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Shotgun DNA microarrays and stage-specific gene expression in *Plasmodium falciparum* malaria

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Summary

Malaria infects over 200 million individuals and kills 2 million young children every year. Understanding the biology of malarial parasites will be facilitated by DNA microarray technology, which can track global changes in gene expression under different physiological conditions. However, genomes of *Plasmodium sp.* (and many other important pathogenic organisms) remain to be fully sequenced so, currently, it is not possible to construct gene-specific microarrays representing complete malarial genomes. In this study, 3648 random inserts from a *Plasmodium falciparum* mung bean nuclease genomic library were used to construct a shotgun DNA microarray. Through differential hybridization and sequencing of relevant clones, large differences in gene expression were identified between the blood stage trophozoite form of the malarial parasite and the sexual stage gametocyte form. The present study lengthens our list of stage-specific transcripts in malaria by at least an order of magnitude above all previous studies combined. The results offer an unprecedented number of leads for developing transmission blocking agents and for developing vaccines directed at blood stage antigens. A significant fraction of the stage-selective transcripts had no sequence homologues in the current genome data bases, thereby underscoring the importance of the shotgun approach. The malarial shotgun microarray will be useful for unravelling additional important aspects of malaria biology and the general approach may be applied to any organism, regardless of how much of its genome is sequenced.

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Introduction

In the fight against malaria, there are only eight commonly used drugs and no reliable vaccines (White, 1996; Holder, 1999). Many strains of the malaria parasite *Plasmodium falciparum* are now resistant to our antimalarial compounds (Peters, 1998) and, in some parts of the world, resistance to new antimalarial agents may be occurring faster than before (Rathod *et al*., 1997). To help overcome these problems, global malaria initiatives have invested heavily in sequencing the *Plasmodium falciparum* genome and the next challenge is to correlate genome sequences to function (Wellems *et al*., 1999). Based on sequencing efforts to date, about half the malarial genome coding regions will have unknown function (Gardner *et al*., 1998; Bowman *et al*., 1999). Relating these genome sequences to malaria biology will be particularly challenging because the experimental tools to study malaria are limited (Wellems *et al*., 1999). First, most species of malarial parasites and most stages of *P. falciparum* cannot be routinely maintained in cell culture. Even the erythrocytic cycle of *P. falciparum,* which can be cultured, is very slow, labour intensive, and expensive to propagate. Second, the experimental power of transfection technology in *P. falciparum* and other malarial species is restricted at present. Although the erythrocytic stages can be transfected, gene disruptions are only possible for non-essential genes, as this part of the parasite life cycle is haploid (Wellems *et al*., 1999). Gene replacement is not possible because there is no negative selection system. Transfection efficiencies in *P. falciparum* are so poor that no gene function has been established purely on the basis of genetic complementation with a library of malarial genes or through a population of random knock outs. Finally, as the complete sexual life cycle of *P. falciparum* can only be studied in mosquitoes and as yet not *in vitro,* classical genetics can only be performed with great difficulty (Walliker *et al*., 1987). Not surprisingly, only two genetic crosses have been performed with malaria parasites and only a handful of traits have been mapped (Walliker *et al*., 1987; Vaidya *et al*., 1995; Wang *et al*., 1997; Wellems *et al*., 1999).

Clearly, there is an urgent need for additional methods for assessing gene function in malaria. Recently, it has become possible to decipher transcriptional programmes of organisms by studying gene expression en masse (Brown and Botstein, 1999). DNA microarray technologies offer an opportunity to look at changes in gene expression in thousands of genes simultaneously under different physiological conditions (DeRisi *et al*., 1997; DeRisi and Iyer, 1999).

Because the malarial genome is not completely sequenced, a variation on the standard array technology was used in this study. Inserts from a malarial genomic library were arrayed randomly to generate 'shotgun' microarrays. To measure variation in expression of genes during the parasite life cycle, the arrays were probed with differentially labelled cDNAs prepared from total RNA isolated from cells at defined developmental stages. PCR products on the array that showed differential hybridization were sequenced.

