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Molecular karyotype of *Plasmodium falciparum*: Conserved linkage groups and expendable histidine-rich protein genes

(malaria/chromosomes/pulsed-field gel electrophoresis/deletions)

D. J. KEMP*, J. K. THOMPSON*, D. WALLIKER†, AND L. M. CORCORAN*

*The Walter and Eliza Hall Institute of Medical Research, Victoria 3050, Australia; and †Department of Genetics, University of Edinburgh, Edinburgh, Scotland EH9 3JN

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ABSTRACT **We describe fractionation of the *Plasmodium falciparum* genome into 14 chromosomal DNA molecules by pulsed-field gel electrophoresis. This number agrees with the number of chromosomes observed by electron microscopic visualization of kinetochores. The assignment of 25 markers to 12 of the 14 chromosomes in three cloned parasite lines demonstrates that chromosomal size variation can greatly change the relative migration of genetically equivalent chromosomes. Deletions that include genes for three different histidine-rich proteins, located on chromosomes 2, 8, and 13, contribute to size differences in some clones. Other karyotypic differences result from chromosome segregation and/or recombination during meiosis.**

The development of pulsed-field gel electrophoresis (PFG electrophoresis) for the fractionation of double-stranded DNA molecules in the megabase range (1) has enabled the study of chromosomes from organisms refractory to other approaches. The genome of *Plasmodium falciparum* was resolved into at least 7 (2–5) chromosomes in the earliest studies. Subsequently, at least 11 chromosomes were resolved (6) and hence the most recent estimate approximates the total of 14 chromosomes obtained by electron microscopic observation of kinetochores (7).

The *P. falciparum* chromosomes are highly polymorphic in size, both in cultured isolates and in parasites obtained directly from patients (2–6). Although the karyotype of a cloned line is stable during the asexual cycle, some size variation occurs *in vitro* that may result from deletions of genes such as the knob-associated histidine-rich protein (KAHRP) gene (4, 8). Other deletions may involve repetitive DNA (3). However, studies on the progeny from a genetic cross of two cloned isolates have shown that polymorphisms can arise during the cross (9), presumably from chromosomal rearrangements during sexual reproduction in the mosquito.

A number of genes encoding malaria antigens currently viewed as candidate vaccine molecules have been assigned to different chromosomes (2–6, 8, 9). For example, the ring-infected erythrocyte surface antigen (RESA) gene is located on chromosome 1 (2, 4), the circumsporozoite protein (CSP) gene is located on chromosome 3 (9), and the gene encoding the precursor to the major merozoite surface protein (PMMSA) is located on a larger chromosome (10). *P. falciparum* is haploid (9) and recombinant genotypes (that could include antigenically novel forms) arise at a high frequency during meiosis, which occurs during the mosquito phase of the life cycle. Further, recent studies on the PMMSA gene have revealed possible intragenic recombination events (11, 12). As this capacity for recombination could have implications for any future molecular vaccine, we have analyzed the variation that occurs in *P. falciparum* chromosomes in further detail. We show here that there are indeed 14 chromosomes and assign a total of 25 markers to 12 of them in each of three different clones. The sizes of some chromosomes vary so much that their relative rates of migration do not necessarily reflect their genetic identity.

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MATERIALS AND METHODS

PFG Electrophoresis. Procedures for preparation of *P. falciparum* chromosomes and for PFG electrophoresis were as described (2, 4) except that the double-focusing electrode array (13) was used. After PFG electrophoresis as described in the figure legends, gels were incubated in 0.25 M HCl for 1 hr at room temperature prior to denaturation. Bidirectional transfer of DNA (14) to Hybond-N nylon membrane (Amersham) was carried out according to manufacturer's instructions. DNA probes were radiolabeled using a random oligonucleotide labeling system (15). Hybridizations with DNA probes were carried out for at least 12 hr at 65°C in 0.9 M NaCl/90 mM sodium citrate, pH 7, 5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.5% NaDodSO$_4$, and 0.02 mg of denatured herring sperm DNA per ml. Filters were washed with 0.3 M NaCl/30 mM sodium citrate at 65°C. The cloned sequences used as probes are described in the text and in the following references: RESA (16); Ag169 (17); S antigen (18); *falciparum* interspersed repeat antigen (FIRA) (19); mature-parasite-infected erythrocyte surface antigen (MESA) (20); Ag44 (21); Ag361 (22); Ag63 (23); Ag23 (24); Ag352 and Ag394 (25).

