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Cloning and characterization of chromosome breakpoints of *Plasmodium falciparum*: breakage and new telomere formation occurs frequently and randomly in subtelomeric genes

Artur Scherf and Denise Mattei
Unité de Parasitologie Expérimentale, CNRS URA 361, Institut Pasteur, 75724 Paris, Cedex 15, France

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ABSTRACT

We analysed the genetic stability of two subtelomeric genes of the human malaria parasite *Plasmodium falciparum*. A PCR based assay, using a telomere and a target-gene specific primer was used to detect potential chromosome rearrangements. We show that chromosome breakage and the formation of new telomeres occur frequently in the two genes coding for histidine rich proteins (HRP I and HRP II) in laboratory isolates, but remains undetectable in clinical parasite isolates. This finding suggests an essential role of these genes *in vivo* and that chromosome breakage is rather an accidental process than a programmed chromosome fragmentation. Cloning and sequencing of 8 chromosome breakpoints of the HRP II gene from one parasite isolate shows that the breakage occurs within a broad region in which new telomere formation appear to take place at random sites. Furthermore, this analysis revealed no obvious sequence similarities of sites of telomere addition. Finally, we show that an irregular pattern of heterogeneous telomere repeats is added at each broken end and that each healed chromosome contains a distinct pattern of repeats. We discuss a model for telomere formation in *P. falciparum*.

INTRODUCTION

Over the last decade, the human malaria parasite *Plasmodium falciparum* has been subjected to intensive investigation using recombinant DNA technologies. One motivation for this investigestion has been the desire to identify parasite antigens for inclusion in a subunit vaccine against malaria. Some of the candidate antigens exhibit considerable diversity in the parasite population (1). Thus, it will be difficult to predict the outcome of a widespread use of vaccines without a fundamental understanding of the basic genetics of malaria parasites.

Fundamental research on the genome of this unicellular parasite has lead to the understanding of the chromosomal organisation (for review see 2, 3). The *P. falciparum* genome is composed of 14 haploid chromosomes which range in size from 650 to 3500 kb (4, 5). The karyotype shows a remarkable size polymorphism. Crossover at meiosis (6, 7), homologous, but unequal recombination and direct deletion of the subtelomeric region of the pPFrep20 repeats have been proposed as possible mechanisms both *in vitro* (8, 9) and *in vivo* (10). Another type of DNA rearrangement that has been described in a limited number of cases, is the deletional inactivation of telomere-proximal malaria genes in laboratory isolates. Mutations in the genes encoding the histidine-rich proteins HRP I and HRP II have been described that were generated by chromosomal breakage followed by repair through the addition of telomere repeats (11). A similar DNA rearrangement leads to the loss of 90% of the Pf11-1 locus (12). Another study showed that inversion of a 5′ segment of the Pf155/RESA gene caused deletion of the chromosome fragment located upstream and that the resulting DNA rearrangement inactivated the Pf155/RESA gene (13).

Developmentally programmed DNA fragmentation and healing by telomere addition has been observed for ciliated protozoan such as *Paramecium* (reviewed in 14) or *Ascaris* (15). However, the biological implication of gene deletions in the unicellular haploid parasite *P. falciparum* is still unknown.

In this report we analysed the stability of chromosomes of *P. falciparum* derived from laboratory as well as clinical isolates. We focussed our studies on subtelomeric genes (HRP I, HRP II) since the ends of chromosomes appear to be more fragile than internal regions. DNA sequence analysis of 9 chromosome breaks demonstrated that the truncated chromosomes acquired a pattern of heterogenous telomere repeats which was distinct for each healed end. Our results show that telomere addition sites are variable within one gene locus and that no obvious sequence similarity has been found among breakpoints of *P. falciparum*. These data suggest that *de novo* addition of telomere sequences to spontaneously broken chromosome ends in *P. falciparum* is probably synthesized by a telomerase enzyme.