Results and discussion

Array construction

The malaria shotgun microarray was constructed by printing 3648 PCR-amplified inserts from a *P. falciparum* DNA library (Fig. 1). To provide as complete a representation of genes as possible, and to minimize bias towards specific sequences, a mung bean nuclease genomic library was used. Mung bean nuclease preferentially cuts malarial DNA in regions flanking coding regions (McCutchan *et al*., 1984; Vernick and McCutchan, 1998). Such digestion was expected to capture long stretches of unique coding regions and avoid over-representation of flanking sequences or introns on the array. Individual colonies from the unamplified library were immediately transferred to a 96-well plate. Amplified inserts from 8000 independent clones were analysed by agarose gel electrophoresis. Only PCR products greater than about 300 bp were applied on the DNA array. The average size of the insert applied to the array was 1–2 kb, but some clones had PCR products as large as 5 kb. In addition to clones from this library, several previously characterized genes encoding stage-specific malarial surface antigens (MSP-1, Pfs25, Pfs28, Pfs48/45) were included in the prototype array (Holder, 1988; Kaslow *et al*., 1988; Duffy *et al*., 1993; Kocken *et al*., 1993).

Transcriptional differences between trophozoites and gametocytes

The usefulness of the shotgun microarray for analysing malarial transcription programmes was evaluated by comparing gene expression between two differentiated forms of *Plasmodium*. Trophozoite-specific RNA was used as a template to generate Cy3-labelled cDNA (green fluorescence) and late-stage gametocyte-specific RNA was used to generate Cy5-labelled cDNA (red fluorescence). Equal amounts of the two labelled cDNA populations were mixed and hybridized to the shotgun microarray. Fluorescence signals from Cy3 and Cy5 label were separately measured at each spot on the array using a

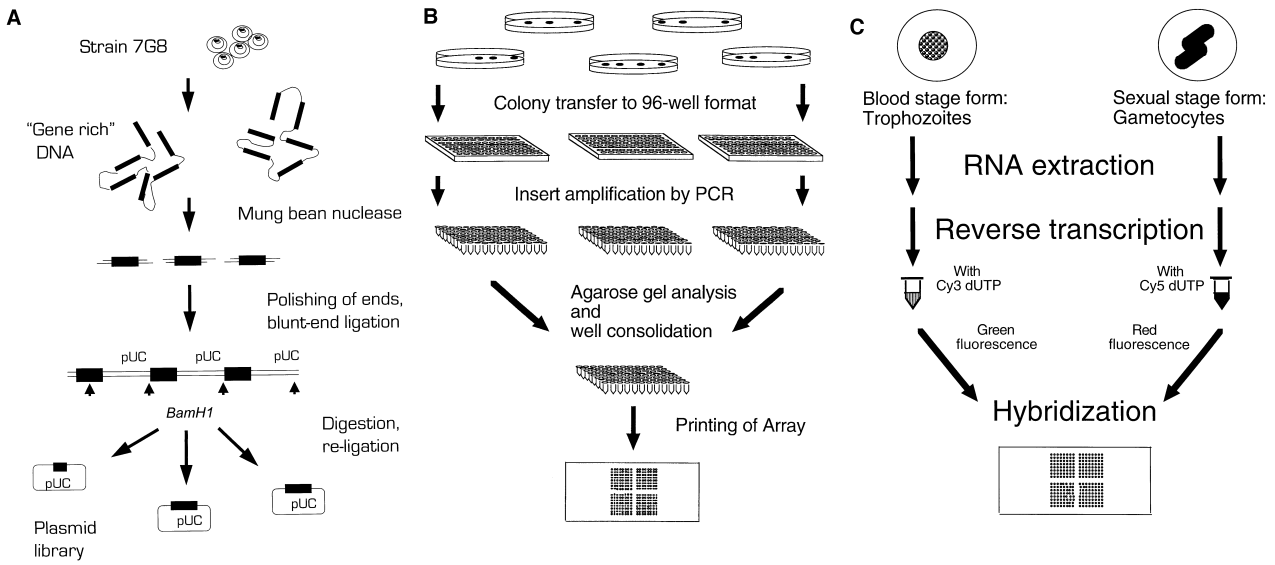

Fig. 1. Construction of a shotgun microarray and identification of stage-specific genes.
A. Preparation of mung bean nuclease-digested malaria genomic DNA.
B. Preparation of PCR products for shotgun DNA microarray.
C. Differential labelling of trophozoite and gametocyte RNA and stage-selective hybridization to the shotgun array.

scanning confocal microscope (DeRisi *et al*., 1997). The red/green fluorescence ratio provided a measure of the relative abundance of transcripts, from each DNA segment represented on the array, in trophozoites compared with late-stage gametocytes (Fig. 2A; the raw data from this hybridization and all the figures in this publication may be accessed on the web at http://derisilab.ucsf.edu/malaria/).