RESULTS

Resolution of 14 Chromosomes from *P. falciparum*. We used a double-focusing PFG electrophoresis apparatus (13) because this promotes more linear migration than earlier models. For resolving the largest chromosomes, increased pulse times and lower voltages were necessary. We chose clone 3D7 as the prototype because it can undergo the full life cycle (9) and therefore is unlikely to lack essential genetic information. Furthermore, the chromosomes of 3D7 were more easily resolved than those of HB3, another cloned line capable of completing the life cycle (9). We also studied clones D10 and E12, both derived from isolate FC27 but unique in several respects, displaying deletions of genes for the KAHRP (4, 8, 26) or a histidine- and alanine-rich protein,

Abbreviations: PFG electrophoresis, pulsed-field gel electrophoresis; KAHRP, knob-associated histidine-rich protein; RESA, ring-infected erythrocyte surface antigen; CSP, circumsporozoite protein; PMMSA, precursor to the major merozoite surface protein; FIRA, *falciparum* interspersed repeat antigen; MESA, mature-parasite-infected erythrocyte surface antigen; HOR, hypervariable octapeptide repeat; SHARP, small histidine- and alanine-rich protein; HRP, histidine-rich protein; GBP, glycophorin binding protein; CRA, circumsporozoite protein-related antigen.

respectively (27, 28). These deletions helped resolve some ambiguities in chromosome identification.

Fractionation of the chromosomes of these clones at pulse times of 210 sec, 420 sec, and 740 sec is shown in Fig. 1. At 210 sec, 10 chromosomes (labeled 1–10 in Fig. 1*a*) are resolved to varying extents in the different clones, but some larger chromosomes (labeled 11–14) remain unresolved. Resolution varies in different areas of the gel (compare the right and left tracks of each isolate in Fig. 1*a*) and also varies between runs, but we consistently observed 10 chromosomes in this region for both D10 and 3D7. Hybridization studies confirm this number and show that it also applies to E12. It is clear in Fig. 1*a* that the phenomenon of size polymorphisms documented previously for chromosomes 1–4 also occurs among the larger chromosomes. The identity of chromosomes 1–10 in each clone is established below. At 420 sec, bands for chromosomes 1–10 were condensed, but chromosomes 11 and 12 were clearly separated (Fig. 1*b*). At 740 sec, chromosomes 13 and 14 were resolved (Fig. 1*c*) and ethidium bromide staining suggested equal stoichiometry of chromosomes 11–14. As there was not sufficient stained material to account for additional chromosomes above the 14th, either on the gel or in the slot, we conclude that we have resolved all of the chromosomes of *P. falciparum*. Runs at intermediate pulse times confirm these results (data not shown). Further, the estimate of 14 chromosomes agrees with that obtained by electron microscopy of kinetochores (7).

Relationships of the Chromosomes in Different Clones. Cloned *P. falciparum* sequences have provided markers to define chromosomes 1–4 in a number of isolates (2–6, 8–10), and a number of other markers, mainly genes for antigens, were used here. Most previous mapping assignments to larger chromosomes are obsolete due to the lack of resolution on earlier gels. We will consider the identification of each chromosome separately. The chromosome numbering (Figs. 1 and 2) is based on the order of 3D7 chromosomes.

Chromosome 1. The fastest-moving chromosome of each clone is genetically equivalent, though highly polymorphic in size (2–5). We have established its identity in D10, E12, and 3D7 with probes for RESA (16) and Ag169 (17), with four other randomly chosen fragments from libraries of chromosome 1 fragments (10) in isolates HB3, K1, NF7 (data not shown), and with ribosomal RNA (3, 6). Furthermore, RESA mapped to the smallest chromosome in five independent isolates taken directly from patients (4, 5).

