Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7

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**ABSTRACT** The resurgence of malaria in recent decades has been accompanied by the worldwide spread of resistance to chloroquine, a drug once uncontested as the first-line antimalarial agent because of its efficacy and low toxicity. Chloroquine-resistant strains of *Plasmodium falciparum* counter the drug by expelling it rapidly via an unknown mechanism. In the absence of explicit biochemical knowledge of this efflux mechanism, reverse genetics provides a powerful approach to the molecular basis of chloroquine resistance. Here we report genetic linkage analysis in which 85 restriction fragment length polymorphism markers were used to examine inheritance of the 14 *P. falciparum* chromosomes in a laboratory cross between a chloroquine-resistant and a chloroquine-sensitive parasite. Inheritance data from 16 independent recombinant progeny show that the rapid efflux, chloroquine-resistant phenotype is governed by a single locus within an ~400-kilobase region of chromosome 7. Identification and characterization of genes within this region should lead to an understanding of the chloroquine-resistance mechanism.

Malaria chemotherapy is increasingly hampered by the spread of chloroquine-resistant *Plasmodium falciparum* strains in Southeast Asia, Africa, and South America (1, 2). Greater reliance on a limited number of less desirable alternative drugs now is necessary, accentuating the need to understand chloroquine resistance and develop measures that circumvent it. Chloroquine-resistant parasites are known to rid themselves of the drug 40 to 50 times faster than sensitive parasites (3), but the biochemical basis of this efflux is unknown. To determine the genetics of chloroquine resistance and to investigate the mechanism of rapid drug efflux, we have completed a laboratory cross between chloroquine-resistant (Dd2) and chloroquine-sensitive (HB3) *P. falciparum* parasites (4). Sixteen progeny clones were obtained that exhibit different combinations of parental markers, indicating that each clone arose from a separate meiotic event. Eight progeny possessed the same absolute levels of resistance and rapid chloroquine-efflux rates as the chloroquine-resistant Dd2 parent; eight others exhibited the chloroquine response and slow-efflux rate of the chloroquine-sensitive HB3 parent. No progeny clone possessed an intermediate or nonparental chloroquine phenotype, thus suggesting that a single locus, perhaps a single gene, governs the rapid-efflux mechanism (4).

Chloroquine resistance and, presumably, the efflux mechanism in *P. falciparum* is partially reversed by verapamil in vitro (5). Because this reversal phenomenon is in some ways analogous to the reversal of multidrug resistance (mdr) in mammalian tumor cell lines, chloroquine resistance has been postulated to involve an energy-driven P-glycoprotein pump similar to that encoded by the mammalian mdr gene (5). Two mdr-like *P. falciparum* genes have been identified (6, 7) of which one, pfmdr1, was proposed to mediate (6) or provide a competent basis for resistance (8). Examination of the HB3 × Dd2 cross showed, however, that pfmdr1 was not linked to the rapid-efflux mechanism (4). Reported sequence data from two chloroquine-resistant strains (Nigeria 60 and P120) also provide exceptions to the conclusion that one of two mutant pfmdr1 alleles (Tyr86 or Cys1034/Asp1042/Tyr1246) is necessary for chloroquine resistance (8). These findings indicate that another molecular mechanism, independent of amplification or mutations in the pfmdr1 gene, accounts for the rapid-efflux, chloroquine-resistant phenotype in *P. falciparum*.

In the absence of explicit biochemical methods to identify the factor responsible for the efflux mechanism, reverse genetics (9) provides an approach to cloning the chloroquine-resistance gene. Because the efflux mechanism appears to be controlled by a single locus (4), restriction fragment length polymorphism (RFLP) analysis (10) can be used to map this locus to a specific chromosome region. Here we report genetic linkage-analysis studies in which RFLP markers have been used to examine inheritance of the 14 parasite chromosomes in the 16 different progeny from the HB3 × Dd2 cross. The rapid-efflux, chloroquine-resistant phenotype is shown to be governed by a single locus within a ~400-kilobase (kb) region of chromosome 7.

**MATERIALS AND METHODS**

*Parasite Cultivation and DNA Extraction.* Parasite clones from the HB3 × Dd2 cross were cultivated in *vitro* by standard methods (11). DNA was purified as described (12).

