MOLECULAR AND CELLULAR BIOLOGY, Dec. 1990, p. 6759–6764
0270-7306/90/126759-06$02.00/0
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Vol. 10, No. 12

Long Insertions within Telomeres Contribute to Chromosome Size Polymorphism in *Plasmodium berghei*

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Received 21 May 1990/Accepted 29 August 1990

During prolonged in vivo mitotic multiplication of a *Plasmodium berghei* ANKA clone (8417HP), parasites that contained an enlarged version of chromosome 4 were observed. Restriction mapping and hybridization results demonstrated that the extra DNA present in the enlarged chromosomes consists of 2.3-kb tandem repeats, known to be normally located in subtelomeric position at several chromosomal ends but absent in the original chromosome. The inserted 2.3-kb units appeared to interrupt one of the original telomeres and to create an internal (~1-kb-long) telomeric sequence.

Among isolates of *Plasmodium* species, considerable variation in the size of homologous chromosomes is revealed by pulsed field electrophoresis (2, 7, 12, 15). Extensive clonal variation of the molecular karyotype was observed even within single isolates (3; A. Saïd, Ph.D. thesis, Vrije Universiteit, Brussels, Belgium, 1987). Novel karyotypes were shown to arise during meiosis in the mosquito midgut (17) or during mitotic multiplication, both in vitro (10, 14) and in vivo (6, 10a).

In cases in which physical mapping of the size variants of the same chromosome was achieved (3, 9, 10, 13, 14, 19), size variations were invariably shown to be confined to subtelomeric regions (identified with the help of telomere-specific probes) (10a, 11, 16), while internal chromosomal organization appeared to be, on the whole, conserved (3, 10a, 13).

In *Plasmodium berghei*, a 400-kb variation in the size of a chromosome (chromosome 7) was completely accounted for by the size variation of the two subtelomeric regions and related to a difference in the copy number of a 2.3-kb, tandem repeat family (10a). These repeats appear to be clustered exclusively in a subtelomeric position on several chromosomes (4, 8). The 2.3-kb unit contains a short stretch (160 bp) of a telomere-related sequence (8), including some perfect tandem repeats of the 27-bp motif CCCTGAA CCATAAA CCTGAA CCCTAAA, composed of two canonical (CCCTGAA, CCCTAAA) and two modified (CCAT AAA, CCTGAA) heptanucleotides typical of plasmodial telomeres (11). The 160-bp stretch was shown (at least in one case) to be directly joined to the telomeric structure (4).

The finding (4) that the telomere-related, 160-bp stretches are regularly spaced in an organization similar to that described for $(C_{1-3}A)_n$ repeats in *Saccharomyces cerevisiae* (1, 18) suggested (4) that these sequences might play a role in recombinational events which lead to the maintenance and dispersal of subtelomeric repeated structures and contribute to chromosome-size polymorphism, even for chromosomes initially not possessing members of the 2.3-kb family. The work described here lends support to this hypothesis by analyzing a case in which the history of the parasite population was known and the temporal trend of the size variations was unequivocally determined.

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A cloned line of *P. berghei* ANKA (clone 8417HP; obtained from A. Saïd, Institut de Médecine Tropicale, Antwerp, Belgium) was mechanically passaged in mice (by injection of infected blood, without mosquito cyclical passages) for extended periods of time. The parasite populations, examined after a number of mechanical passages, exhibited extensive modifications of the initial karyotype and differed from experiment to experiment (6, 10a). Furthermore, they were generally heterogeneous, since different-size versions of the same chromosome (identified through chromosome-specific probes) were often present in the same population (10a). Size variants both larger and smaller than the corresponding parental chromosomes could be observed (10a).