Reliability

The faithfulness of the shotgun DNA microarray for reporting stage-specific gene expression was apparent in four ways.

First, three separate hybridizations from three independent cDNA preparations showed virtually identical differential hybridization patterns (Fig. 2B). Second, genes such as Pfs25, Pfs28, Pfs48/45 and MSP1, which were known to be expressed in a stage-selective fashion and which were applied to the microarray as controls, exhibited the expected stage-specific expression (Table 1, standard genes). Third, the 50 arrayed genes showing the highest red/green fluorescence and the 35 genes with the highest green/red fluorescence were sequenced, they were found to include several previously known stage-specific genes (Table 2A and B).

Among the trophozoite-selective gene transcripts identified in this way, MSP-1 was represented twice (Table 2A). Other transcripts such as HRP-1 (histidine-rich protein-1), RAP-1 (rhoptry-associated protein-1) and PfEMP-3 (*P. falciparum* erythrocyte membrane protein 3) were also found to be trophozoite-specific in comparison to stage IV–V gametocytes. The stage-specific expression of these proteins is consistent with association of knob proteins, rhoptry proteins, PfEMP 3 and merozoite function in asexual stage parasites (Holder *et al*., 1985; Ellis *et al*., 1987; Holder, 1988; Pasloske *et al*., 1993), but not in late stage (III–V) gametocytes (Day *et al*., 1998).

Among the sexual stage-selective transcripts, we identified sequences coding for the known gametocyte-specific genes Pfg377 and Pfs2400 (11.1 gene) (Table 2B, Fig. 3A;

