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Subtelomeric chromosome deletions in field isolates of *Plasmodium falciparum* and their relationship to loss of cytoadherence *in vitro*

(malaria/phenotypic variation/two-dimensional pulsed-field gradient electrophoresis)

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ABSTRACT **Subtelomeric deletions are responsible for the loss of expression of several *Plasmodium falciparum* antigens, including the knob-associated histidine-rich protein (KAHRP). Such deletions are detectable by two-dimensional pulsed-field gradient electrophoresis (PFGE) in which the chromosomes separated in dimension 1 are cleaved with *Apa* I, and the sizes of telomeric fragments are determined in dimension 2. This sensitive technique has enabled us to examine the role of subtelomeric deletions in two aspects of the biology of *Plasmodium falciparum*. First, we show that similar subtelomeric deletions to those that occur *in vitro* also occur in field isolates. Second, we demonstrate a correlation between subtelomeric deletions and loss of the phenotype of "cytoadherence" in cultured isolates. Subclones were generated from the cytoadherent cloned isolate ItG2F6, and their phenotypes were examined with respect to cytoadherence, the expression of "knobs," and agglutination of infected erythrocytes with rabbit antiserum. The only chromosomal change detectable by two-dimensional PFGE among subclones that differ from wild type in each of these three characteristics is a deletion of approximately 100 kilobases at one end of chromosome 2. This deletion includes the gene coding for KAHRP and the subtelomeric repeat designated rep20.**

Subtelomeric deletions in cultured isolates are known to result in the loss of a number of functional genes of *Plasmodium falciparum* (1–7). To examine whether this phenomenon occurs in nature or is an artifact of growth *in vitro*, we used two-dimensional pulsed-field gradient electrophoresis (PFGE) to examine chromosomal DNA from fresh field isolates. This technique allows resolution of telomeric fragments after digestion of separated chromosomes with the restriction endonuclease *Apa* I. It relies on the observation that a conserved complex consisting of telomeric repeats, an *Apa* I site(s), and a block of rep20 subtelomeric repeats is common to all chromosomes of *P. falciparum* (Fig. 1) (2–4). If the *Apa* I site between the telomere and rep20 has been deleted at one end of a chromosome, the resulting telomeric *Apa* I fragment is usually larger than the fragment at the other end (2). Nondeleted telomeric *Apa* I fragments are only 12–15 kilobases (kb) in length (2), and so the fragments from chromosomes with deletions are obviously larger; rep20 deletions can also be detected by failure of a rep20 probe to hybridize with the larger telomeric fragment. All subtelomeric deletions that include functional genes studied so far delete rep20 (2). These include the three histidine-rich protein genes (5–7) and the ring-infected erythrocyte surface antigen gene of isolate FCR3 (Robert Cappai, personal communication).

P. falciparum lines grown *in vitro* often rapidly lose the ability to adhere to endothelial cells (8, 9). In natural infections this property enables erythrocytes infected with mature parasites to sequester in deep vascular beds, thereby avoiding destruction in the peripheral circulation. Electron-microscopic examination of autopsy material from patients who had died from malaria suggests that attachment to vascular endothelial cells occurs at areas of thickening in the erythrocyte membrane called "knobs" (10). The knob-positive phenotype is often retained in cultured isolates that have lost the ability to adhere to endothelial cells, suggesting the presence of a closely linked but discrete cytoadherence molecule (9). The most likely candidate molecule is a polymorphic high molecular weight protein (11). We propose that the frequent loss of the cytoadherence-positive phenotype *in vitro* occurs because the cytoadherence locus is near the telomere and subject to subtelomeric deletions.

In this paper we show that subtelomeric deletions similar to those that occur *in vitro* also occur in field isolates. Further, we have examined whether subtelomeric deletions correlate with the loss of cytoadherence in a cloned isolate, ItG2F6.

MATERIALS AND METHODS

Parasite Culture, Cloning, Cytoadherence, and Agglutination Assays. Field isolates were collected from patients in Madang, Papua New Guinea, and cultured until schizont stage (1), and chromosome blocks were prepared as described (12). A twice-cloned Brazilian isolate, ItG2F6 (9), which had been in long-term culture, was recloned by limiting dilution (13) and by using a fluorescence-activated cell sorter. Parasites were maintained in culture by methods previously reported (14). Cytoadherence assays using fixed C32 melanoma cells (American Tissue Type Collection) were performed when 5–12% of erythrocytes were infected with mature parasites. Results were expressed as the number of infected cells adherent to 100 melanoma cells (15, 16). Agglutination assays were performed with antisera raised by immunizing rabbits with erythrocytes infected with trophozoites or schizonts of ItG2F6 (15, 17).