A particularly interesting case is that of chromosome 4. In a population subjected to 62 mechanical passages (10a), it exhibited a significant increase in size (about 50 kb) and became susceptible of hybridization to a 2.3-kb probe (de-

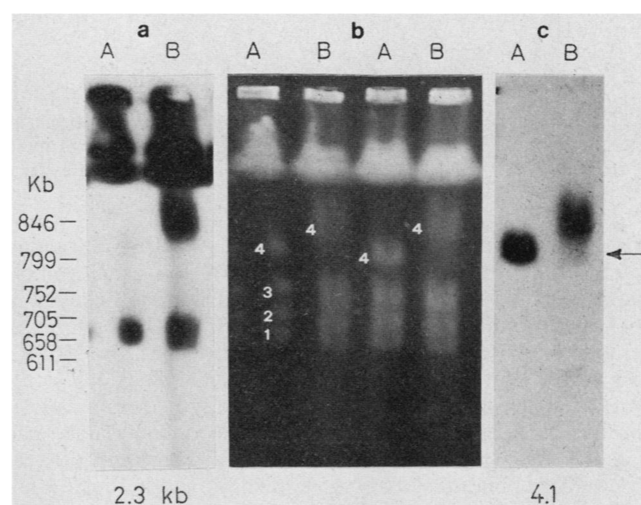

FIG. 1. (b) Contour-clamped hexagonal field electrophoresis separation of chromosomes 1 to 4 for *P. berghei* clone 8417HP (lanes A) and line HPA62, recovered after 62 mechanical passages (lanes B). The gel was blotted, and the two halves were hybridized respectively to the 2.3-kb probe (a) and to probe 4.1, specific for chromosome 4 (c). The arrow indicates material of the original size present in the heterogeneous HPA62 population.