MATERIALS AND METHODS

DNA probes

The HRP I and HRP II genes were amplified by the polymerase chain reaction (16) (PCR). The following oligodeoxynucleotides are based on the 5′ end (HRP I-3) and on the 3′ end (HRP I-2)

of the HRP I gene of the FCR-3 strain (17) HRP I-3 5′ CCGGGATCCATGAAAAGTTTTAAGAACAA and HRP I-2 5′ GGAAGCTTCCTCTTGACATTACTTGTTCAC. The primers for the HRP II gene PCR-amplification are based on the 7G8 strain (18) primer HRP II-2 (5′ end) 5′ CCGGATCCAATAATTCCGCATTTAAT and HRP II-1 (3′ end) 5′ GGAAGCTTTTAATGGCGTAGGCAATGTGTG. Restriction sites introduced at the 5′ end of the primers are underlined (*Bam*HI and *Eco*RI).

Cloning and sequence analysis of chromosomal breakpoints

Chromosome breakpoints were amplified by PCR using either a specific primer located at the 5′ end of the HRP I gene (HRP I-3) or a primer located at the 3′ end of the HRP II gene (HRP II-1)) and a second primer corresponding to the telomere repeats of *P. falciparum* (5′ GGAATTCCCTAAACCCT AAACCCTAAACCC) (19). Amplification of genomic DNA of *P. falciparum* (approximately 100 ng) was done in a Hybaid Thermal Cycler using Amplitaq (Cetus). The following conditions were used 1s, 94°C; 60 s, 55°C; 120 s, 75°C; mode tube; 30 cycles. The PCR-products were digested by *Bam*HI-*Eco*RI and cloned into *Bam*HI-*Eco*RI digested pUC19. Positive clones were selected by colony screening using HRP I or HRP II specific probes. Double stranded sequencing (20) was performed using the Sequenase kit (USB).

Southern blotting

Electrophoresis was routinely carried out on 0.8% agarose gels and transferred to nylon membranes (Hybond nylon$^+$, Amersham) essentially as described (21). DNA probes were prepared by random primer (Amersham) and used for hybridization in 6×SSC, 0.1%SDS, 3% non-fat milk powder at 65°C. The membranes were washed in 0.2×SSC, 0.1%SDS at 65°C.

Parasites

The parasite strains used in this work Banjul (Gambia), FCC1 (China), ITG2G1 (Brazil), FCR3 (West Africa) and Palo Alto (Uganda) were as described (22); the clinical *P. falciparum* isolates from Senegal as described recently (23). The Palo Alto knob$^+$ strain was cultured with frequent selection for the K$^+$ phenotype using the gelatin-floatation method as described (24).

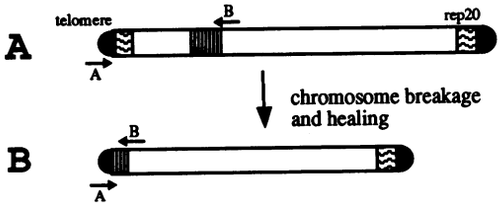

Figure 1. Applied strategy to detect DNA rearrangements in subtelomeric genes. A) Schematic of a *P. falciparum* chromosome. A subtelomerically located gene is indicated by a shaded lines, telomeres are shown as blackened ends and pPFrep20 as wavey lines. B. Chromosome breakage within the indicated gene leads to the loss of the distal portion of the right chromosome arm and healing by addition of telomere repeats. Sense (telomere) and antisense (gene) PCR primers are indicated by arrows labeled A and B. The 3′ end HRP I gene and the 5′ end of the HRP II gene are proximal to the telomere.