Chromosome 2. Previous studies defined chromosome 2 as a polymorphic chromosome bearing the KAHRP gene (4, 8, 26). In clone E12, isolate V1 (4), and isolates FcR-3 and FV0 (8), it is subject to large deletions that include all or part of the KAHRP gene. In D10, chromosome 2 is slightly larger than chromosome 3. However, the KAHRP probe hybridized to the second smallest chromosome in several independent cultured isolates and in each of the five field isolates described above (4, 5). Ag513 (J. Smythe, personal communication) also hybridized to chromosome 2 in D10, E12, and 3D7 (data not shown).

Chromosome 3. A number of randomly chosen cloned DNA segments from libraries of chromosome 3 fragments were first used to identify chromosome 3 (2), and one of these, pF3.3, was used to relate the chromosomes here (data not shown). The CSP gene has subsequently provided a well-characterized marker in clones 3D7 and HB3 (9). Note that chromosome 3 of 3D7 is larger than chromosome 4 of D10 or E12.

Chromosome 4. Two randomly chosen cloned DNA segments from libraries of chromosome 4 fragments were first used to define chromosome 4 (5, 10), and one of these, pF4.3, was used here (data not shown). A cloned sequence that apparently encodes a hypervariable octapeptide repeat (HOR; H. D. Stahl and L.M.C., unpublished data) hybridizes to chromosome 4 in D10 and E12 as well as 3D7 and HB3 (data not shown). These data show that chromosome 4 in 3D7 is much larger than its homologue in D10 and E12 (Fig. 1*a*), corresponding in size to the fifth smallest chromosome in the latter two clones.

Chromosomes 5 and 8. The relatively simple size relationship between chromosomes 1–4 of different clones is not apparent for these larger chromosomes. The heat shock gene probe Ag63 (hsp70-1; ref. 23) hybridized to chromosomes 5 and 8 in 3D7 (Fig. 3*b*). In D10, a pattern similar to that of 3D7 was obtained, but in E12 a single broad, intense band the size of 3D7 chromosome 8 was obtained (Fig. 3*b*). At low stringency, probe Ag57 (27) also hybridized to chromosome 8 in 3D7 but not to chromosome 5 (it also hybridized to chromosome 13; see below). As expected, Ag57 hybridized to the chromosome 8-sized band in E12 but, surprisingly, it hybridized only to the smaller band in D10 (Fig. 3*c*). Hence, chromosome 5 of D10 is of similar size to chromosome 8 of 3D7 and *vice versa*. The sizes of chromosomes 5 and 8 coincided in E12, accounting for the relatively greater staining with ethidium bromide and poorer resolution in this region and for the single intense band hybridizing for Ag63 (Fig. 3).

Ag57 encodes the small histidine- and alanine-rich protein [SHARP (27); also called HRPIII (30)] and cross-hybridizes to a related gene (27) designated HRPII (30). Ag57 hybridized to chromosome 8 and chromosome 13 (Fig. 3*c*). The hybrids on chromosome 8 of each clone melted at 30 mM NaCl/3 mM sodium citrate, 65°C, implying that this is the cross-hybridizing gene that encodes HRPII (27), whereas the gene on