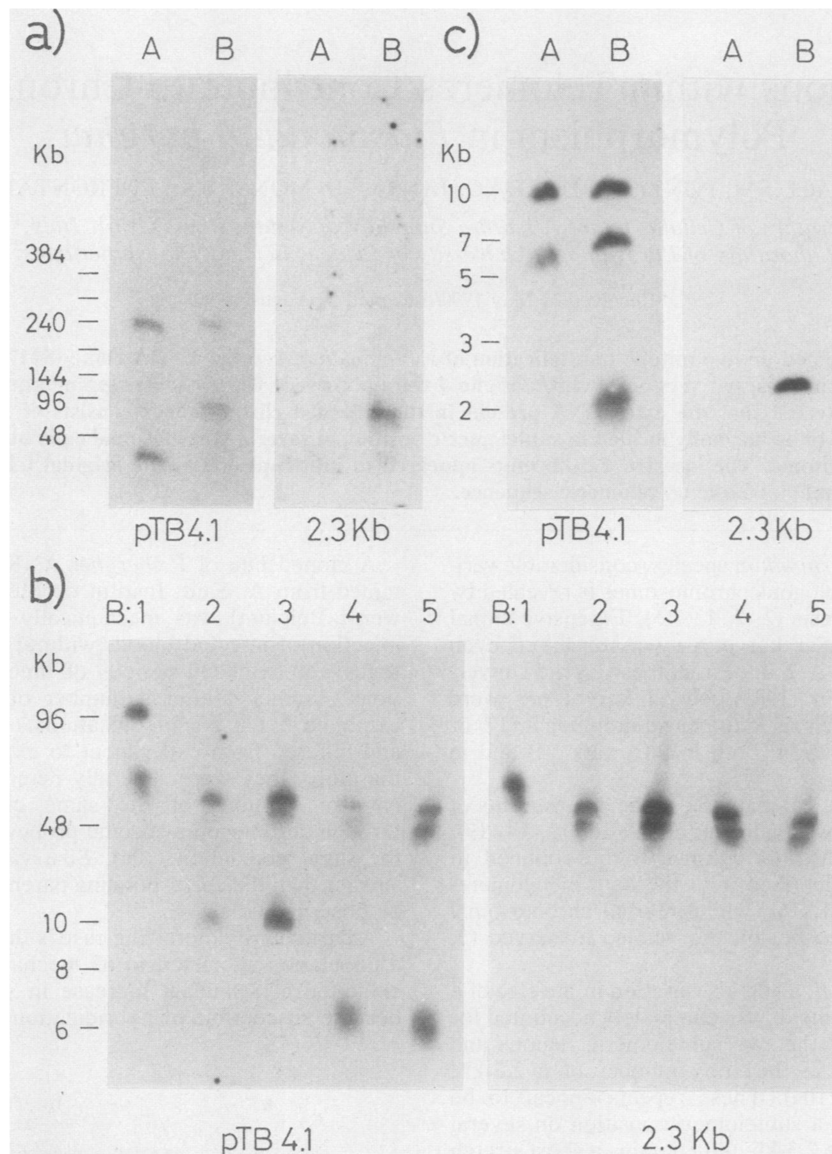

FIG. 2. Samples of chromosome 4 DNA, purified from clone 8417HP (lanes A) and from line HPA62 (lanes B), digested with different restriction enzymes and hybridized to the pTB4.1 telomeric probe (left) and to the 2.3-kb probe (right). (a) *Apa*I. (b) Lanes 1, *Bgl*I; lanes 2, *Eco*RI; lanes 3, *Eco*RI plus *Bgl*I; lanes 4, *Hae*III; lanes 5, *Hae*III plus *Bgl*I. Only the data for HPA62 (B) are shown. (c) *Cla*I.

scribed in reference 4), while the smaller (~800-kb), original version did not contain 2.3-kb repeats (Fig. 1). Chromosomes were separated by using contour-clamped hexagonal field electrophoresis under conditions (LKB Pulsaphor apparatus equipped with hexagonal electrode array; 250 V, 35-s pulse; 19 h run in 0.5× TBE [0.0445 M Tris, 0.0445 M boric acid, 0.1 mM EDTA]) such that only the four smallest chromosomes appeared in the ethidium bromide-stained gel (Fig. 1b), both in the original (8417HP) and in the passaged (HPA62) population. After hybridization with the 2.3-kb probe or with a probe specific for chromosome 4 (described in reference 10a), the autoradiograms were overexposed (at the expense of optical density linearity) so that chromosome 1 of clone 8417HP, which contains only one to three copies of the 2.3-kb repeat (10a), gave a sufficiently strong signal. It can thus be safely stated that in its original version, this chromosome does not contain 2.3-kb repeats.

Parasites with different-size versions of chromosome 4 coexisted in the population obtained after 62 weeks (Fig. 1). The band corresponding to chromosome 4 was broader and less intensely fluorescent than the others; when labeled with the probe specific for chromosome 4, it extended downward to include DNA molecules of the original size (arrow in Fig. 1). This result indicates that a small population endowed with the original, 2.3-kb-negative version of chromosome 4 was still present, even if it did not show up in the (less sensitive) fluorescent pattern. The enlarged versions of chromosome 4, unlike the original one, were heavily labeled by the 2.3-kb probe (Fig. 1a).

To investigate the chromosomal location of the extra DNA present in the enlarged chromosomal versions and positive for the 2.3-kb probe, the fluorescent gel region corresponding to chromosome 4 was recovered from the gel for both the initial and the passaged populations. The excised agarose