RESULTS

DNA rearrangements occur frequently at several *P. falciparum* gene loci during propagation *in vitro*

We investigated if chromosome breakage and healing is a rare event, or if it occurs commonly in subtelomeric genes of *P. falciparum*. To address this question we used the polymerase chain reaction (PCR) to study potential DNA rearrangements within the genes HRP-I and HRP-II. These genes reside on chromosome 2 and 8, respectively, and each is located at least 100 kb from the telomere (8, 11, 25). Oligonucleotide primers based on the gene of interest and a trimer of the *P. falciparum* telomere repeat were used to prime PCR with genomic DNA of several laboratory strains and the resulting PCR-amplified fragments were analyzed by Southern hybridization with the respective gene-specific probe. This methodology, shown schematically in Figure 1, should only detect that subpopulation of HRP-I and HRP-II genes that have undergone a rearrangement which moves them suitably close to a telomere for efficient amplification by PCR.

In most of the laboratory strains examined a multitude of heterogeneously-sized fragments hybridize with each of the probes and the pattern of fragments size differed among each of the strains. Conversely, no hybridizing fragments were observed in control experiments using single oligonucleotides to prime PCR.

Figure 2 A shows an example of the PCR-Southern analysis described above. In this case a sense oligonucleotide specific for *P. falciparum* telomeres and an antisense oligonucleotide specific for the 3′ end of the HRP-II gene were used to prime PCR with DNA isolated from laboratory strains Palo Alto K+, Banjul, FCBR, ITG2G1 and FCC1 (lanes 1−5). The HRP-II probe hybridized with amplification products derived from each of the strains and identified several major fragments within a smear of hybridizing fragments ranging in size from ca. 0.1 to 0.7 kb.

Although the entire HRP-II gene is present in these strains (Figure 2B, lane 1−5), the PCR result of Figure 2 A strongly suggests, that a fraction of the parasite population has deleted part of the the HRP-II gene. In order to confirm the validity and specificity of our PCR approach we cloned and sequenced PCR

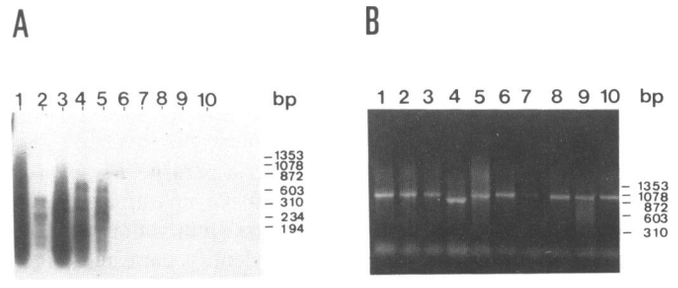

Figure 2. Gene deletion occur in a subpopulation of laboratory isolates of *P. falciparum*. A) Southern blot analysis of genomic DNA of 5 laboratory strains and 5 clinical isolates (Senegal) following PCR amplification (30 cycles) with HRP-II (antisense) and telomere (sense) specific primers as shown in figure 1. The blot was hybridized with a HRP-II DNA probe and washed under stringent conditions (0.2×SSC, 0.1% SDS, 65°C). B) Ethidium bromide stained 1% agarose gel showing the same DNA samples as in figure 2A after PCR using primers specific for the 5′ and 3′ end of the HRP-II gene. Lane 1 Palo Alto K+, lane 2 Banjul, lane 3 FCR3, lane 4 ITG2G1, lane 5 FCC1, lane 6 S1, lane 7 S2, lane 8 S3, lane 9 S4 and lane 10 S5.

products derived from the Palo Alto K^+ strain. All 8 clones analysed had identical HRP-II 3′ end DNA sequences. However, an abrupt transition from HRP-II gene sequences to consensus *P. falciparum* telomere repeats (19) was observed at 5 distinct positions within the gene locus as shown in Figure 3 A. Three clones, HRP-IIΔ2/3/5 had the same breakpoints at bp 941 and two other clones, HRP-IIΔ4/7 at bp 930. Interestingly, the comparative analysis of the telomere repeat sequences found at the breakpoints shows that an irregular pattern of heterogeneous repeats has been added to each broken chromosome end. It is noteworthy that each healed end displayed a distinct repeat pattern, even in the case where the breaks occured at the same point (Figure 3 B).