Electron Microscopy. Infected erythrocytes were processed by using 1.25% glutaraldehyde/1% formaldehyde fixation for 1 hr at room temperature, osmium postfixation, and uranyl acetate staining (18).

Immunoblots. Trophozoite-rich parasite preparations were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Filters were probed with affinity-purified human antibodies to the knob-associated histidine-rich protein (KAHRP) (18) or S antigen (19) as described (20).

PFGE. Chromosome blocks were prepared from 10 ItG2F6 clones by methods previously described (12). Chromosomes 1 and 2 were separated by one-dimensional PFGE (21–23) in a contour-clamped homogenous electric field for 48 hr with 90-sec pulses at 150 V. Gels were stained with ethidium

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Abbreviations: KAHRP, knob-associated histidine-rich protein; PFGE, pulsed-field gradient electrophoresis; MSA2, merozoite surface antigen 2.

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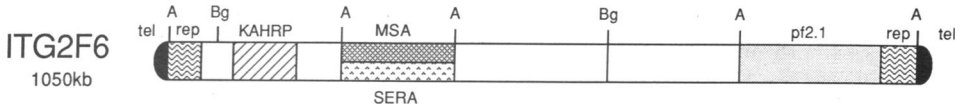

FIG. 1. Map of chromosome 2 showing the positions of the telomeric and rep20 fragments, the KAHRP gene, and restriction endonuclease sites (A = *Apa* I, Bg = *Bgl* I). The positions of the genes coding for MSA2, the serine-rich antigen (SERA, previously p126), and pf2.1 are assumed from chromosome mapping of other isolates (2, 24).

bromide, photographed, and transferred to Hybond-N (Amersham). Hybridizations with DNA probes were performed at 65°C in 6× standard saline citrate (SSC; 1× = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) containing 5× Denhardt's solution (1× = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate, and 20 μg of denatured herring sperm DNA per ml. Filters were washed in 2× SSC at 65°C. The merozoite surface antigen 2 (MSA2) and KAHRP probes have been described elsewhere (24, 25).

Two-Dimensional PFGE. Chromosomes were separated in the first dimension for 44 hr with 270-sec pulses at 120 V and then for 24 hr with 30-min pulses at 70 V. Strips of gel containing the chromosomes for each isolate were excised and stored in 10 mM Tris/50 mM EDTA at 4°C. Ethidium bromide was removed from the strips by washing with butanol-saturated 1 M NaCl/10 mM Tris/1 mM EDTA. Each strip was digested with the restriction endonuclease *Apa* I (300 units/ml; New England Biolabs), and the fragments were separated by PFGE with 20-sec pulses at 160 V for 19 hr. Hybridization conditions are described above. The telomere and rep20 DNA probes have been described elsewhere (2).

RESULTS

Subtelomeric Deletions Can Occur in Chromosomes from Field Isolates of *P. falciparum*. To examine whether rep20 deletions are present in parasites causing natural infections, chromosomes were obtained from field isolates that had been cultured to the schizont stage. The parasites were harvested before reinvasion occurred (i.e., cultured for less than one generation), thus precluding genetic selection *in vitro*. After fractionation by two-dimensional PFGE, hybridization with a telomeric probe showed that the telomeric *Apa* I fragments from most chromosomes were of the small size expected (12–15 kb) and therefore migrated as a poorly resolved smear across the gels (Fig. 2 *a* and *b*). However, in both field isolates (*a* and *b* of Fig. 2), about six of the telomeric *Apa* I fragments were larger, migrating as distinct spots well behind the smear. Both telomeric fragments from the unusually small chromosome 2 of the first isolate migrated more slowly than the smear (arrows on the left in Fig. 2*a*). We attribute material in the 12- to 15-kb region from chromosome 2 to the crossing of the smears that are evident in each dimension. Hybridization with a rep20 probe after removal of the telomere probe showed that neither of these fragments contained detectable rep20 sequences (arrows in Fig. 2*c*), and indeed, there was no detectable rep20 on chromosome 2. The gene for KAHRP was still present on chromosome 2 of this field isolate (data not shown). Of the other large telomeric *Apa* I fragments, all but one coincided in position with fragments containing rep20. Whether these represent small deletions involving the *Apa* I site is not clear, but one end of chromosome 1 from another *P. falciparum* isolate, 3D7, has been shown to have a similar structure (2). The large telomeric *Apa* I fragment on