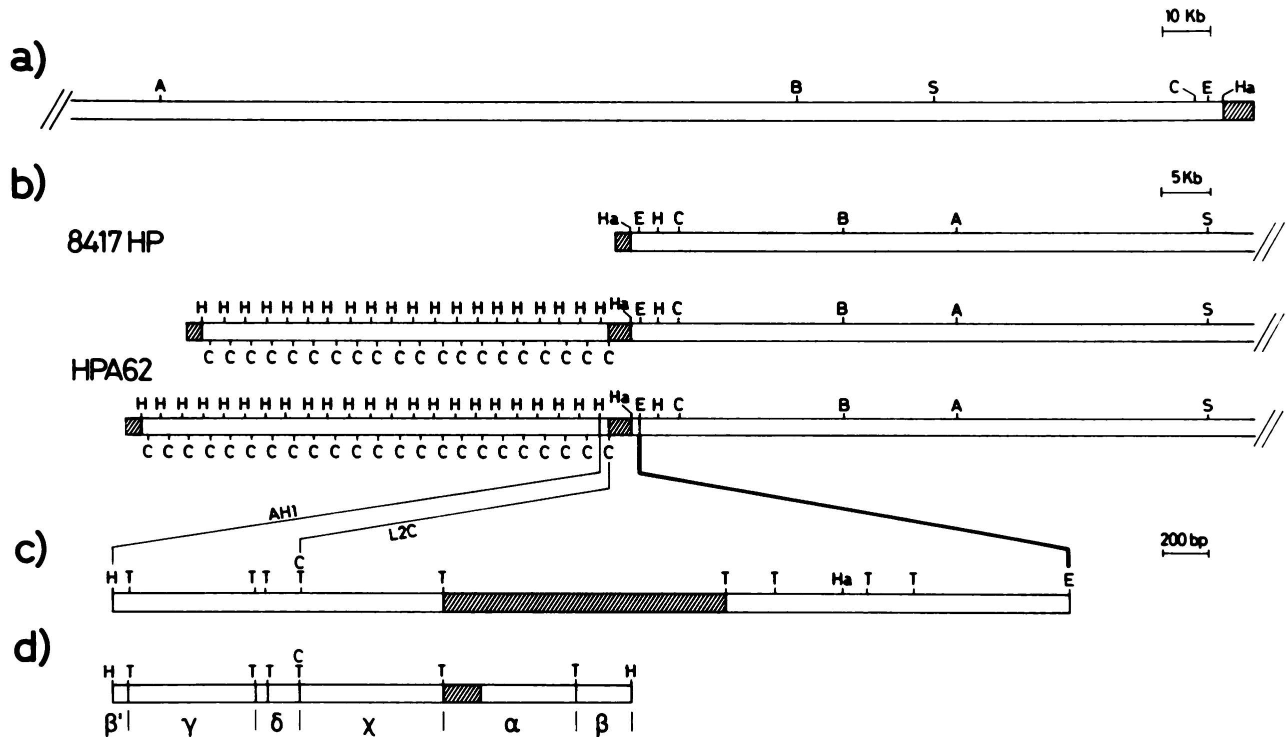

FIG. 3. Restriction maps of chromosome 4 extremities in the original 8417HP clone and in two enlarged versions present in the heterogeneous HPA62 population recovered after 62 mechanical passages. (a) Unmodified extremity, not containing 2.3-kb repeats, identical in the three examined versions. (b) 8417HP and HPA62. The original (8417HP) version lacks 2.3-kb repeats, which appear in different copy number in two versions present in the heterogeneous HPA62 population. (c) Restriction map of clone AH1 (*Hin*dIII-*Eco*RI). Clone L2C (*Cla*I-*Eco*RI) is perfectly superimposable starting from the common *Eco*RI site. Hatched regions in panels a to c indicate the smallest restriction fragments recognized by the telomeric (pTB4.1) probe. (d) *Taq*I restriction map of the (*Hin*dIII-cloned) 2.3-kb repeat (8). The hatched region indicates the stretch of 160-bp telomere-related sequence. Restriction sites: A, *Apa*I; B, *Bgl*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; S, *Sma*I; T, *Taq*I.

blocks were digested with different restriction enzymes and run under suitable resolution conditions, according to the cutting frequencies of the various enzymes. Blots were hybridized first to a purely telomeric probe (10a) and then to the 2.3-kb probe. Examples of restriction data are shown in Fig. 2, and their organization is summarized in the restriction maps shown in Fig. 3a and b. In the original (8417HP) chromosome, the most distal restriction sites typical of the 2.3-kb repeat (such as *Sau*3A, *Cla*I, and *Hin*dIII) are located more than 5 kb from the chromosome ends, which confirms that subtelomeric 2.3-kb repeats are not present in the original version.

