*I site at the 5′-end and a *Not*I as well as a *Cla*I site at the 3′-end of the gene (Fig. 1B).

Because MSP-1^{S2} (Fig. 1B and C) lacks the signal peptide and the anchoring signal, it should be synthesized and remain in the cytoplasm. We, therefore, prepared two further genes with modifications to the 5′- and 3′-ends. In one (*msp-1*^{S}), the original signal peptide at the 5′-end and the GPI anchor signal at the 3′-end flank the coding sequence for the mature protein. In the other (*msp-1*^{SI}), the signal sequence, but not the anchor sequence, which should permit secretion of the protein but not membrane retention, flank the coding sequence for the mature protein. While modifications at the 5′-end of the gene are facilitated by a unique *Hin*dIII site at position 116, those at the 3′-end are best achieved by synthesizing variants of p19 which can be introduced via the unique *Sph*I site.

The polynucleotide encoding MSP-1, complete with a signal peptide and GPI anchor signal, comprises 4917 bp whereas the



C

Sequences at the Termini

	N-terminus	C-terminus
MSP-1^{S}		
DNA	GCACGCGTATGAAAATC ----------	AGCTTCATCTAATAGATCGATGGC
Protein	MIu I Met Lys Ile ----------	Ser Phe Ile stop stop Cla I
	1 2 3 ----------	1637 1638 1639
MSP-1^{S1}		
DNA	GCACGCGTATGAAAATC ----------	AGCTCTAATTAATAGGCGGCCGCATCGATGGC
Protein	MIu I Met Lys Ile ----------	Ser Ser Asn stop stop Not I Cla I
	1 2 3 ----------	1619 1620 1621
MSP-1^{S2}		
DNA	GGGATCCTATGGTGACC ----------	AGCTCTAATTAATAGGCGGCCGCATCGATGGC
Protein	BamHI Met Val Thr ----------	Ser Ser Asn stop stop Not I Cla I
	20 21 ----------	1619 1620 1621

Figure 1. Schematic outline of the primary structure of MSP-1 of *P.falciparum* (FCB-1) and strategy for the synthesis of its coding sequence. (**A**) The protein comprises 1639 amino acids including the signal peptide (SP) and the signal for GPI anchoring (GA). Conserved regions are depicted in white, dimorphic regions in grey. The two blocks showing the highest variability are hatched. Upper arrows delineate the major processing products corresponding to p83–p19 as well as, according to the nomenclature of Stafford *et al.* (34), SP and GA. The lower arrows indicate unique cleavage sites in the synthetic gene. They permit subdivision of the gene into segments encoding the individual processing products. The processing site between p83 and p30 has not been defined experimentally. The sizes of the sequences in bp encoding the various processing products of MSP-1 are depicted below. They allow calculation of molecular weights which for various reasons can significantly deviate from those derived previously by electrophoretic mobility of the respective proteins (2). (**B**) Flow chart of the gene synthesis. Five DNA fragments (83–19) were synthesized which encode the major processing products. They overlap their adjacent fragment by an average of 18 bp, which allows the fusion of neighbouring fragments via common unique endonuclease cleavage sites as indicated. Fragment 83 encoding the signal peptide contains at its 5′-end an *Mlu*I cleavage site (>). The 5′-end of all other fragments contains a *Bam*HI site (◇). The 3′-end of p19 not encoding GA is followed by a *Not*I (O) and a *Cla*I (●) site whereas the version of p19 encoding GA contains only a *Cla*I site. The fragments were fused stepwise as indicated to yield *msp-1*^{S1}. The GA signal of the parasite was introduced by an appropriately modified fragment 19 to yield *msp-1*^{S}, whereas the SP sequence was eliminated from *msp-1*^{S1} by inserting the appropriate oligonucleotide between the *Mlu*I and a unique *Hin*dIII site at position 116. The resulting *msp-1*^{S2} gene can be inserted into expression vectors via its unique *Bam*HI and *Cla*I (or *Not*I) cleavage sites. (**C**) N- and C-terminal sequences of MSP-1^{S}, MSP-1^{S1} and MSP-1^{S2} at the nucleotide and amino acid levels. The numbering of the amino acid positions is according to Heidrich *et al.* (24).