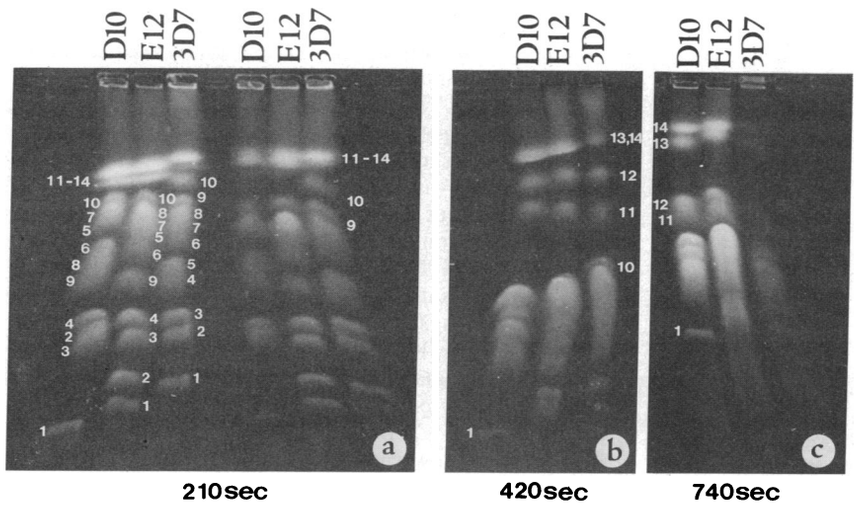

FIG. 1. Fractionation of the *P. falciparum* genome into 14 chromosomes by PFG electrophoresis. Chromosomes from *P. falciparum* clones D10, E12, and 3D7 immobilized in agarose were prepared as described (2, 4), fractionated on a 1% gel in a double-focusing PFG electrophoresis apparatus (13), and stained with ethidium bromide. (*a*) Fractionation was at 8.7 V/cm for 48 hr with a 210-sec pulse interval. (*b*) Fractionation was at 6.9 V/cm for 72 hr with a 420-sec pulse interval. (*c*) Fractionation was at 5.0 V/cm for 72 hr with a 740-sec pulse interval.

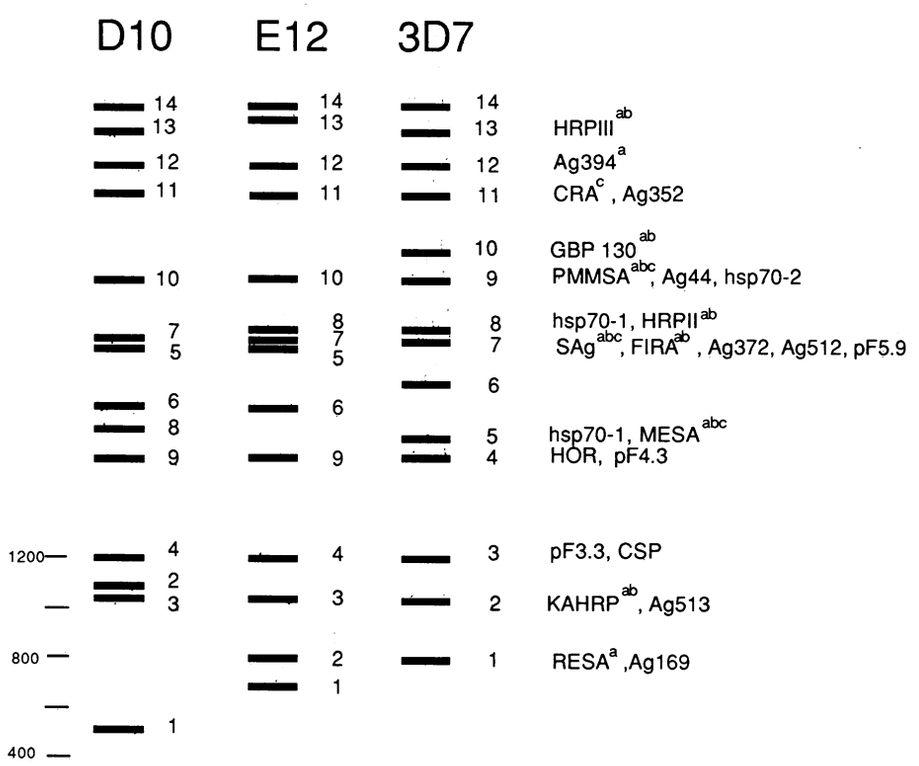

FIG. 2. Karyotypes of three clones of *P. falciparum*. The chromosomes are represented as bars on a vertical scale calibrated up to 1200 kilobases (kb) (bottom left) using chromosomes from yeast strains YP80 and YP148 (29). Above 1200 kb, a schematic diagram of the relative orders of migration is given, but the sizes are not known accurately. The chromosomes of 3D7 were designated 1–14 in decreasing order of mobility. The relationships of chromosomes of D10 and E12 to those of 3D7 were established by hybridization. The identities of chromosomes 6 and 14 in the three isolates are assumed. a, Restriction fragment length polymorphism; b, electrophoretic variants; c, antigenic variants. Chromosomal assignments shown on the right are aligned with the relevant chromosome of 3D7.