Enzymes such as *Apa*I and *Sma*I, which cut *P. berghei* DNA only rarely, yielded the kind of pattern shown in Fig. 2a. In the original (8417HP) version of chromosome 4, *Apa*I cut two telomeric fragments (230 and 35 kb, respectively) which, as expected, did not hybridize with the 2.3-kb probe. In the enlarged version (HPA62), whereas the larger telomeric fragment was not modified, the smaller telomeric fragment exhibited a significantly larger size (85 kb) and fluoresced with the 2.3-kb probe. It can thus be concluded that sequences recognized by the 2.3-kb probe became inserted within 85 kb from that particular chromosomal end. Similar restriction data obtained by using other enzymes showed the latter figure to be as low as 50 kb.

Enzymes that cut more frequently (*Bgl*I, *Eco*RI, and *Hae*III) yielded the pattern shown for the passaged population in Fig. 2b. In the lower range of molecular weights that was analyzed in this case, the 2.3-kb-positive telomeric portion was resolved in a doublet, a result which supports the view that this population consists of two main subpopulations carrying insertions of different lengths at the same extremity. Results of double digestions (Fig. 2b, lanes 3 and 5) lend further support to this interpretation, since they yielded fragment length data incompatible with alternative interpretations based on the colinear arrangement of two insertions of unequal length along the same chromosome.

Restriction enzymes known to have a single site inside the 2.3-kb repeat, such as *Cla*I or *Hin*dIII, produced the interesting pattern shown in Fig. 2c. Instead of the two telomeric fragments normally revealed in digests of isolated chromosomes under high-stringency conditions (4), three bands were produced by the telomeric probe in the enlarged version of chromosome 4 after digestion with *Cla*I. The upper one, which closely corresponds to one of the original *Cla*I telomeric fragments (~12 kb), clearly represents the unmodified extremity. The lowest band (~2.2 kb) falls in a molecular weight range in which the heterogeneity in length typical of telomeric fragments becomes evident, so that there is little doubt that it represents the other chromosomal end. The third telomeric band (~7 kb) was shown through double digestions to map internally (Fig. 3b).

These results suggest the occurrence of a recombinational event which results in the splitting of the telomeric structure by the insertion of a number of 2.3-kb units. Partial *Hin*dIII and *Cla*I digestions (data not shown) confirm the tandem arrangement of these units in HPA62 chromosome(s) 4. Combined restriction and hybridization data indicate that the

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a
β'-γ- AA TTTCATCAAATAAAGAAACATATTGCGTTATGCATAATTCGATGTAACCACATATTA   2198
      ** *********************************************************
AH1-  AATTTCATCAAATAAAGAAACATATTGCGTTATGCATAATTCGATGTAACCACATATTA     60

      ACAAATTTTATATAATAGGAATTATAGCATGATATGAATGCATATGTAGTCAATATATAT  2138
      ************************************************************
      ACAAATTTTATATAATAGGAATTATAGCATGATATGAATGCATATGTAGTCAATATATAT   120

      ATTAATATATACAATACAAATAT......                                 2115
      ***********************
      ATTAATATATACAATACAAATAT......                                  143

b
χ-    TCGATAAAACTACTAATTAATTTATTTACTAGTGCTGACCCTGATAATGTGATGTTAGAA  1391
      ******** *** * **********************************************
L2C-  TCGATAAA CTA TNATTAATTTATTTACTAGTGCTGACCCTGATAATGTGATGTTAGAA    58

      CTTCGGTTCAGAACTTTGTGCAGGATTTTGTATTGTGTTTATCGGTGTAATGGATGGAAT  1331
      * ********************************************** ************
      C TCGGTTCAGAACTTTGTGCAGGATTTTGTATTGTGTTTATCGGTGAAATGGATGGAAT   117