A similar approach was applied to the HRP-I locus. In this case a 5′ gene specific primer for the HRP I gene (nucleotide 1−22) and a telomere specific primer were used to PCR amplifiy genomic DNA of five laboratory isolates (Palo Alto K+, Palo Alto K−, FCC1, Banjul and FCR3). A HRP I gene probe

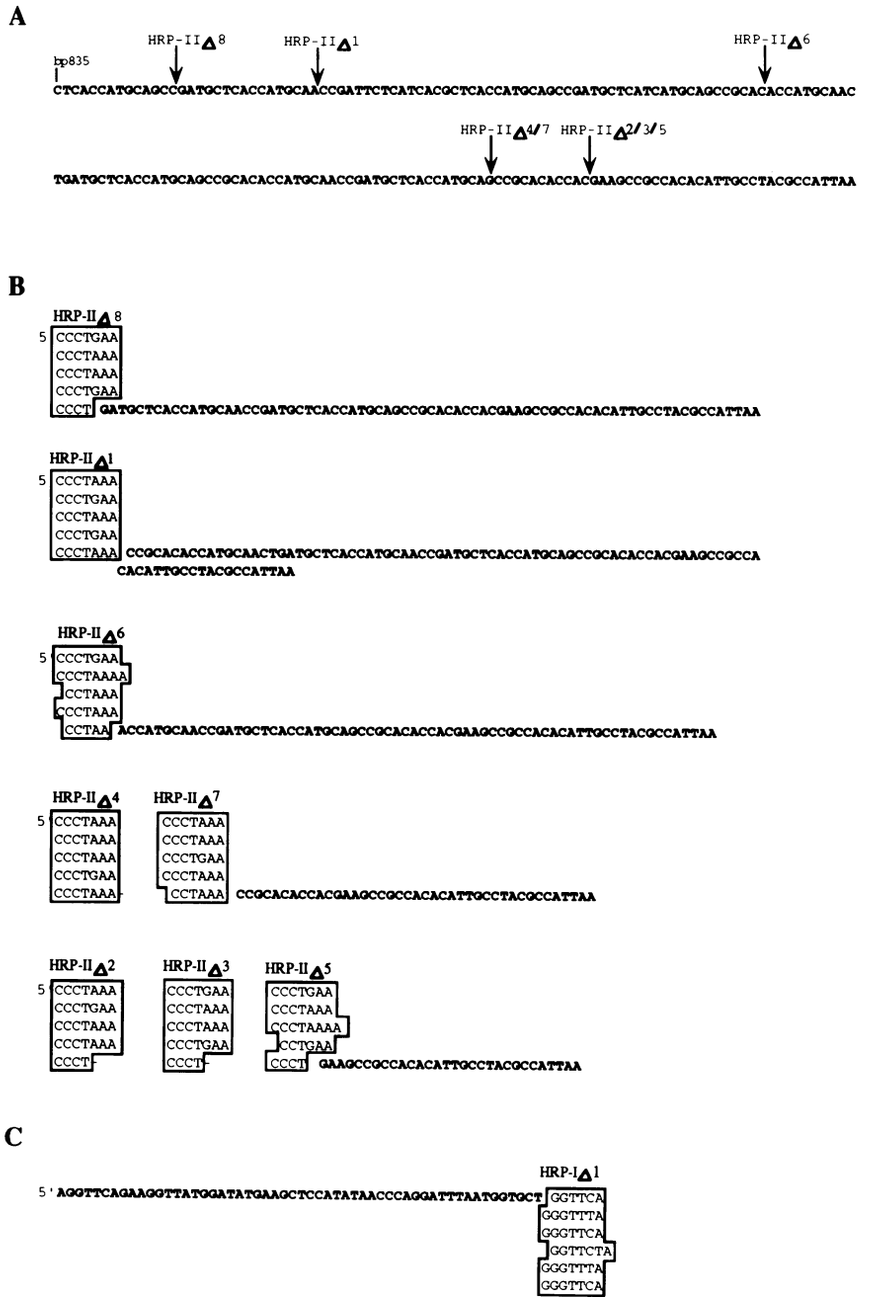

Figure 3. Chromosome breakpoints in the HRP I and HRP-II gene. The PCR-fragments generated by the telomere primer and HRP I or HRP-II primers, respectively, were cloned into pUC19 and sequenced. A) The breakpoints, indicated by arrows, occur in five distinct sites proximate to the 3′ end of the HRP-II gene (bp 835−971). B) The DNA sequence spanning the breakpoints of 8 different HRP-II deletion clones (HRP-IIΔ1−8) is shown. The last codon represents the stop codon of the HRP-II gene. C) The HRP-I breakpoint (HRP-IΔ1, bp 930) of the FCC1 strain is shown. The first 5 telomere repeats upstream each gene breakpoint (HRP-II) or downstream (HRP-I) are aligned. Note that each breaksite is followed by a distinct pattern of telomere repeats.

hybridized strongly to PCR products of four out of five parasite strains, yielding several major bands ranging between 0.5 and 1.5 kb in size (data not shown). Only one strain, that had been selected recently for the presence of knobs (Palo Alto K^+), did not yield any detectable bands with this probe. We cloned and sequenced the PCR product of strain FCC1. Nucleotides 876–930 were identical with the HRP I gene of the FCR-3 strain (17), and then abruptly changes to the sequence of the telomere repeats (Figure 3 C).

Chromosome breakage and healing occurs randomly in a site unspecific manner

An alignment of the wild-type sequences flanking each of the experimentally determined breakpoints is shown in Figure 4 and compares them with those of HRP I, HRP II genes (11), RESA (13) and the Pf11-1 gene (12). *De novo* telomere addition appears to occur in a DNA sequence independant manner in the described breakpoints, suggesting that the chromosome repair mechanism of *P. falciparum* can heal any free chromosome end. The characterized breaks are found within three distinct regions of the HRP I (Figure 5 A) and 6 distinct regions of the HRP II gene (Figure 5 B), demonstrating the random location of deletions in *P. falciparum*, rather than site specific ones.