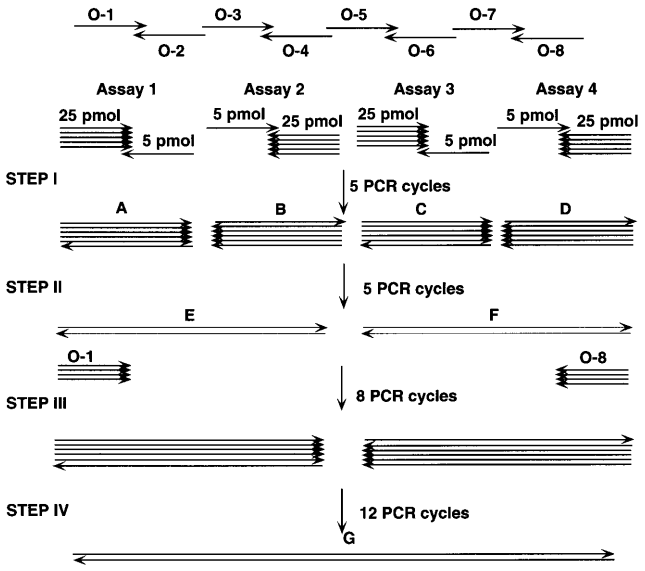


Figure 2. Flow chart for the synthesis of a polynucleotide of 600–1100 bp in length. Eight synthetic oligonucleotides (O1–O8) which overlap their respective neighbouring sequences by an average of 18 nt were mixed pairwise in four assays at the stoichiometry indicated. After five amplification cycles, products A–D are obtained. Fragment A was combined with B and C with D, producing E and F, respectively, after another five additional amplification cycles. Asymmetry in DNA strand composition was reintroduced by amplifying E in the presence of O1 and F in the presence of O8. The final product G was prepared by combining the asymmetric mixtures of E and F and amplifying for 12 cycles. The product was purified from agarose gels. For fragments where 10 or 12 oligonucleotides are required as starting material, as in the synthesis of oligonucleotides encoding p83, purified amplification products corresponding to G and D or F were subjected to amplification steps III and IV to yield the final products such as p83 DNA fragments I and II.

total sequence synthesized including two stop codons and flanking restriction cleavage sites comprised 4940 bp.

Synthesis and cloning of polynucleotides encoding MSP-1 or portions thereof

The full-length *msp-1* gene sequence, designed above, was subdivided into five overlapping fragments each corresponding to one of the major processing products of MSP-1, namely p83, p30, p38, p29 and p19 (Fig. 1B). They were synthesized using the PCR-based procedure outlined in Figure 2 that allows efficient production of double-stranded DNA fragments of up to 1200 bp long. With the exception of DNA encoding p83, each fragment was prepared from four pairs of overlapping synthetic oligonucleotides that covered the fragment as part of the upper or lower strand. To facilitate later assembly of adjacent fragments, the terminal O1 oligonucleotides, which encoded the fragment N-termini, also contained the unique 3′ cleavage sites from the upstream adjacent fragment and the terminal O8 oligonucleotides, encoding the fragment C-termini, contained the unique 5′ cleavage sites from the downstream adjacent fragments. Moreover, every O1 oligonucleotide contained a *Bam*HI site upstream of its unique 5′ cleavage site. Similarly, every O8 oligonucleotide contained two tandem stop codons and a *Cla*I site or a *Not*I and a *Cla*I site downstream of the unique cleavage site (Fig. 1B and C). These features allow the cloning of each individual fragment encoding a processing product of MSP-1 via *Bam*HI/*Cla*I cleavage. An exception to this is the DNA fragment encoding p83, which contained the N-terminal signal peptide. In this case, the 5′-end of O1 contains a *Mlu*I site immediately upstream of the start codon of the full-size gene. This *Mlu*I site allows us to transfer the assembled MSP-1-encoding DNA into any vector via the unique *Mlu*I/*Cla*I or *Mlu*I/*Not*I sites. The synthesis of the DNA fragment encoding p83 and the N-terminal signal sequence required another modification of the scheme due to the size of 2025 bp of the oligonucleotide. The more N-terminal fragment I was synthesized from a total of 12 oligonucleotides of 106–126 bp. Eight of these produced the 5′ portion (778 bp) of fragment I according to Figure 2. The 445 bp 3′ portion was synthesized from four oligonucleotides according to steps I and II in Figure 2. The complete fragment I was then generated by joining these two fragments by PCR as outlined for steps III and IV in Figure 2. The p83 DNA fragment II (954 bp) was generated from 10 oligonucleotides in an analogous procedure. Both p83 DNA fragments I and II were, after sequence verification, combined via a unique *Sca*I site at position 1145.