chromosome 13 must encode SHARP. In D10 the hybridization to chromosome 8 relative to chromosome 13 was much lower than in E12 and 3D7. HRPII is not produced by D10 (27). We therefore conclude that the lower amount of hybridization to chromosome 8 and the size difference between chromosome 8 of E12 and D10 result from a deletion during culture of the clone *in vitro* that removes part but not all of the HRPII gene.

The MESA (20) provided another marker to confirm the relative sizes of chromosome 5 in E12 and 3D7 (data not shown).

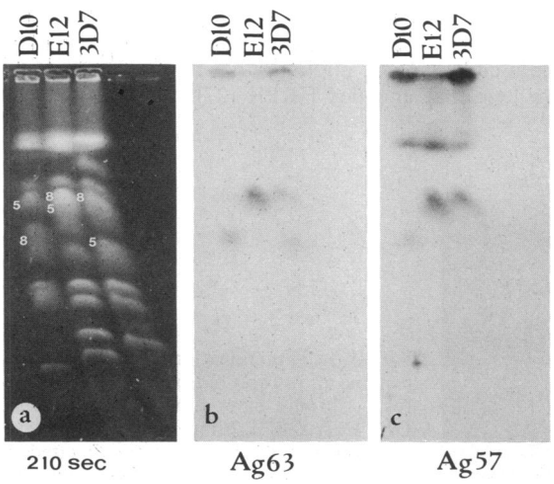

FIG. 3. Size polymorphism of chromosomes 5 and 8. The gel shown in *a* (conditions as in Fig. 1*a*) was blotted to a Hybond-N membrane and hybridized to the following probes. (*b*) An Ag63 probe (23). (*c*) An Ag57 probe (27). Ethidium-bromide stained bands corresponding to the hybrids are indicated.

Chromosome 6. Chromosome 6 can be seen in the stained pattern shown on the left of Fig. 1*a* and is slightly larger in 3D7 than in D10 and E12. We have no marker for this chromosome. The assignment shown in Fig. 2 is based on the presence in each clone of a chromosome in this size range that lacks known markers and is surrounded by chromosomes identified with several probes.

Chromosome 7. There are four genes for *P. falciparum* antigens that hybridize to chromosome 7—namely, the genes for the S antigen (18), the FIRA (19), and Ags 372 (25) and 512 (J. Smythe, personal communication)—as well as a random fragment, pF5.9. Chromosome 7 shows no size polymorphisms among these clones and is very similar in size to chromosome 8 in 3D7 and E12. However, the chromosome 8 deletion in D10 allowed us to assign these markers unambiguously to chromosome 7.

Chromosome 9. The genes for three antigens—namely, the rhoptry protein Ag44 (20) (Fig. 4*b*), the PMMSA (11, 12) (Fig. 4*c*), and the heat shock protein designated hsp70-2 in Fig. 2 (Ag361; ref. 22)—are located on chromosome 9 of 3D7. Remarkably, all of these probes hybridized to the fifth smallest chromosome of D10 and E12 (Fig. 4). To check whether D10 and E12 (both derived from isolate FC27) carried an aberrantly small form of chromosome 9, we hybridized the Ag361 probe to chromosomes from two additional isolates, K1 and NF7 (data not shown). Chromosome 9 is intermediate in size in the other two isolates and therefore is highly polymorphic.

Chromosome 10. One well-characterized marker, Ag23 (24), otherwise known as the "glycophorin binding protein" GBP130 (31), defines this chromosome (data not shown).