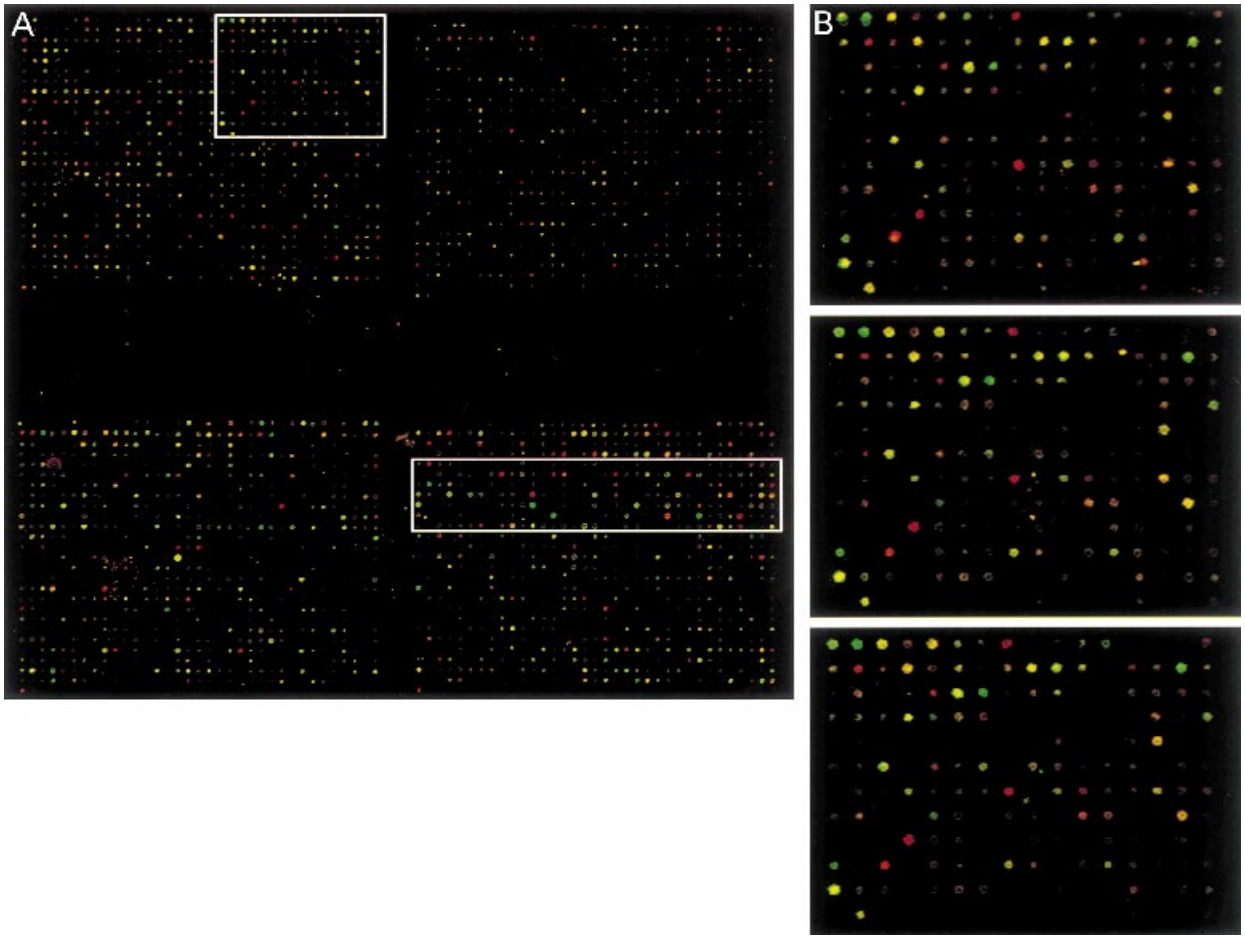

Fig. 2. Fluorescence image of differential hybridization from trophozoite-specific cDNA (green) compared with gametocyte-specific cDNA (red).
A. The whole shotgun microarray. Lower right strip is shown in detail in Fig. 3A.
B. Reproducible hybridization of three independent pairs of cDNA preparations from identical RNA preparations to three shotgun microarrays.

Table 1. Hybridization characteristics of standard clones applied on the shotgun microarray and of related sequences identified on the array.

Clone name	Source	Clone ID	Trophozoite (Cy3)/gametocyte (Cy5) fluorescence ratio
MSP-1	Standard PCR product	3642	5.02
MSP-1	Shotgun array	2047	5.47
MSP-1	Shotgun array	2848	4.96
Pfs28	Standard PCR product	3645	0.38
Pfs 48/45	Standard PCR product	3646	0.39
Pfs 25	Standard PCR product	3644	0.67

Feng *et al*., 1993; Alano *et al*., 1995). Other gametocyte-specific genes, Pfs25, Pfs28 and Pfs48/45, were not found among the 50 sexual stage-specific clones chosen for sequencing. The cDNAs for these genes were, however, present in the fluorescent probe mix as the control clones corresponding to these genes exhibited the appropriate expression profile (Table 1, standard PCR products).

Fourth, for several of the new genes of unknown function in malaria that were shown by the DNA microarray to be expressed selectively in trophozoites or selectively in gametocytes, stage-specific expression was assessed, and in each case confirmed by conventional Northern blotting (Fig. 3A and B).

Advantages of the shotgun approach

The benefits of the shotgun microarray for understanding malaria biology were apparent even from a limited analysis. For instance, functionally interesting malarial ESTs (including several enzymes and a gene resembling *Toxoplasma gondii* myosin C), which were previously not known to be expressed in a stage-specific manner, were shown to be overexpressed in trophozoites (Table 2A). Similarly, several ESTs (including key enzymes such as a lysophospholipase) were found to be preferentially expressed in gametocytes for the first time (Table 2B). To date, very few sexual stage-specific proteins have been identified in *P. falciparum.* This has been due, in part, to the impractical quantities of parasites needed to obtain native proteins in sufficient quantity for microsequencing. Many years work has revealed just a dozen or so genes in total that are expressed only in the sexual stage. However, even from this small pool, three genes encoding surface proteins Pfs48/45, Pfs230 and Pfs27/25 present feasible candidates for transmission-blocking malaria vaccines (Quakyi *et al*., 1987; Kaslow *et al*., 1988; Duffy *et al*., 1993). In the present study, a single microarray hybridization has added substantially to our list of candidate genes for testing as transmission blocking agents. It is noteworthy that MSP-1, a leading candidate for malaria vaccines (Holder, 1988; Riley *et al*., 1992), is one of the most prominent blood stage-specific transcripts identified by microarray hybridization (Table 2A). Other transcripts from Table 2A may prove to be important new targets for asexual stage vaccines. Some stage-specific sequences from the present work revealed identity to genes in malarial genome data bases whose functions until now were completely unknown (Table 2A and B). As more hybridizations are performed, using RNA extracted from parasites under different physiological conditions, clues to the physiological roles of the 'orphan genes' are likely to emerge (Brown and Botstein, 1999). Finally, the shotgun microarray revealed a large number of stage-specific sequences that were new. Of the 51 stage-specific unique sequences examined, 23 clones (45%) had no homology to sequences currently in Genebank or in the malaria data bases (Table 2A and B). Until the genome sequencing project is complete, the random array provides a way to identify biologically important variation in gene expression that would be missed if arrays were constructed strictly using sequences available in the present data bases.