The full-size gene encoding the entire MSP-1 was obtained by successively joining the various DNA fragments corresponding to the processing products as outlined in Figure 1B. The polynucleotide encoding the entire MSP-1^{S1} was generated by combining the fragment encoding p83 and p30 with the fusion products encoding p38 and p42 via the *Eco*47III cleavage site. Sequences encoding signals for GPI anchoring were attached by inserting properly modified polynucleotides via the *Sph*I/*Cla*I cleavage site whereas modifications of the N-terminal signal peptide were generated by exchanging sequences upstream of the unique *Hin*dIII site (data not shown). The synthetic gene which includes the coding sequence for the authentic signal peptide and for the GPI-mediated anchoring signal of the parasite is designated *msp-1*^{S} (Fig. 1C). For expression studies in *E.coli*, the sequence encoding the signal peptide was removed, yielding *msp-1*^{S2}. This was achieved by replacing the *Mlu*I–*Hin*dIII fragment by an appropriately synthesized *Bam*HI–*Hin*dIII oligonucleotide as outlined in Figure 1B and C. The resulting gene starts with an ATG followed by the codon for amino acid 21 (Fig. 1C). All the sequences described herein were submitted to the EMBL database under accession no. AJ131294.

Controlled expression of *msp-1*^{S2} DNA in *E.coli* and isolation of the products

The *msp-1*^{S2} as well as portions thereof encoding p83, p30, p38, p42 and p19 were placed under the control of an IPTG-inducible promoter in a vector that fuses six histidines to the N-terminus of the expression products. Upon induction, all six proteins were produced in *E.coli* and cell extracts were subjected to Ni^{2+} chelate chromatography under denaturing conditions. Renaturation of the adsorbed material via a urea gradient and subsequent elution with imidazole hydrochloride yielded the six proteins in highly purified and soluble form. The induction of MSP-1^{S2} synthesis in *E.coli* and electrophoretic characterization of purified p19–p83 is shown in Figure 3A. The isolation procedure via an N-terminal histidine tag was chosen since several fragments do not contain the epitope recognized by the 5.2 mAb antibody most suitable for immunoaffinity chromatography. Moreover, high expression levels may lead to inclusion of bodies from which the respective proteins can be readily recovered under denaturing conditions. Adsorption of such proteins to Ni^{2+} chelate supports facilitates their renaturation.