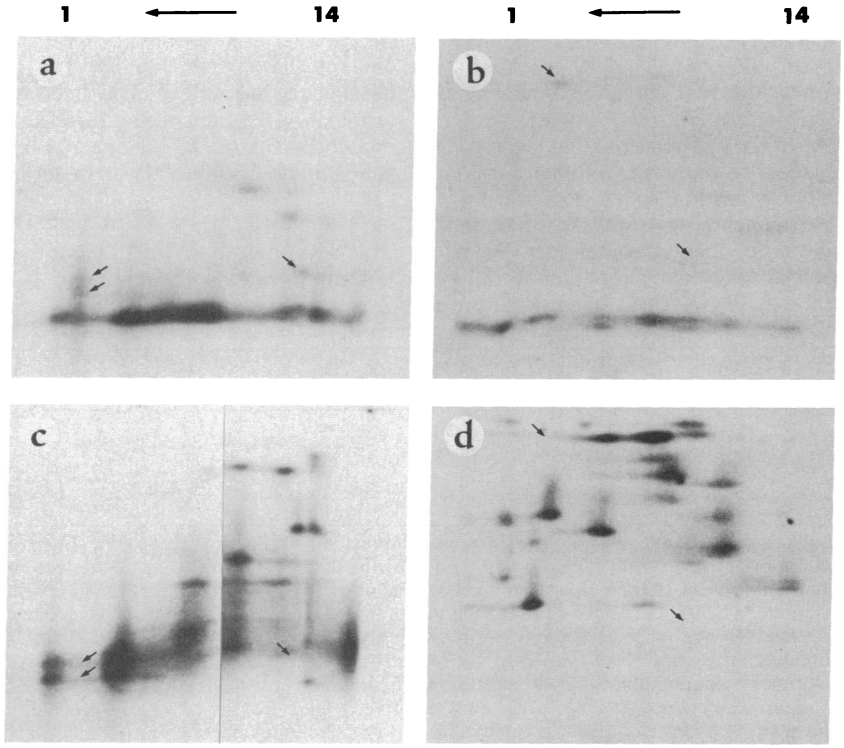

FIG. 2. Two-dimensional PFGE of chromosomes from parasites causing natural infection. *Apa* I fragments from two field isolates are shown hybridized with a telomere (*a* and *b*) and then with a rep20 probe (*c* and *d*); *c* is a composite made from two exposures of a single filter to provide greater clarity. Approximately six large telomeric fragments are apparent in each isolate. The telomeric fragments indicated by arrows in *a* and *b* do not have corresponding rep20 fragments (expected positions are indicated by arrows in *c* and *d*), suggesting that a deletion has occurred that includes both the terminal *Apa* I site and rep20. At the top, 1 ← 14 indicates the separation of the chromosomes in the first dimension.

chromosome 12 did not hybridize detectably to rep20 (arrows on left Fig. 2 *a* and *c*), and so this field isolate has at least three rep20 deletions.

In the second field isolate, about six slowly migrating telomeric fragments were again evident (Fig. 2*b*). One such telomere from chromosome 3 and one telomere from a larger chromosome, probably 9 or 10, did not contain detectable rep20 sequences (arrows in Fig. 2 *b* and *d*). In Fig. 2*d* it is particularly evident that chromosome 3 contains only one rep20 fragment. We conclude that deletions that remove all detectable rep20 sequences can occur on at least one end of four of the chromosomes of natural isolates. These results demonstrate that subtelomeric chromosome deletions occur in the field and are not just laboratory artifacts.

Subtelomeric Deletions in Chromosomes from Cytoadherent and Noncytoadherent Clones. To determine whether subtelomeric deletions account for the loss of proteins responsible for cytoadherence, we generated a number of subclones from the cytoadherent cloned isolate ItG2F6 that differed in cytoadherence phenotype. As ItG2F6 had been cloned twice before, we could be confident that all of these subclones originated from the same parent despite their differences in phenotype (Table 1). This was confirmed by showing that all of the subclones were identical with respect to the most hypervariable marker known, the S antigen (Table 1). Ten of the subclones were examined for chromosome variation by using one-dimensional PFGE. Variability was very marked on chromosome 2 (Fig. 3 *a* and *b*). Eight clones were found to have a partial deletion of this chromosome, and in seven this included the gene for KAHRP (Fig. 3*c*). Clone 5 was a cytoadherent clone in which the deletion on chromosome 2 did not remove all of the KAHRP gene. Clone 1 had a partial deletion of chromosome 1.