*Chromosome Markers.* Fourteen probes obtained in plasmid vectors were used to detect genes encoding *P. falciparum* proteins: glucose phosphate isomerase (13); the *Mr* 25,000 sexual-stage-specific antigen (PfS25) (14); thrombospondin-related anonymous protein (15); the *Mr* 130,000 putative glycophrin-binding protein (GBP-130) (16); gp195 precursor to the major merozoite surface antigen (17); a candidate glucose-regulated protein (grp) (18); dihydrofolate reductase/thymidylate synthase (19); the knob-associated histidine-rich protein (20); the circumsporozoite protein (21); ribosomal RNA-encoding DNA (22); histidine-rich proteins II and III (23); and two parasite homologues of mammalian P-glycoproteins (4). Antisense oligonucleotides were purchased from Synthecell (Rockville, MD) to detect genes encoding other proteins: 5'-GGT-GCG-AGG-GCA-TCC-ACT-TCA-GCT-TC-3' for the acidic basic repeat antigen (24); 5'-CGG-TGG-ATA-CTA-CAC-CTA-ATA-AAC-CTG-C-3' for exported protein 1 (Exp-1) (25); 5'-GAT-GGG-ATG-GGA-CAA-AGC-AGT-AGT-AGC-3' for apical membrane antigen 1 (26); 5'-GTC-ATT-ATC-ATT-ACC-TCC-TCC-TTC-TCC-3' for the S-antigen (27); 5'-AGC-AAG-TCT-CTC-TTC-TTC-TAA-ATC-GCT-3' for a liver-stage-specific antigen (28); 5'-AGT-ATT-TTC-AGA-TTC-ACC-AGT-ATT-TTC-3' for the mature-parasite-infected erythrocyte surface antigen (29); 5'-AAC-ACA-AGA-ACC-TGT-GAC-AAC-ACA-

Abbreviations: RFLP, restriction fragment length polymorphism; PFG, pulsed-field gradient.

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AGA-3' for the *falciparum* interspersed repeat antigen (30); 5' - TGA- ACT- TGA- ACT- AGA- ACT- TGA- ACT- TGA- ACT-3' for the serine repeat antigen (31); 5' - AGG- AGT- AAT- TGG- TGA- TTC- TCC-3' for the Mr 45,000 merozoite surface antigen (32); 5' - GGT- CTC- TCC- ATT- CAC- TAG- GTA- TGT- GGA-3' for the clustered-asparagine-rich protein (33), and 5' - AGC- ATC- AGT- TTC- TCC- ATT- TTC- TTC-3' for the ring-inkaged erythrocyte surface antigen (34). A 0.2-kb fragment specific for the S-antigen gene was also amplified from Dd2 DNA by a PCR using the above S-antigen oligonucleotide and the forward S-antigen primer 5'- ATG- AAT- AGA- ATA- TGG- TGC- TTT- TCA- T-3'.

**RFLP Markers.** Restriction fragment length polymorphisms distinguishing the HB3 and Dd2 parents were identified from DNA digested with selected endonucleases. RFLP markers included gene probes (above) and anonymous probes picked randomly from genomic DNA libraries in plasmid vectors (13, 35) or Agt11 (36). Plasmids were prepared by the alkaline lysis method (37); inserts from recombinant bacteriophage were amplified in PCR reactions by using Agt11 insert primers (Clonetech). Oligonucleotides were 32P-end-labeled by using polynucleotide kinase (Bethesda Research Laboratories). Plasmids and PCR products were 32P-labeled by a random-hexamer-priming system (Boehringer Mannheim). DNA was fractionated by agarose gel electrophoresis, transferred to Nytran, and probed with labeled oligonucleotides or random-primer probes as recommended (Schleicher & Schuell). Blots were stripped between hybridizations with NaOH (12) or with 2× standard saline phosphate/EDTA (SSPE; 0.18 M NaCl/10 mM phosphate, pH 7.4/1.0 mM EDTA)/50% formamide at 65°C (37).

**Chromosome Mapping.** Markers were assigned to *P. falciparum* chromosomes by pulsed-field gradient (PFG) electrophoresis (12). Chromosomal DNA of the Dd2 clone was separated by preparative PFG electrophoresis, excised in agarose blocks, and mapped with BglI, BstIII, SmaI, and ApaI by described methods (12, 35). For Dd2 chromosome 7, DNA of both chromosomes 7 and 9 was recovered as a doublet from the preparative PFG gels. Chromosome 7 fragments were distinguished from chromosome 9 fragments by chromosome-specific probes (Table 1) and by comparison with digests of chromosome 7 from the *P. falciparum* XP5 clone (12, 40). Where assignment of BglI or ApaI fragments was uncertain, their locations were determined by excision of BstIII or SmaI fragments from preparative gels and digestion with BglI or ApaI. Relative arrangement of restriction sites was established from Southern blot analysis of double digests and partial digests of chromosome DNA. Telomeric sequences were detected with the pc4H11 probe (35).