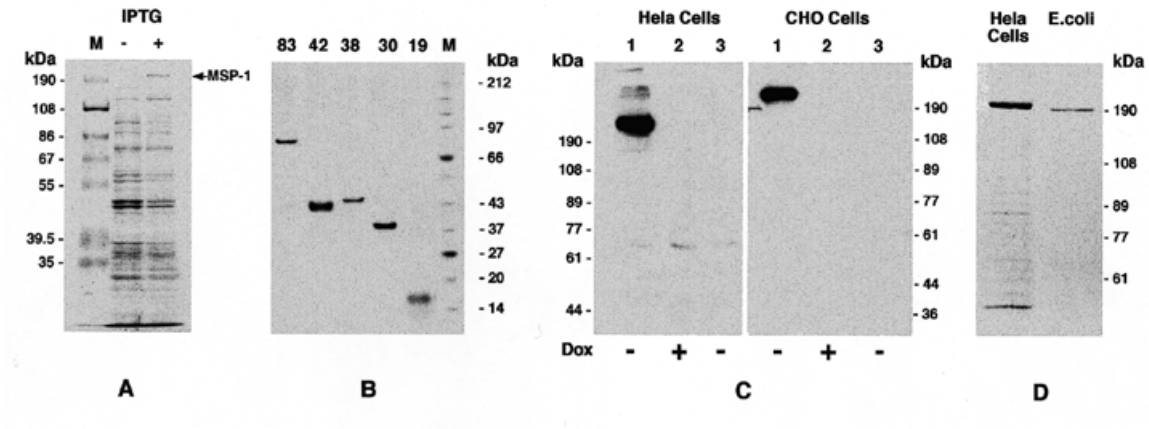


Figure 3. Expression of *msp-1*^{S2} and *msp-1* fragments in *E.coli* and in mammalian cells. (**A**) The *msp-1*^{S2} sequence was inserted into expression vector pDSS6 where it is under control of an IPTG inducible promoter. Upon transfer in *E.coli* strain SG13009, the synthesis of MSP-1^{S2} can be induced (IPTG+). The arrow identifies an induced product with a molecular weight of ~190 kDa. M denotes a molecular weight marker. (**B**) Electrophoretic analysis of IPTG-induced MSP-1 fragments produced in *E.coli* and purified by Ni^{2+} chelate chromatography. The various fragments are indicated above the lane. The designation of the protein fragments (34) is not identical with their molecular mass calculated from the respective sequences (Fig. 1). In addition, some (p38, p30 and p19) show a migration behaviour that does not allow a strict correlation with the molecular weight standard. M denotes a broad range molecular weight marker (New England Biolabs). Coomassie stained 4–12% PAA gradient gel. (**C**) Expression of MSP-1 in HeLa and CHO cells. HtTA-93/9 and CtTA27/29 cells were grown to 40% confluency in the presence of Dox (100 ng/ml) before the antibiotic was removed. After 24 h, cell extracts were prepared and analysed by western blot using mAb 5.2. Lanes 1 and 2 show extracts from induced (–Dox) and uninduced (+Dox) cultures, lane 3 shows extracts from HtTA-1 and CtTA cells. (**D**) Electrophoresis of purified MSP-1 from HeLa cells and from *E.coli*. The left lane shows a MSP-1^{S1} preparation obtained from cultures of HtTA-93/9 cells after immunoaffinity purification using the 5.2 mAb and the right lane shows full-size material from *E.coli* obtained by Ni^{2+} chelate chromatography. Coomassie stained PAA gels.

Production of MSP-1^{S1} in mammalian cells

It may be difficult to obtain in *E.coli* the properly folded form of a protein like MSP-1 that is normally secreted and membrane anchored. We therefore also studied the expression of the synthetic genes encoding MSP-1^{S1} and MSP-1^{S2} in HeLa and CHO cells. Since preliminary results suggested that MSP-1 may interfere with the cellular metabolism (data not shown), its synthesis was controlled via the tetracycline regulatory system (31). Thus, the coding sequences were placed under the control of a bidirectional promoter (30) that is responsive to the tetracycline-controlled transcriptional transactivator (tTA). In these constructs, the expression of the *msp-1* gene is co-regulated with the luciferase reporter gene which is used as a convenient screening tool for identifying stably transformed cell lines. HeLa and CHO cell lines for the controlled expression of the *msp-1*^{S1} gene were generated by transfecting HtTA-1 and CtTA-1 cells, which constitutively produce tTA (31), with the appropriate plasmids. Clones that showed good tetracycline-dependent regulation of luciferase were selected and examined for MSP-1^{S1} synthesis. Several HeLa and CHO cell lines such as HtTA-93/9 and CtTA-27/29 were established. They exhibited high regulation factors for luciferase (up to 10^4-fold) and also co-regulate well the synthesis of MSP-1^{S1} (Fig. 3B). Interestingly, although the *msp-1* gene encodes the genuine signal peptide, no protein could be recovered from the culture supernatant of both HtTA-93/9 and CtTA-27/29 cells, suggesting that the protein is not liberated under these conditions. HtTA-93/9 cells grown at preparative scale allowed us to isolate full-length MSP-1 from cell extracts via immunoaffinity chromatography (Fig. 3D) on columns prepared with mAb 5.2.