Limitations of the shotgun approach

The principal drawbacks of the shotgun microarray are, first, that the sequence of each array element is not known from the start. Therefore, to identify a DNA fragment that shows an interesting variation in gene expression, the clone must be sequenced. A second limitation is that the arrayed DNA segments do not necessarily correspond to unique transcripts.

Representation of the malarial genome

It is estimated that the prototype microarray reported here represents $\approx$ 40% of the coding sequences of the parasite. This estimate is based on the following consideration. Approximately 36% of the highly stage-specific expression sequences were represented more than once on the microarray. If this ratio holds for all genes, 64% of the 3648 clones making up the microarray are likely to be unique. Thus, 2335 unique genes, out of an estimated total of 6000 genes in the *P. falciparum* genome, would be represented on the array. Based on these calculations, we estimate that a shotgun microarray with an additional 10 000 clones would be sufficient to cover almost all of the malarial genes. Such a comprehensive array is under construction and evaluation in our laboratories.

Table 2. Identification of stage-selective gene expression on the microarray. Subsets of individual clones contained within the microarray that hybridized to (A) trophozoite cDNAs or (B) gametocyte cDNAs are shown with their sequence similarity to *P. falciparum* genes entered in nucleic acid and protein data bases. In parentheses are known genes from other organisms that share sequence similarity to the clones listed.

	Sequences producing high-scoring segment pairs			
Clone ID	Known genes	GenBank entry	New sequence	*P. falciparum* accession number
A. Trophozoite stage gene transcripts				
856	HRP-1			Y00060
1318			Unknown	AF192539
2973			Unknown	Small insert
2913			Unknown	AF192540
1172, 2552, 371, 2596		HTGS (chromosome 4)		AL034557
3594	PfEMP3			AE001371
1522			Unknown	AF192541
132, 77		HTGS		PF14_png32_7631..8270
1008		HTGS (chromosome 4)		AL010241
1679, 3334, 2835	RAP-1			U41077
366			Unknown	AF192542
159, 1552		EST(serine/threonine phosphatase alpha)		N97612
730			Unknown	AF192543
2047, 2848	MSP-1			M19143
3272			Unknown	AF192971
3501			Unknown	AF192544
2202			Unknown	Small insert
2567, 2671		EST		N97785
2585, 2012	Pfg27/25			X849041
645	*P. falciparum* hypothetical protein			AE001428
2897			Unknown (serine/threonine kinase isologue)	AF192545
2195			Unknown (myosin C)	AF192546
544	R45 gene			M83793

Table 2. Continued

Clone ID	Sequences producing high-scoring segment pairs: Known genes	GenBank entry	New sequence	*P. falciparum* accession number
B. Gametocyte transcripts				
2959, 3167, 156, 2153		HTGS (chromosome 12)		AC004157
108, 2388, 3571		EST		N98072
2009			Unknown	AF192972
982, 948	Pfg377			L04161
1532	Var gene			AE001366
1223, 1390, 2887			Unknown	AF192973
3339, 2912		EST (lysophospholipase)		AA550106
60, 1421, 1962, 740			Unknown	AF193273
1449		HTGS		PF14_png124_27131..27574
1321		HTGS (chromosome 3)		AI010142
2334, 2123, 1476, 3075, 150, 288			Unknown	AF193623
2905, 743		EST		N98067
786			Unknown	AF193622
694	Pf11.1 gene			X07454
1205		HTGS		PF14_png75_15519..15911
1364			Unknown	AF193621
1345, 1240			Unknown	AF193620
3220		HTGS		PF14_png106_77121..77569
1830		HTGS (PEP carboxykinase)		PF14_png47_3684..4127
1299		EST		N98408
2302			Unknown	AF193619
3583, 824, 2696		HTGS (chromosome 12) (dihydrolipomide dehydrogenase)		AC004710
2871		HTGS		PF14_png11_34755..34951
753			Unknown	AF193274