      ATCACTACAATCAACTTTATATTACAAT.......                           1303
      ****************************
      ATCACTACAATCAACTTTATATTACAAT.......                            145

c
α     TCGAACAACCATAGACCATAAA CCCTAAACCACAAACCCTAAA|CCCTGAACCATAAACC   758
      ********************** *********************|****************
T1    TCGAACAACCATAGACCATAAACCCCTAAACCACAAACCCTAAA|CCCTGAACCATAAACC    60

      TGAACCCTAAA|CCCTGAACCATAAACCTGAACCCTAAA|CCCTGAACCATAAACCTGAAC   699
      ***********|***************************|*********************
      TGAACCCTAAA|CCCTGAACCATAAACCTGAACCCTAAA|CCCTGAACCATAAACCTGAAC   119

      CCTAAA| CCAAAAACCTAAACCTGAACCATAAA CCCT AAACCAAATTAACATATTTCA   646
      ******| **  ** * ************ **** **** ***** **     * *   *
      CCTAAA|CCCTGAACCATAAACCTGAACCCTAAA|CCCTGAACCCTAAACCCTAAACCCTA   178

      GTTTTAGTCATTGATACAATATTATATAT...                               617
          *  *  *   *
      AACCCTAAACCTAAACCCTAAACCCTGAA X X Y X Y Y Y X Y X Y Y X X Y X   319
                                X   Y
      Y Y X Y X Y Y Y X Y Y X Y Y Y X X Y X Y X Y Y X X X Y X Y X X   536

      X Y X X X Y Y Y X Y X Y Y X Y Y X Y Y X Y X Y X Y Y X Y X Y Y   753

      Y Y Y Y Y X X Y Y X X Y X X X Y X Y X Y X X Y X X X Y X Y Y Y   970

      Y X Y X X Y X X Y X Y X Y X X Y X X X Y X Y X Y CCCTATAATCTTC  1151

      CTCTATATGTTTCTATATACTTAATAATACCATTTTCTTATTAACTCAATTTATAATTATT  1212

      CCATTGATTATTTTCGA                                             1229
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FIG. 4. Partial sequencing of the extremities of clones AH1 and L2C and complete sequencing of the pTB4.1-positive T1 subfragment. (a) The distal extremity of clone AH1 is compared with subfragments β′ and γ of the 2.3-kb unit (8); 99% homology is found over the 143 bp sequenced. (b) The distal extremity of clone L2C is compared with subfragment χ of the 2.3-kb unit (8); 97% homology is found over the 145 bp sequenced. (c) The complete sequence of fragment T1 (1,229 bp) is compared with the first (distal; 4) 206 bp of subfragment α; 95% homology is found over the 160-bp stretch of telomere-related sequence (from nucleotides 817 to 657 of the α sequence). X and Y indicate telomeric heptanucleotides CCCTAAA and CCCTGAA, respectively. The 27-bp motifs are boxed. Nucleotide numbering for subfragments β′, γ, χ, and α corresponds to the deposited sequence of the 2.3-kb unit (GenBank accession number M19300).

length of the insertions (42 to 49 kb) corresponds to the overall size increase of chromosome 4, estimated to be in the order of magnitude of 50 kb.

To examine the structure and the environment of the internal telomeric region in more detail, we used the telomeric probe to select, from HPA62 genomic libraries constructed in pUC8, the *Hin*dIII-*Eco*RI and *Cla*I-*Eco*RI recombinants shown in Fig. 3c (clones AH1 and L2C). *Taq*I maps of the inserts of these clones are superimposed in Fig. 3c. The known (8) *Taq*I map of the *Hin*dIII-cloned, 2.3-kb unit is also reported for purposes of comparison (Fig. 3d). *Taq*I subfragments of the 2.3-kb unit, ordered in a telomere → centromere direction, are indicated as β′, γ, δ, χ, α, and β.