Interaction of heterologously produced MSP-1 with monoclonal antibodies directed against the native protein

To gain a first insight into conformational properties of MSP-1 as isolated from *E.coli* and from mammalian cells, purified antigen was reacted with a panel of MSP-1-specific monoclonal antibodies. Of these antibodies, five are specific for the K1 prototype as represented by the FCB-1 sequence, six are known to recognize epitopes within the conserved parts of the molecule and two are specific for MAD20 sequences. Nine of the antibodies react with conformational rather than with sequential epitopes (35,36). When preparations of MSP-1^{S1} from CHO cells and MSP-1^{S2} from *E.coli* were probed with the various antibodies in western blots, a rather striking result was obtained. MSP-1^{S2} isolated from *E.coli* interacted with all 11 antibodies that are specific for the K1 prototype. No interaction was seen with antibody 9.7 specific for the MAD20 prototype or with antibody 12.1, which recognizes an oligomorphic sequence of block IV which is not present in MSP-1 of the FCB-1 strain (Fig. 4 and Table 2). In contrast, MSP-1^{S1} isolated from CHO cells while interacting with most K1-specific antibodies was not recognized by three of the mAbs that bind to MSP-1^{S2} isolated from *E.coli*. Again, as expected, there was no interaction between CHO-derived MSP-1^{S1} and monoclonal antibodies 9.7 and 12.1 (Fig. 4 and Table 2).

DISCUSSION

DNA of *P.falciparum* has an extraordinaryily high AT content which can exceed 90% in intragenic regions and may reach 75% in coding sequences. The reasons for the strong preference of AT over GC, most clearly revealed at the wobble position of codons (Table 1), are not understood. A consequence of the high AT

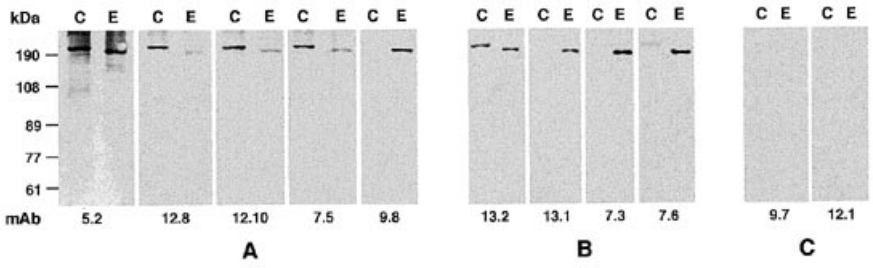


Figure 4. Interaction of MSP-1 from *E.coli* and CHO cells with monoclonal antibodies. MSP-1^{S2} and MSP-1^{S1} isolated from *E.coli* and CHTA-27/29 cells, respectively, were subjected to electrophoresis under non-reducing conditions and analysed by western blot using the monoclonal antibodies indicated. MSP-1^{S1} from CHO cells (C) migrates slower than MSP-1^{S2} from *E.coli* (E) due to glycosylation in the eukaryotic expression system. (**A**) Antibodies directed towards conserved portions of MSP-1. Interactions with antibodies specific for dimorphic regions are shown in (**B**) (K1) and (**C**) (MAD20).

Table 2. MSP-1 purified from *E.coli* and CHO cells, respectively, was subjected to gel electrophoresis under non-reducing conditions

MSP-1 specific antibodies					IFA	Western blot	
Specificity	mAb No.	Epitope Type	Region of interaction	Ref.	*P.f.* FCB-1	*E.coli*	CHO
Conserved	5.2	conformational	p19	32	++++	+	+
	12.8		p42	35,36	++	+	+
	12.10	conformational	p19	35	++++	+	+
	7.5	conformational	p19	35	++++	+	+
	9.8	conformational		35,36	++++	+	-
	2.2	conformational	p19	35	++++	+	+
K1	13.2		p83	35,36	++++	+	+
	13.1		p42	35,36	++++	+	-
	7.3	conformational		35	++++	+	-
	7.6	conformational		35	++++	+	+
	6.1		p42	35,36	++++	+	ND
MAD20	9.7	conformational		36	-	-	-
	12.1		p83 (block IV)	36	-	-	-

Immunoblots with a panel of well-characterized monoclonal antibodies (32,35,36) were prepared and visually evaluated (Fig. 4). Indirect immunofluorescence assays (IFA) were performed as described in Harlow and Lane (33). The number of + reflects the intensity of immunofluorescence. ND, experiment not done due to limiting amounts of mAb 6.1. The western blots for mAb 2.2 and 6.1 are not shown in Figure 4 since limiting amounts of the antibodies did not allow us to carry out this experiment more than once.

content of *P.falciparum* DNA is the failure of cloning and stably maintaining large genes in *E.coli* rendering in-depth studies of the respective gene functions most difficult. In some cases, the biased codon composition was even believed to hamper the expression of *P.falciparum* genes in heterologous systems (37,38).