**Chromosome Segment Libraries.** Segment-specific recombinant DNA libraries were constructed from the DNA of SmaI, BstIII, or BglI chromosome restriction fragments. The restriction fragments were purified by PFG electrophoresis, excised, and digested directly in the agarose blocks with *Alu*.

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**Table 1. Linkage of *P. falciparum* RFLP markers to chloroquine phenotype in HB3 × Dd2 cross**

<table>
<thead>
<tr>
<th>Chromosome*</th>
<th>Chromosome identification probes1</th>
<th>Number of RFLP markers examined per chromosome</th>
<th>Linkage ratios2 of individual RFLP markers, range</th>
</tr>
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<tbody>
<tr>
<td>14 (3.5 Mb)</td>
<td>pfmdr2, pfpgi</td>
<td>8</td>
<td>6/16-10/16</td>
</tr>
<tr>
<td>13 (3.2 Mb)</td>
<td>TRAP, HRP-III</td>
<td>6</td>
<td>6/16-10/16</td>
</tr>
<tr>
<td>12 (2.7 Mb)</td>
<td>ABRA</td>
<td>9</td>
<td>6/16-10/16</td>
</tr>
<tr>
<td>11 (2.5 Mb)</td>
<td>Exp-1, AMA1</td>
<td>14</td>
<td>7/16-12/16</td>
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<td>10 (2.1 Mb)</td>
<td>PfS25, SAg, LSA, GBP-130</td>
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<td>7/16-9/16</td>
</tr>
<tr>
<td>9 (1.7 Mb)</td>
<td>gp195, grp</td>
<td>2</td>
<td>9/16-10/16</td>
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<tr>
<td>8 (1.6 Mb)</td>
<td>HRP-III, pH 9.45</td>
<td>5</td>
<td>3/16-6/16</td>
</tr>
<tr>
<td>7 (1.7 Mb)</td>
<td>pS590.7</td>
<td>11</td>
<td>11/16-16/16</td>
</tr>
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<td>6 (1.6 Mb)</td>
<td>pSc11.63</td>
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<td>8/16-10/16</td>
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<tr>
<td>5 (2.1 Mb)</td>
<td>pfmdr1, MESA, FIRA</td>
<td>5</td>
<td>6/16-9/16</td>
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<td>4 (1.4 Mb)</td>
<td>DHFR/TS</td>
<td>6</td>
<td>7/16-9/16</td>
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<tr>
<td>3 (1.4 Mb)</td>
<td>CSP</td>
<td>2</td>
<td>4/16-5/16</td>
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<tr>
<td>2 (1.1 Mb)</td>
<td>KAHRP, SERA, CARP, AG513</td>
<td>5</td>
<td>7/16-8/16</td>
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<tr>
<td>1 (0.8 Mb)</td>
<td>RESA</td>
<td>1</td>
<td>7/16</td>
</tr>
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</table>

1Abbreviations indicate genes encoding *P. falciparum* proteins: pfpgi, glucose phosphate isomerase; TRAP, thrombospondin-related anonymous protein; HRP-II and -III, histidine-rich proteins II and III; ABRA, acidic basic repeat antigen; Exp-1, exported protein 1; AMA-1, apical membrane antigen 1; PfS25, M, 25,000 sexual stage-specific antigen; SAg, S-antigen; LSA, liver-stage-specific antigen; GBP-130, Mr, 130,000 putative glycoporphin-binding protein; gp195, precursor to the major merozoite surface antigens; grp, candidate glucose-regulated protein; pH9.45, pS590.7, and pSc11.63, anonymous probes for chromosomes 8, 7, and 6, respectively; MESA, mature-parasite-infected erythrocyte surface antigen; FIRA, *falciparum* interspersed repeat antigen; DHFR/TS, dihydrofolate reductase/thymidylate synthase; CSP, circumsporozoite protein; KAHRP, knob-associated histidine-rich protein I; SERA, serine repeat antigen; CARP, clustered-asparagine-rich protein; AG513, Mr, 45,000 merozoite surface antigen; and RESA, ring-infected erythrocyte surface antigen.

2Values in parentheses indicate estimated sizes of Dd2 chromosomes in megabase (Mb) pairs.