The close correspondence between the left-hand parts of the 2.3-kb unit and of clones AH1 and L2C is evident from Fig. 3c and d and was confirmed by partial sequencing (Fig. 4). A 97 to 99% sequence homology was found (Fig. 4a and b) when β, part of γ, and part of χ were compared with the corresponding regions of clones AH1 and L2C. Identical, nonrepetitive sequences were obtained for both clones when they were sequenced from the proximal *Eco*RI site (data not shown). The proximal *Eco*RI-*Taq*I fragment was subcloned and shown to recognize the *Hin*dIII telomeric band (Fig. 3b) in digests of isolated chromosome 4 from 8417HP.

The central *Taq*I subfragment, T1 (hatched in Fig. 3c), was completely sequenced (Fig. 4c). Canonical telomeric heptanucleotide repeats (X = CCCTAAA and Y = CCCT GAA) occupy the main part of the fragment, whose total length is 1,229 bp. Only the last, proximally located 90 bp are free from telomeric repeats. In Fig. 4c, the T1 sequence, which is read on the C-rich strand in the telomere → centromere direction, is compared with the first 200 bp of subfragment α (read in the same direction). An almost perfect homology extends over the region of α containing telomere-related motifs (8); 27-bp repeats (boxes in Fig. 4c) start exactly at the same position in both sequences, but four perfect copies of the repeat are found in clone T1 instead of the three known to be present in α. The degree of homology decreases sharply thereafter. It can thus be concluded that the inserted 2.3-kb units are joined to the internal telomeric region through the 27-bp repeat region of the adjacent 2.3-kb unit.

In conclusion, we report here the insertion of a number of 2.3-kb tandem repeats into an originally 2.3-kb-negative chromosome during mitotic multiplication in vivo. The modified chromosomal version undergoes a corresponding increase in size, amounting to about 50 kb. Occasional losses of some 2.3-kb units, taking place after the insertion event, can explain the presence of parasite subpopulations with slightly different numbers of inserted units. The insertion appears to split one of the original telomeres, leaving an internal telomeric sequence (about 1 kb in length). This is exactly the effect predicted for a recombinational event involving one of the short (160-bp) internal telomeric sequences, periodically positioned in the *P. berghei* subtelomeric repeat structure (4), and the actual telomere of a nonhomologous chromosome. Since a reciprocal change of ~50 kb would be undetectable in one of the larger chromosomes, we cannot distinguish between reciprocal and nonreciprocal recombinational mechanisms of sequence transfer, such as unequal crossing over or strand invasion and copy (5). Alternatively, a cluster of 2.3-kb repeats might be present, at least transiently, in an episomal form not easy to detect.

In any case, our results suggest that the initial pairing event of the postulated recombinational mechanism should involve a relatively short homology region within the 27-bp motifs through which 2.3-kb units became joined to the bulk chromosome. A similar mechanism might be expected to contribute to size polymorphisms in the case of *S. cerevisiae* chromosomes, whose subtelomeric organization, with telomeric stretches separating Y′ repeats (1, 18), closely corresponds to that of *P. berghei*.

The sequence reported for the 2.3-kb unit has been deposited in GenBank (accession number M19300).

Thanks are due to Elisabetta Pizzi for help in sequence treatment and to Raffaella Scotti for skillful technical assistance.

This work was supported by the Commission of European Communities in the framework of the program Science and Technology for Development under contract TS2 0082 and in part under contract TS2 0126.