The synthesis of a 4917 bp long polynucleotide encoding the 190 kDa MSP-1 of the FCB-1 strain in a codon composition that reduces the AT content to 55% has opened up new possibilities for the study of this intriguing protein since the synthetic gene can now be stably cloned and expressed in its entirety in *E.coli* as well as in a variety of other heterologous systems. Several parameters were reconciled in the design of the synthetic *msp-1* gene. Thus, it appeared sensible to place unique endonuclease cleavage sites at or near positions where the protein is processed at the surface of the mature schizont which permits separate cloning and expression of the portions of the *msp-1* gene that encode the different processing products (Fig. 1). Although, with the exception of the GPI anchor at the C-terminus, native MSP-1 of *P.falciparum* appears not to be glycosylated (39) we have conserved potential glycosylation sites as any change in the amino acid composition may destroy epitopes important in the host–parasite interaction. Moreover, choosing proper hosts for MSP-1 synthesis can prevent the modification of such sites and, finally, it will not be difficult to eliminate glycosylation sites at a later stage should it become desirable.

The unique *Hin*dIII site near the 5′-end and the *Sph*I site near the 3′-end of the gene allow for switching signal peptides or membrane anchoring signals. Indeed, besides the sequence

modifications shown here (MSP-1^{S} to MSP-1^{S2}, Fig. 1), we have fused sequences encoding several other specific signal peptides or anchoring signals with the gene (data not shown). Further parameters that were considered are discussed in Results and Materials and Methods.

For synthesizing an oligonucleotide of the size of the *msp-1* gene, we have examined several strategies. The approach described here is based on an asymmetric amplification process that starts out with eight overlapping oligonucleotides and leads to an end product of 600–800 bp, without the requirement of isolating intermediates. Following this strategy, it is essential to limit the size of the starting oligonucleotides to <120, optimally to 70–90 nt, since the error rate of the PCR products increases for longer oligonucleotides, most likely due to incomplete deprotection or to modifications of nucleotides during chemical synthesis. The complete *msp-1*^{S2} gene and all the synthetic intermediates obtained by this procedure were stably cloned in *E.coli* confirming that the parameter responsible for the instability of large *P.falciparum* genes in *E.coli* is the high AT content.

First expression studies with the synthetic sequences in *E.coli* revealed that MSP-1^{S2} was readily produced as an intracellular protein. Moreover, N-terminal fusion of a histidine tag allows its rapid isolation in soluble form via affinity chromatography. Encouragingly, the examination of such MSP-1^{S2} preparations with a panel of monoclonal antibodies, of which several are considered to recognize conformational epitopes, indicates that at least some portions of the protein are properly folded under the conditions used. Production and purification of MSP-1 fragments corresponding to the various processing products of the native protein are even more efficient and all the fragments are obtained as soluble proteins, a prerequisite for structural and functional studies.

Despite the promising results in *E.coli*, proper folding of a complex protein like MSP-1 that is transported to the surface of the parasite may be more readily achieved in a eukaryotic system under conditions of secretion and possibly membrane anchoring. We have, therefore, begun to study the expression of the *msp-1*^{S1} gene in mammalian cells. After initial studies suggested that synthesis of MSP-1 may negatively affect the growth of HeLa cells, we placed the gene under tetracycline control and generated stable HeLa and CHO cell lines where MSP-1^{S1} synthesis is stringently controlled and can be induced over several orders of magnitude. Full-size protein is recovered from cell extracts upon induction. Since the *msp-1*^{S1} gene encodes the genuine signal peptide of the parasite, which is quite similar to other eukaryotic signal sequences, one might anticipate the secretion of the protein into the culture supernatant. We failed, however, to detect any secreted material (data not shown) and are presently analysing in which compartment of the cell the protein accumulates. The isolated protein migrates distinctly slower in an electric field than the protein produced in *E.coli* and thus it can be assumed that it enters the endoplasmic reticulum and the Golgi pathway where it is glycosylated. Interestingly, full-size MSP-1 can be isolated from cell extracts by affinity chromatography with mAb 5.2, i.e. by an antibody that recognizes a conformational epitope near the C-terminus. This indicates again that at least this rather critical domain of the protein may be in a conformation identical to the native one. This conclusion is supported by the reactivity of the antigen with mAbs 12.10, 7.5 and 2.2, all of which have been mapped to conformation-dependent epitopes within p19 of MSP-1 (Table 2).