Chromosome breakage and healing remains undetectable in clinical isolates of *P. falciparum*

The studies described above demonstrate that gene rearrangements of *P. falciparum* reflect the lability of chromosome ends during *in vitro* culture of parasite strains. In order to investigate the possible *in vivo* biological function of this type of deletion, parasite DNA was isolated directly from blood of malaria patients. Although the HRP-II gene is present in these isolates (Figure 2 B, lane 6–10), no PCR bands indicating chromosome breakage and healing events in the HRP-II locus were detectable (Figure 2 A, lane 6–10). Similar results were observed using the same approach for HRP-I gene. These results imply that host selective pressure acts against parasites with deletions in genes coding for proteins that are not essential during *in vitro* culture whereas deletions in non-coding, subtelomeric DNA has been reported (10). Alternatively, cultivation conditions *in vitro* could render the parasite genome more fragile than those of developing in the natural host.

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                    ↓
HRPIIΔ-1)      ATGCTCACCATGCAA CCGATTCTCAT
HRPIIΔ-2)      GCAGCCGCACACCAC GAAGCCGCCAC
HRPIIΔ-4)      ATGCTCACCATGCAG CCGCACACCAC
HRPIIΔ-6)      ATCATGCAGCCGCAC ACCATGCAACT
HRPIIΔ-8)      TCTCACCATGCAGCC GATGCTCACCA
HRPIIΔ(D10)    GCATGATGAGCATCA GCTGCATGGTG
HRPIΔ-1)       GGATTTAATGGTGCT AGTAATGGTAT
HRPIΔ(V)       AGCACCACAGGTTCA CCAACAAGTAC
HRPIΔ(G)       ATTATATATTTTACA AAATTTATATA
Pf11-1Δ(H12)   AGAAACATTTGTGCA AGATGTTTCAG
RESAΔ(FCR3)    GATTTTTTCTCAACA ATATATGGGTA
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Figure 4. Comparison of wild-type sequences flanking the 5′ and 3′ sides of the deletion breakpoints (arrow). The breakpoint positions of the HRP-I (V and G), HRP-II (D10), RESA and Pf11-1 are as described (11, 13, 12) and the other breakpoint positions are described in this work. The dinucleotide 'CA' found 5′ upstream the breakpoints is shown as bold letters.