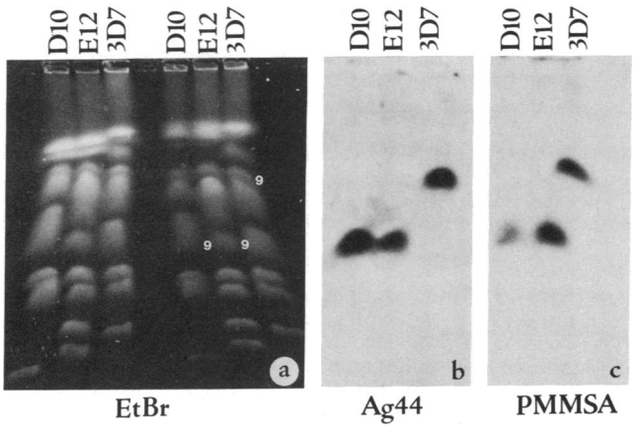

FIG. 4. Size polymorphism of chromosome 9. The gel in *a* (conditions as in Fig. 1*a*) was blotted and the right and left halves were hybridized with probes derived from Ag44 (21) (*b*) and the PMMSA (12) (*c*), respectively. Ethidium-bromide (EtBr)-stained bands corresponding to the hybrids are indicated.

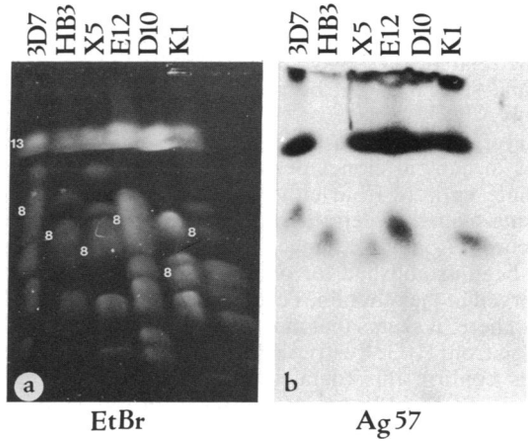

FIG. 5. Karyotypes of clones from a defined genetic cross. The gel in *a* (pulse time = 300 sec, 6.9 V/cm, 72 hr) was blotted and hybridized to an Ag57 probe (*b*). As well as the parental clones 3D7 and HB3, progeny clone X5 and unrelated clones E12, D10, and K1 were loaded as indicated. EtBr, ethidium bromide.

Chromosome 10 in 3D7 is significantly larger than its homologue in D10 or E12.

Chromosome 11. One marker for chromosome 11 is a gene for the circumsporozoite protein-related antigen (CRA; ref. 32), also called exp-1 (33). The other marker is antigen-positive clone Ag352 (25) (data not shown). Chromosome 11 is equivalent in size in each clone.

Chromosome 12. The gene for antigen-positive clone Ag394 (25) provides a marker for chromosome 12 (data not shown): it is equivalent in size in each clone.

Chromosome 13. The gene for SHARP/HRPIII defines chromosome 13 (see above). This was confirmed by hybridization to the 740-sec pulse gel shown in Fig. 1*c* (data not shown). It appears larger in E12 than in the other two clones.

Chromosome 14. Although we have no marker for chromosome 14, we presume that it is equivalent in each isolate as it is the only chromosome of this size without a marker.

Sizes of *P. falciparum* Chromosomes. Chromosomes 1–4 were sized using yeast strains YP80 and YP148 (29) as markers. These values (Fig. 2) were reproducible within ±5% between runs. The sizes of the larger chromosomes are not yet accurately known.

Chromosomes in Clones from a Defined Genetic Cross. We have used the data summarized in Fig. 2 to karyotype HB3, the parental line used together with 3D7 for the defined genetic cross of *P. falciparum*, and one of the progeny, X5. Previously, chromosomes 1–4 in HB3 and X5 were identified using various probes (9). With a 300-sec pulse (Fig. 5), chromosomes 2–4 and chromosomes 5–9 migrate in groups that are less well-resolved than in 3D7. The pattern for X5 is much more similar to HB3 than to 3D7. However, chromosomes 9 (identified with the PMMSA probe; data not shown) and 10 resemble their 3D7 homologues in size. Using the Ag57 probe, it is clear that chromosome 8 in X5 is similar in size to the HB3 parent (Fig. 5*b*). Further, the SHARP/HRPIII gene is deleted from chromosome 13 in HB3, in accord with our report that HB3 lacked this antigen (28). However, chromosome 13 of X5 contains the SHARP/HRPIII gene (Fig. 5*b*) and this can only derive from 3D7.