EST, expressed sequence tag; HTGS, high throughput genome sequence; MSP-1, merozoite surface protein 1; RAP-1, rhoptry-associated protein 1; HRP-1, histidine-rich protein 1. 'Small insert' refers to reads of less than 50 bp. Longer versions of these two sequences will be put on the website (http://derisilab.ucsf.edu/malaria/).

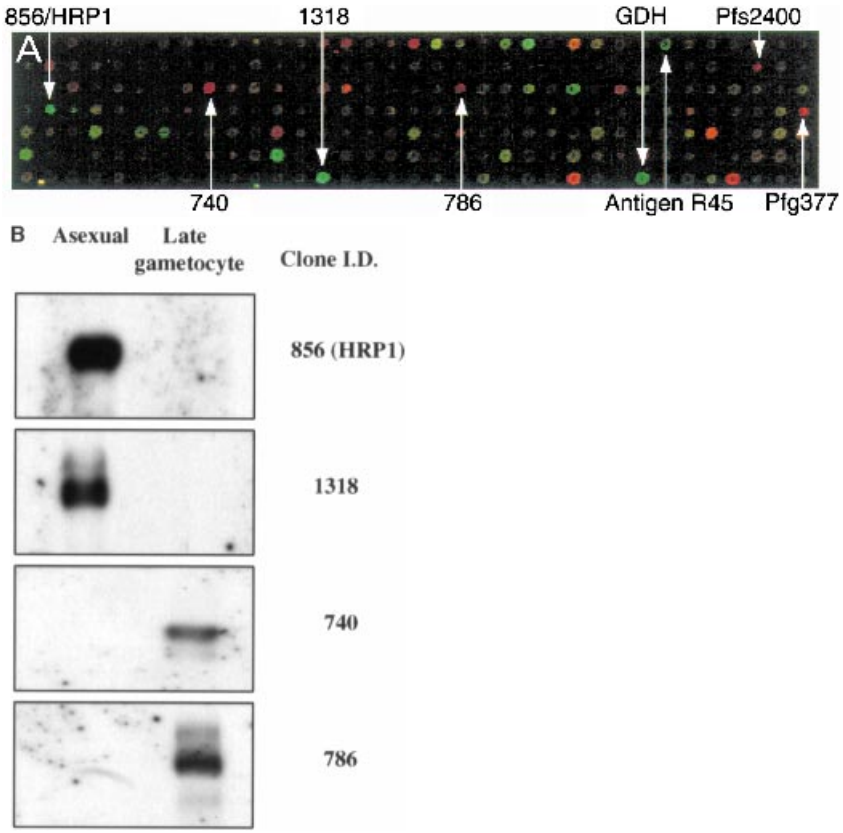

Fig. 3. A. Fluorescence image of some representative known and unknown genes that showed stage-specific gene expression.
B. Northern blots confirm stage-specific upregulation of four transcripts at different stages of *P. falciparum* development as measured by the shotgun microarray. Clone 856 (HRP-1) and clone 1318 are trophozoite specific, and clones 740 and 786 are gametocyte specific.

Shotgun microarrays for other organisms

The shotgun approach to microarray construction may be useful to study differential gene expression in many other important organisms whose genome sequences remain unavailable particularly because the 'genase' activity of mung bean nuclease is not unique to malaria but is also applicable to DNA of other organisms (Brown *et al.*, 1986; Vernick and McCutchan, 1998). For organisms having small intragenic regions, arrays may also be constructed from genomic libraries prepared using common restriction enzymes instead of mung bean nuclease.

Experimental procedures

Mung bean nuclease library

DNA from *P. falciparum* strain 7G8 was digested with mung bean nuclease (Kaslow and Hill, 1990). The digested fragments were blunt ended and initially cloned into a *Sma*I site of pUC13 vector. To avoid tandem repeats, the initial library was digested with *Bam*HI and resealed under conditions that favour intramolecular ligation (see Fig. 1A). A ligation mix containing about 25 ng of DNA was electroporated into 20 μl of DH10B competent cells (Gibco BRL) and the transformants were incubated overnight on agar plates in the presence of 100 μg ml^{-1} of ampicillin.