The availability of a *msp-1* gene that can be transferred, stably maintained and expressed in various biological systems will advance the elucidation of its role in the parasite's life cycle. This will include structural analysis of the intact protein as well as of its processing products. Of particular interest will be the analysis of the interaction of MSP-1 and its processing products with erythrocytes.

Several approaches are under way in our laboratory. For example, we have successfully placed full-size MSP-1 as well as portions thereof onto the surface of HeLa cells (P.Burghaus, manuscript in preparation) and *Toxoplasma gondii* where they are anchored by a GPI moiety (I.Türbachova *et al*., manuscript in preparation). Interaction with our collection of mAbs suggests that the surface-exposed proteins have assumed the natural conformation. These systems are opening up a variety of experimental strategies aimed at the analysis of MSP-1 function. This will include not only the interaction of MSP-1 or any portion thereof with the surface of erythrocytes but also questions concerning the maturation of the MSP-1 precursor. Thus, heterologously produced MSP-1 or portions thereof may constitute useful substrates for proteases involved in this process (41). Finally, it will be possible to study the interaction between purified MSP-1 fragments representing the natural processing products which may allow reassembly of the MSP-1 complex *in vitro*. Together, such studies may lead to new insights into the early phases of erythrocyte invasion and reveal new targets for interfering with *P.falciparum* infection at the blood stage.

The synthetic *msp-1* gene will also facilitate the rigorous examination of the protective potential of MSP-1 or any portion thereof when used as an experimental vaccine. Most of the recent MSP-1-based vaccination trials focused on the C-terminal portion of the protein, in part for technical reasons. While such studies have clearly identified this region of the molecule as promising, the analysis can now be extended throughout the entire molecule as there is little reason to exclude any portion of this surface protein from such examination, particularly considering the contribution of cellular responses towards malaria immunity where MSP-1 could play a role (40). The FCB-1-derived amino acid sequence encoded in our synthetic gene is highly homologous to MSP-1 of the Colombian FVO strain which is well adapted to *Aotus* monkeys. Novel experimental vaccines that include the entire protein or any portion thereof can now be tested in this animal model and first monkey trials with vaccines based on heterologously expressed proteins as well as a recombinant attenuated *T.gondii* are under way. The sequence chosen here belongs to the K1 prototype. It diverges maximally from the MSP-1 sequence of the 3D7 strain, a representative of the MAD20 prototype. The synthesis of the gene encoding the 3D7 MSP-1 is presently being completed in our laboratory. Together, the two genes will allow comparative structural and immunologic studies. In particular, they will permit vaccination studies with heterologous challenge infections in *Aotus* monkeys. Moreover, the availability of unlimited amounts of MSP-1 proteins representing the processing products or any other portion of the K1- and the MAD20-derived MSP-1 will make a detailed analysis of the humoral response in populations where *P.falciparum* is endemic feasible. Such analyses may lead to more reliable correlations between patterns of humoral response and susceptibility towards infection and disease as was suggested in earlier studies (10,11), possibly allowing for the development of diagnostic tools with

predictive value. Indeed, analysing sera from recent immunization trials with *Aotus* monkeys using the various MSP-1 fragments have revealed clear correlations between antibodies directed towards certain areas of the protein and protection (R.Tolle *et al*., manuscript in preparation).

Finally, since the well-characterized 3D7 strain is being used in human trials for challenge infections, immunization of humans followed by homologous or heterologous challenge appears feasible in the near future. Together, such studies should increase our understanding of the role of MSP-1 in the parasite's life cycle, the basis of its dimorphic nature and its potential as a component in a subunit vaccine.

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