3Single-copy sequences that identify each chromosome. Chromosome assignments agree with those of markers in common with Foote and Kemp (38), except for the thrombospondin-related anonymous protein-encoding gene, which identifies chromosome 13 (39) and the S-antigen, which identifies chromosome 10 (this study). Anonymous probes pH9.45, pS590.7, and pSc11.63 were used to identify chromosomes 8, 7, and 6 in the absence of available gene sequences that identify these chromosomes (the histidine-rich protein II gene is deleted from Dd2 and certain progeny of the cross).

4Linkage ratios of individual RFLP markers were determined as the number of parental polymorphisms that correlate with chloroquine phenotype per 16 progeny. Because the erythrocytic stage of *P. falciparum* is haploid (40), individual polymorphisms were detected from only one parent in each progeny clone.
I, HindII, Rsa I, or Ssp I. DNA was recovered by electrophoresion, precipitated in the presence of glycon, and blunted with T4 DNA polymerase (New England Biolabs). After attachment of nonpalindromic BstXI linkers, the DNA was size fractionated by gel electrophoresis [500–8000 base pairs (bp)], recovered by electrophoresion, and ligated between the BstXI sites of vector pcDNAII, as recommended by the supplier (Invitrogen, San Diego). Transformants were obtained by electroporation into Escherichia coli DH10B ElectroMAX cells (GIBCO/BRL). Anonymous RFLP markers were identified by hybridizing plasmids from individual colonies to blots of restricted HB3 and Dd2 DNA. Approximately 5–10% of the plasmids identified useful Alu I, HindII, Rsa I, or Ssp I polymorphisms.

**Nucleotide Sequencing, Oligonucleotide Synthesis, and PCRs.** Dideoxynucleotide sequence data were obtained from double-stranded plasmid templates (pcDNAII vector) by the Sequenase system (United States Biochemical) with the use of M13 primers (5'-CAG-GAA-ACA-GCT-ATG-AC-3' and 5'-GTA-AAA-CGA-CGG-CAA-GT-3'). To generate a sequence-tagged (41) probe for the chloroquine-resistance locus, forward and reverse PCR primers were designed from the p590.7 nucleotide sequence and synthesized as recommended with an Applied Biosystems 391 DNA synthesizer. Primers were combined with 0.2 μg of Dd2 DNA in a standard PCR mixture (Perkin–Elmer/Cetus). After 35 PCR cycles (93°C for 30 sec, 58°C for 30 sec, and 72°C for 120 sec), the 0.5-kb product was recovered and purified by preparative gel electrophoresis.

**RESULTS AND DISCUSSION**

Eighty-five RFLP markers that distinguish the chloroquine-resistant and chloroquine-sensitive parents were used to survey inheritance of the 14 parasite chromosomes among the 16 independent recombinant progeny of the cross. Markers were assigned to the *P. falciparum* chromosomes by PFG electrophoresion, and linkage was assessed by comparing the inheritance pattern of each RFLP marker with inheritance of the chloroquine phenotype. Table 1 shows the results of this survey. Of RFLP markers to the 14 chromosomes, only those on chromosomes 7 and 11 detected polymorphisms suggestive of linkage to the chloroquine phenotype (linkage ratio ≥ 11/16), whereas markers to all other chromosomes showed random correlations. Full-length restriction maps of chromosomes 7 and 11 were, therefore, constructed from the Dd2 clone and analyzed for chromosome crossovers among progeny. Subchromosomal libraries of selected chromosome restriction fragments were used to obtain RFLP markers to specific segments along the length of each chromosome.

**Table 2.** Inheritance of chromosome 7 RFLP markers in the HB3 × Dd2 cross*

<table>
<thead>
<tr>
<th>RFLP probe</th>
<th>B1-SD</th>
<th>QC-13</th>
<th>3B-B1</th>
<th>QC-01</th>
<th>B4-R3</th>
<th>SC-05</th>
<th>TC-08</th>
<th>GC-03</th>
<th>3B-A6</th>
<th>1B-B5</th>
<th>3B-D5</th>
<th>SC-01</th>
<th>QC-34</th>
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<td>D</td>
<td>D</td>
<td>D</td>
<td>Rsa I</td>
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<td>pS590.20</td>
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<td>H</td>
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<td>Rsa I</td>
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</tbody>
</table>

Crossovers 1 0 0 1 0 0 0 1 2 0 0 0 1 0 3 1

*H and D, inherited polymorphisms from the HB3 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) parents of the cross, respectively; ND, not determined.