DISCUSSION

We have been able to resolve 14 chromosomal DNA molecules in *P. falciparum* clones 3D7 and D10. They all appear to be present in equimolar amounts and there is insufficient material at the top of the gel to account for any more chromosomes. The concordance of this estimate with that obtained by counting kinetochores (7) suggests that 14 is the definitive number of chromosomes in *P. falciparum*.

Using the conditions described here, we no longer observe the unexplained apparent duplication of chromosomes previously designated 5 and 6 (2). A similar apparent duplication in *Leishmania* (34) has also disappeared and so we conclude that the duplications were artefactual. Despite the extensive size polymorphisms of most chromosomes, there is only a single form of each chromosome in each parasite clone. We conclude that there is only a single form of each of the 14 chromosomes of *P. falciparum*, as would be expected from the previous finding that the parasite is genetically haploid (9). A number of the markers exhibit restriction fragment length polymorphisms, antigenic differences, and/or polypeptide size differences, usually resulting from varying numbers, lengths, or sequences of repeat sequences common to malaria antigens. These polymorphisms (indicated in Fig. 2) will be useful markers for typing *P. falciparum* isolates.

Genes for the Three HRPs Are Located on Different Chromosomes and Can Each Be Deleted. Cloned lines that lack KAHRP together with large segments of chromosome 2 are characterized by a knobless phenotype (4, 8, 26). There are two other HRPs (HRPII and SHARP/HRPIII) that are highly homologous in sequence and consequently most probably have arisen from a gene duplication event (27, 30). Our results show that HRPII and SHARP/HRPIII are located on chromosomes 8 and 13, respectively. Chromosome 8 from D10 is considerably shorter than that from E12, suggesting that a major deletion has occurred *in vitro*, as for chromosome 2 in E12. The low level of hybridization to chromosome 8 in D10 suggests that the end point of the deletion may be located within the probe sequence (the HRPII coding sequence), as was true for the KAHRP gene in some isolates (8). Chromosome 8 of 3D7 is as small as that of D10, but there is no evidence for a similar deletion of the HRPII gene. In clone HB3, which lacks SHARP/HRPIII, the gene for SHARP/HRPIII is absent from chromosome 13. The data for HB3 therefore confirm the chromosomal locations for each of the three HRP genes.

It is remarkable that each of the three known HRP genes of *P. falciparum* can be absent without loss of parasite viability (although simultaneous loss of all three HRP genes has not been observed). KAHRP is known to be a component of knobs, structures that mediate cytoadherence *in vitro* (26). As clone HB3 can undergo the complete life cycle, SHARP/HRPIII may not serve an essential function. It remains to be

seen whether HRPII, which is lacking from D10, is essential at any stage. Indeed, as HRPII and SHARP/HRPIII are so closely related, they may complement one another in function and so it may not be possible to delete both simultaneously.

Karyotypic Variation During Meiosis. Individual chromosomes in haploid clones derived from a genetic cross may originate entirely from either parent or be the product of crossing-over between them. We have seen no evidence of interchromosomal exchange. Indeed, where linkage groups have been established for one clone (3D7), they have been preserved in the other lines examined. From the size patterns shown here, it seems that most small chromosomes in X5 are derived from HB3. Restriction fragment patterns of the three clones confirm this (data not shown). However, chromosomes 9, 10, and 13 appear to derive from 3D7. Chromosome 2 is larger than its counterpart in either parent (9) and so may represent a crossover product. Obviously, the size alone cannot define the origin of each chromosome. However, the polymorphic markers that we have so far assigned to eight of the chromosomes will enable us to analyze their origins in much greater detail.

In a concurrent study using some of the same *P. falciparum* clones used here, Wellems *et al.* (35) have also reported the separation of 14 chromosomes from *P. falciparum* and localized the HRPII and HRPIII genes to chromosomes 8 and 13, respectively.

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