DISCUSSION

In this study we demonstrate, that chromosome rearrangements occur more frequently in subtelomeric genes of *P. falciparum* than predicted from previous reports. The deletion of genes or gene fragments coding for antigens has been so far described only for some few parasite lines of *P. falciparum*. We have summarized in Table I those genes that have been shown to undergo deletional inactivation. Interestingly, all of them have in common a telomere-proximal location on distinct chromosomes. Our PCR-based study allowed us to detect in all *P. falciparum* laboratory strains examined parasite subpopulations, that have undergone chromosome breakage and healing events within the HRP-I and HRP-II genes. A similar observation has been made for a sexual stage specific gene called Pf11-1 (12). With the exception of HRP I gene deletions, observed with cultured parasites (11, 25, 26), these deletions remain undetectable by conventional genomic Southern blot analysis because of the presence of the intact gene in the majority of the parasites. This finding suggests that the mutant subpopulations are numerically small and have no selective growth advantage over wild-type parasites. In order to quantify the number of *in vitro* cultured parasites that has undergone chromosome breakage and healing we compared the intensities of PCR generated bands of the genomic DNA of laboratory strains with decreasing amounts of genomic DNA of a mutant parasite clone that partially deleted the Pf11-1 gene (12). 30 and 150 pg of mutant parasite DNA gave PCR intensities after hybridization with a Pf11-1 probe

Table I

Inactivation of genes of *P. falciparum* by genome rearrangements.

Gene	Chromosome location	Type of DNA rearrangement	References
HRP I	2; subtelomeric	chromosome breakage and healing	11 and this work
HRP II	8; subtelomeric	chromosome breakage and healing	11 and this work
HRP III	13; subtelomeric	not characterised	5
MESA	5; subtelomeric	not characterised	34
RESA	1; subtelomeric	partial inversion of the first exon	13
PF11-1	10; subtelomeric	chromosome breakage and healing	12

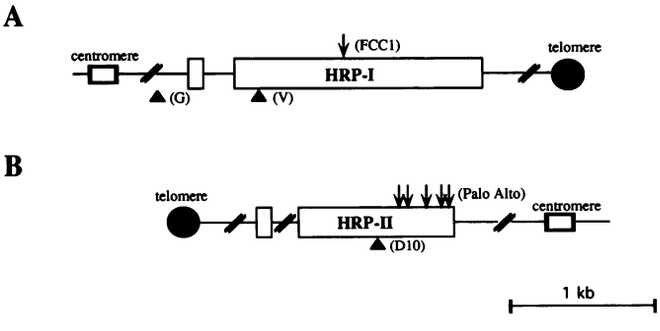

Figure 5. Summary of the breakpoints of the HRP-I and HRP-II genes. A schematic drawing of the HRP-I (A) and the HRP-II gene (B) is shown. The breakpoints described in this work for the HRP-I and HRP-II gens are indicated by arrows; those described earlier (11) are indicated by triangles. Open rectangles represent transcribed sequences, broken lines indicate undetermined distances. Abbreviations used for strains are Vietnam (V); Gambian, (G).

which ranged between those given by 100 ng of DNA of the laboratory strains (A. Scherf, unpublished results). This suggests that approximately 0.03–0.15% of the cultured parasite population have deleted part of the Pf11-1 gene. When the same PCR-Southern methodology was applied to parasite DNA isolated directly from infected patients we were unable to detect deletions in any of these genes. Thus, it appears that the corresponding gene products are functionally important to the malaria parasites developing in their natural environment. If this is true, any parasites harboring deletions in these genes would be subject to severe negative selection, at least *in vivo*. In the light of malaria vaccine development, it is important to note that the HRP II gene product, which has been successfully used in a vaccine trial to protect *Aotus* monkeys from *P. falciparum* infection (27), appears to be essential for the parasite survival in its natural host.