Four clones representing different cytoadherent and agglutinating phenotypes (Table 1) were examined for subtelomeric deletions by using two-dimensional PFGE. The only difference in telomeric fragment size between clones occurred on chromosome 2, although a number of other chromosomes had typical subtelomeric deletions (Fig. 4 *a* and *b*). In knob-positive, cytoadherent clones, the two telomeric fragments were of the small size (arrows in Fig. 4*a*). In knobless, noncytoadherent clones, a deletion had occurred that included the terminal *Apa* I site, producing one larger telomeric fragment (arrow in Fig. 4*b*). Hybridization with a rep20 probe confirmed that both rep20 fragments were present on chromosome 2 in knob-positive cytoadherent clones (arrows in Fig. 4*c*). However, in knobless noncytoadherent clones, only one rep20 fragment was apparent (arrow in Fig. 4*d*). The missing rep20 fragment had been deleted together with the terminal *Apa* I site and the KAHRP gene—a total of approximately 100 kb of DNA. A nearby chromosome 2 marker, the gene coding for MSA2, was intact in all clones (Fig. 3*b*).

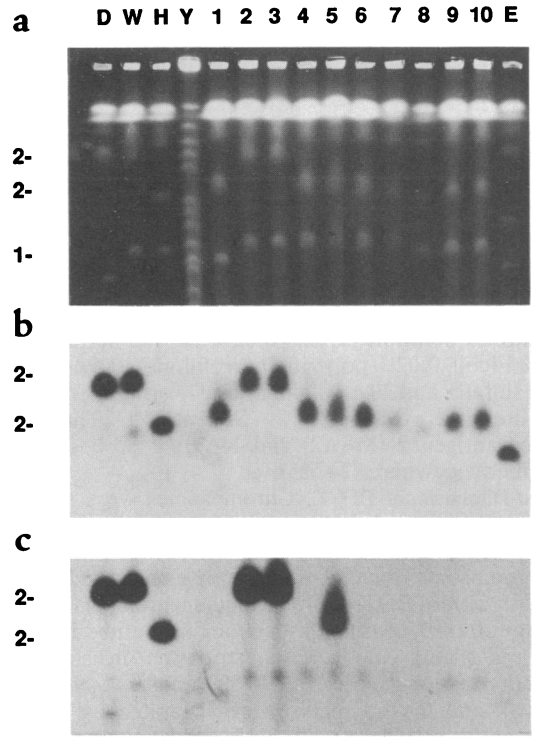

FIG. 3. PFGE of variant ItG2F6 clones. (*a*) An ethidium bromide-stained gel of ItG2F6 clones 1–10 showing the variability in the size of chromosome 2. Control isolates D and W and clones 2 and 3 show the undeleted form of chromosome 2 (identified by upper "2") compared with deleted forms in control isolates H and E and clones 1 and 4–10 (identified by lower "2"). Yeast chromosome markers are shown in lane Y; "1" identifies chromosome 1. (*b*) Hybridization with a chromosome 2-specific probe, the gene coding for MSA2, highlights this size variation. (*c*) KAHRP cDNA hybridizes to clones 2, 3, and 5 and to the controls D, W, and H.

DISCUSSION

This approach to understanding the mechanisms of cytoadherence used the technique of two-dimensional PFGE to

Table 1. Phenotypic variation of ItG2F6 clones examined by two-dimensional PFGE

ItG2F6 clone*	Cytoadherence†	Agglutination‡	Knobs§	KAHRP¶	KAHRP gene‖	Chromosome 2 deletion**
1	60	+/−	−	−	−	+
3	838	+++	+	+	+	−
6	77	−	−	−	−	+
F2	2367	+	+	+	+	−

*Immunoblots showed that all of the ItG2F6 clones harvested had the same S antigen, consistent with their clonal origin. Clones 1, 3, and 6 correspond to the clones shown in Fig. 3. F2 was the most strongly cytoadherent ItG2F6 clone and has been included for comparison.

†Cytoadherence results are expressed as the number of infected erythrocytes bound per 100 melanoma cells. Cytoadherence was designated + if >100 erythrocytes bound to 100 melanoma cells. Noncytoadherent clones were shown to be knob-negative and/or KAHRP-negative by electron microscopy or by immunoblotting with affinity-purified human anti-KAHRP antibodies (see below).

‡Agglutination results are expressed as follows: negative, − and +/− = <3 small clumps of infected cells; positive, + = >3 clumps of infected cells, ++ = >3 clumps per high-power field, and +++ = many clumps seen in each field, some containing >20 infected cells.

§Knobs were identified by electron microscopy of trophozoite-rich preparations.

¶KAHRP was identified by immunoblotting with affinity-purified human antibodies.

‖The KAHRP gene was detected by DNA hybridization.

**A chromosome 2 deletion was presumed to have occurred if this chromosome migrated more rapidly than a control using one-dimensional PFGE.