**Fig. 1.** Long-range restriction map of chromosome 7 from the Dd2 *P. falciparum* clone. The chloroquine-resistance locus (CQR) maps within a ~400-kb segment as determined from chromosome crossovers detected in progeny clones. RFLP markers are shown to the right of the map; linkage among the 16 progeny is indicated in brackets. Restriction endonuclease sites: B, Bgl I; A, Apa I; H, HindIII; S, Sma I. Apa I sites were not determined at bottom of the map (A•••). Telo, telomere.

Mapping studies and RFLP analysis were completed with 14 RFLP markers spanning the length of chromosome 11 (Table 1; map and inheritance data not shown). Although four separate markers to a ~400-kb region in the middle of chromosome 11 were seen to track with the chloroquine phenotype in 12 progeny (all except QC-13, 3B-D5, SC-01, and QC-34), markers flanking this region correlated with the chloroquine phenotype in only 7–11 of the progeny; thus, no
region on chromosome 11 could be found that was linked to inheritance of the chloroquine phenotype in all 16 progeny. Analysis of chromosome 7, however, identified a region fully linked to the chloroquine phenotype. Fig. 1 presents the map of chromosome 7 and indicates the segments recognized by 11 different RFLP markers. Inheritance patterns of the individual RFLP markers are given in Table 2. Results show that sequences detected by three adjacent markers (pS590.7, pBP25, and pB20.3) are perfectly linked to the chloroquine phenotype—i.e., the markers distinguish appropriate HB3 and Dd2 polymorphisms in all chloroquine-sensitive and chloroquine-resistant progeny, respectively. To either side of these markers, linkage ratios are reduced because of chromosome crossover events among the progeny. For example, toward the middle of chromosome 7, the linkage detected by probes pH270.2 and pS90.30 is reduced to 14/16 by crossovers in progeny 3B-A6 and QC-34. In the other direction, linkage detected by probe pS590.17 is 15/16 because of a crossover in progeny TC-05. Boundaries set by these crossovers delimit the locus governing the chloroquine phenotype. Although exact positions of these crossovers are not known, an estimate from this data places an upper limit of ~400 kb on the distance between them (<1.5% of the 30,000-kb P. falciparum genome).

To develop a sequenced-tagged probe for the chloroquine-resistance locus on chromosome 7, forward and reverse oligonucleotide primers 5'-GGT-GAG-GAG-GAA-GAG-GTA-GAG-TCC-3' and 5'-TGC-AAT-TCT-TGC-AAC-TTG-TCT-ATG-3' were designed from the pS590.7 nucleotide sequence and combined with Dd2 DNA in a standard PCR reaction. The resulting 0.5-kb product was purified by preparative gel electrophoresis and labeled by random hexamer priming. Fig. 2 shows that the PCR product detects 670- and 710-bp Rsa I polymorphisms with perfect linkage to the chloroquine phenotype in the cross.

What is the probability that an association of the chloroquine phenotype with a chromosome marker might have occurred by chance in all 16 progeny? Long-range restriction analyses and chromosome crossover studies of two P. falciparum crosses have shown that chromosome polymorphisms in the progeny are generally tightly linked when they are within 400 kb of one another, whereas probes farther apart can often be inherited from different parents (35, 39; this study, data not shown). Consider a rough model of inheritance in which linkage is treated in terms of regions having ~400-kb size. To the extent that polymorphisms are inherited with equal likelihood from either parent, the probability that some chromosome region could by chance contain an RFLP with the same inheritance pattern as the chloroquine phenotype is estimated by a Poisson distribution: $P \leq 1 - \exp \left( -30,000 \text{ kb} / 400 \text{ kb} \times 0.5^{16} \right) \approx 0.001$ (the probability would be reduced further were account made of chromosome regions where inheritance is skewed in favor of one or the other parent). Such a small value indicates that the probability of a chance association of a chromosome marker with chloroquine resistance is negligible.

Results of this study show that the rapid-efflux, chloroquine-resistant phenotype is governed by a single genetic locus on chromosome 7 rather than multiple loci, as have been proposed elsewhere (8, 42). The isolation of candidate chloroquine-resistance genes by positional cloning methods should now be possible. Linkage to the chloroquine-resistance locus will also serve as a stringent test for candidate genes obtained by other approaches.

Chloroquine resistance has spread throughout the world from one or two initial loci (43, 44), and rapid chloroquine efflux has been found characteristic of all resistant parasites (3). These observations suggest the parasite has a limited repertoire of mechanisms for countering the drug. Elucidation of the gene and protein responsible for rapid chloroquine efflux may point the way to other approaches against chloroquine-resistant malaria.

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