Malaria microarray

Individual clones from the fresh mung bean library were transferred to 50 μl solution of Luria–Bertani (LB) medium (with 100 μg ml^{-1} of ampicillin) in individual wells of a 96-well plate and incubated overnight at 37°C. About 1 μl from each well was used for PCR amplification of the inserts using the pUC13 flanking sequences PUCFOR (CCCAGTC ACGACGTTGTAAAACG) and PUCRVS (AGCGGATAA CAATTTCA CACAGG) as universal primers. The PCR products were analysed on 0.8% agarose gels and those showing a product larger than 300 bp were consolidated into 38 new 96-well plates. The PCR products from 3648 wells were precipitated, washed and printed on glass slides as previously described (DeRisi *et al.*, 1997).

Stage-specific malarial RNA

Plasmodium falciparum clone 3D7 was maintained in continuous culture and mature asexual trophozoites were isolated from highly synchronous cultures using gelatine

flotation (Pasvol *et al*., 1978). Sexual stage gametocytes were induced and grown to stages III–V of development (Day *et al*., 1998). Gametocyte cultures were layered on discontinuous gradients of 6%, 11% and 16% Accudenz (Accurate) and centrifuged for 10 min at 10 000 × *g*. Late gametocytes, stages III–V, were retrieved from the 11/16% interface and washed once in warm RPMI–Hepes medium before RNA extraction (Quakyi *et al*., 1987). Purity of asexual and late sexual stages was checked by Giemsa-stained blood film and by immunofluorescence assay using a monoclonal antibody against gametocyte-specific protein Pfs48/45. Total RNA was extracted immediately from enriched parasites using Trizol reagent (Gibco BRL).

Differential hybridization

Fluorescent cDNA was made by incorporation of Cy3-dUTP (for blood stage RNA) or Cy5-dUTP (for gametocyte RNA) during single-stranded synthesis with reverse transcriptase (DeRisi *et al*., 1997). The hybridized cDNA on the glass arrays was processed and analysed for fluorescence as previously described (DeRisi *et al*., 1997).

Sequence analysis

Individual clones were sequenced using dye terminator cycle sequencing reactions (Perkin-Elmer) and an automated ABI Prism 377 DNA sequencer. Sequences were compared with others through searches of the non-redundant protein and DNA sequence data bases at the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD, USA) using the gapped BLAST program and the PSI-BLAST program. Sequences were also compared with those entered as high throughput genome sequence (HTGS) from the *P. falciparum* genome project consortium at The Institute for Genome Research, Stanford University, USA, and the Sanger Centre using their online BLAST servers.

Sequence data for *P. falciparum* chromosomes 3 and 4 was obtained from The Sanger Centre website at http://www.sanger.ac.uk/Projects/P_falciparum/. Sequencing of *P. falciparum* chromosome 3 and 4 was accomplished as part of the Malaria Genome Project with support by The Wellcome Trust. Sequence data for *P. falciparum* chromosome 12 was obtained from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/malaria. Sequencing of *P. falciparum* chromosome 12 was accomplished as part of the Malaria Genome Project with support by the Burroughs Wellcome Fund. Preliminary sequence data for *P. falciparum* chromosome 14 was obtained from The Institute for Genomic Research website (www.tigr.org). Sequencing of chromosome 14 was part of the International Malaria Genome Sequencing Project and was supported by awards from the Burroughs Wellcome Fund and the US Department of Defense.

Northern blotting

Total RNA, 22 μg from each parasite life stage, was subjected to electrophoresis in 1.4% agarose/formaldehyde gels and transferred to nylon membrane (Hybond-N^+, Amersham) by capillary blotting overnight using standard techniques. DNA probes were generated by restriction digestion of mung bean inserts from pUC 13 clones followed by random priming with [^{32}P]-dATP. Filters were incubated with probe overnight at 42°C and then washed at the same temperature in 0.1× SSC and 0.5% SDS for 30 min and then at 55°C for 30 min in the same wash solution. Blots were exposed to autoradiographic film (BioMax MR, Kodak) overnight.

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