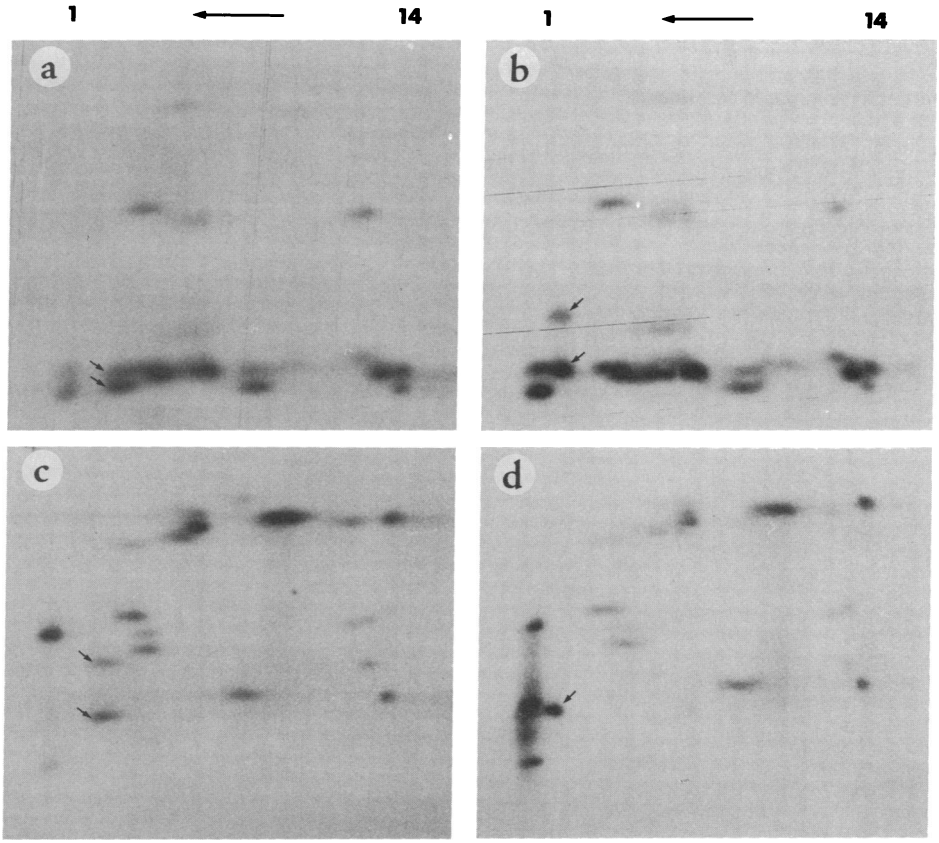

FIG. 4. Two-dimensional PFGE of variant ItG2F6 clones. (*a* and *c*) In knob-positive cytoadherent clones (clone 3 is shown), two small telomeric fragments are evident on chromosome 2 (see arrows in *a*). The corresponding rep20 fragments are shown by arrows in *c*. (*b* and *d*) In knobless noncytoadherent clones (clone 6 is shown), the terminal *Apa* I site has been deleted from one end of chromosome 2, resulting in different-size telomeric fragments (arrows in *b*). One rep20 sequence block has been included in this deletion, and so a single rep20 fragment is apparent on chromosome 2 (arrow in *d*). At the top, 1 ← 14 indicates the separation of the chromosomes in the first dimension.

compare subtelomeric deletions in phenotypically distinct clones of a cytoadherent cloned isolate, ItG2F6. Surprisingly, the only detectable difference between knob-positive cytoadherent and knobless noncytoadherent clones was a 100-kb deletion at one end of chromosome 2 that involves the KAHRP gene and the subtelomeric repeat rep20. It has been reported (5) that this region is deleted from knobless clones. We have now been able to examine the larger chromosomes to show that the chromosome 2 deletion is the only correlate found with the knobless phenotype. The location of the cytoadherence gene within this deleted region of chromosome 2 would be in keeping with the observation that the putative cytoadherence protein is not expressed in knobless lines (26, 27).

It is also possible that the loss of knobs itself results in a noncytoadherent phenotype, even in the presence of an intact cytoadherence gene(s). To exclude this possibility, it will be necessary to generate knob-positive noncytoadherent clones and examine them for subtelomeric deletions on other chromosomes. Another approach could be to characterize the products of genes contained in the deleted region of chromosome 2 to see if one or more encode cytoadherence proteins.

We also have shown that subtelomeric deletions involving rep20 are a feature of natural field isolates grown in cell culture for less than one life cycle. It is unknown whether such deletions result in the loss of genes coding for malaria antigens or whether such isolates would continue to replicate with altered pathogenicity.

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