Our study addresses the issue of whether the breakage sites contain conserved sequences that could function as chromosome breakage site signals as has been described for some ciliated protozoa (for review see 14). The 9 breaks characterized in this work compared to those ones described earlier occur in a broad region of the HRP I and HRP II gene (Figure 5A and B) and comparison of sequences near the breakpoints do not reveal any significant consensus sequence (Figure 4). However, the dinucleotide 'CA' is found at or near the breakpoints of most cases analysed. The function of this short sequence motif is yet unknown, but one might speculate that 'CA' is a preferential substrate for the cellular healing machinery. One obvious question that arises is the biological meaning of the described chromosome rearrangements in *P. falciparum*. Are they part of a programmed gene deletion process that offers the parasite some advantage such as antigenic variation? A recent report argues against chromosomal deletions of coding gene sequences as a genetic mechanism for antigenic variation (28). They failed to detect any change in the genomic make-up of antigenically variant parasites derived from a cloned strain of *P. falciparum*. However, the role of genome rearrangements in antigenic variation is not clear, since only a very small proportion of the genome was examined using a repetitive DNA probe which hybridizes with subtelomeric regions of chromosomes. Two lines of evidence suggest that, at least for the genes studied in this paper, chromosome breakage is an accidental occuring process. First, the malaria parasite is an unicellular organism with a haploid genome in its human host and consequently, gene deletion would lead to an irreversible loss of coding sequences. Second, although we present evidence that subtelomeric chromosome breakage occurs frequently during *in vitro* culture, the fact that these rearrangements remain undetectable in the parasites natural host, makes it unlikely that it plays an important role in the parasite escape mechanism. Indeed, sporadic chromosome breakage has been observed in other organisms like maize, yeast and humans (reviewed in 29).

An interesting question is how the broken chromosome ends of *P. falciparum* acquire new telomeres? Recombination-mediated telomere acquisition in yeast has been reported (30). However, this mechanism is unlikely to be adequate for chromosome healing in *P. falciparum*, since no telomere repeats were found close to the breakpoints that could mediate telomere-telomere recombinantion. An excellent candidate for *de novo* telomere formation has been identified in ciliates and humans (29). A ribonucleoprotein enzyme, termed telomerase, can extend nontelomeric DNA ends in the absence of a DNA template in both *in vitro* (31, 32) and *in vivo* (33). The RNA portion of the telomerase has been shown to serve as a template for synthesis of telomeric DNA (29). Given that both microbial and multicellular eukaryotes have been shown to contain a 'telomerase' enzyme activity that synthesizes repetitive telomere sequences *de novo* it is likely that a similar enzyme activity is responsible for the events described in this report. Preliminary experiments indicate the existence of a telomerase like activity in crude extracts of *P. falciparum* (Scherf, A. unpublished data). However, two aspects of *de novo* telomere formation in *P. falciparum* is not easily reconciled with the current model of telomerase (33) in which a RNA template is responsible for the DNA sequence specificity of newly synthesized telomeres. First, the telomere repeats added to broken chromosome ends of malaria parasites are degenerated heptanucleotides of the type G_3T_3A or G_3T_2CA with occasionally interspearsed hexa- or octanucleotide repeats. We found at least five different types of repeats in this study. Second, each broken end contains a specific distinct mixture of heterogeneous repeats. This finding suggests that in *P. falciparum* a family of telomerase associated RNA template molecules exists that primes telomere synthesis. Furthermore, it could imply that after synthesis of each repeat the telomerase complex dissociates from the chromosome end and another complex reinitiates the next repeat. Alternatively, a less stringent telomerase enzyme activity could be responsible for the events described in this report.

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