LITERATURE CITED

1. **Chan, C. S. M., and B. K. Tye.** 1983. Organization of DNA sequences and replication origins at yeast telomeres. Cell **33:** 563–573.
2. **Corcoran, L. M., K. P. Forsyth, A. E. Bianco, G. V. Brown, and D. J. Kemp.** 1986. Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite populations. Cell **44:**87–95.
3. **Corcoran, L. M., J. K. Thompson, D. Walliker, and D. J. Kemp.** 1988. Homologous recombination within subtelomeric repeat sequences generates chromosome size polymorphisms in *P. falciparum*. Cell **53:**807–813.
4. **Dore, E., T. Pace, M. Ponzi, L. Picci, and C. Frontali.** 1990. Organization of subtelomeric repeats in *Plasmodium berghei*. Mol. Cell. Biol. **10:**2423–2427.
5. **Dunn, B., P. Szauter, M. L. Pardue, and J. W. Szostak.** 1984. Transfer of yeast telomeres to linear plasmids by recombination. Cell **39:**191–201.
6. **Janse, C. J., E. G. Boorsma, J. Ramesar, P. van Vianen, R. van der Meer, P. Zenobi, O. Casaglia, B. Mons, and F. M. van der Berg.** 1989. *Plasmodium berghei*: gametocyte production, DNA content and chromosome-size polymorphisms during asexual multiplication *in vivo*. Exp. Parasitol. **68:**274–282.
7. **Langsley, G., J. Patarapotikul, S. Handunnetti, E. Khouri, K. N. Mendis, and P. H. David.** 1988. *Plasmodium vivax*: karyotype polymorphism of field isolates. Exp. Parasitol. **67:**301–306.
8. **Pace, T., M. Ponzi, E. Dore, and C. Frontali.** 1987. Telomeric motifs are present in a highly repetitive element in the *Plasmodium berghei* genome. Mol. Biochem. Parasitol. **24:**193–202.
9. **Patarapotikul, J., and G. Langsley.** 1988. Chromosome size polymorphism in *Plasmodium falciparum* can involve deletions of the subtelomeric pPFrep20 sequence. Nucleic Acids Res. **16:**4331–4340.
10. **Pologe, L. G., and J. V. Ravetch.** 1988. Large deletions result from breakage and healing of *P. falciparum* chromosomes. Cell **55:**869–874.

10a. **Ponzi, M., C. J. Janse, E. Doze, R. Scotti, T. Pace, T. J. F. Reterink, F. M. van der Berg, and B. Mons.** 1990. Generation of chromosome size polymorphism during in vivo mitotic multiplication of *P. berghei* involves both loss and addition of subtelomeric repeat sequences. Mol. Biochem. Parasitol. **41:**73–82.

11. **Ponzi, M., T. Pace, E. Dore, and C. Frontali.** 1985. Identification of a telomeric DNA sequence in *Plasmodium berghei*. EMBO J. **11:**2991–2995.
12. **Sheppard, M., J. K. Thompson, R. F. Anders, D. J. Kemp, and A. M. Lew.** 1989. Molecular karyotyping of the rodent malarias *Plasmodium chabaudi*, *Plasmodium berghei* and *Plasmodium vinckei*. Mol. Biochem. Parasitol. **34:**45–52.
13. **Sinnis, P., and T. E. Wellems.** 1988. Long-range restriction maps of *Plasmodium falciparum* chromosomes: crossingover and size variation among geographically distant isolates. Genomics **3:**287–295.

14. **Tan-Ariya, P., Y. F. Yang, and A. Kilejian.** 1988. *Plasmodium falciparum*: comparison of the genomic organization of the knob protein gene in knobby and knobless variants. Exp. Parasitol. **67**:129–136.
15. **Van der Ploeg, L. H. T., M. Smits, T. Ponnudurai, A. Vermeulen, J. H. E. T. Meuwissen, and G. Langsley.** 1985. Chromosome-sized DNA molecules of *Plasmodium falciparum*. Science **229**:658–661.
16. **Vernick, K. D., and T. F. McCutchan.** 1988. Sequence and structure of *Plasmodium falciparum* telomere. Mol. Biochem. Parasitol. **28**:85–94.
17. **Walliker, D., I. A. Quakyi, T. E. Wellems, T. F. McCutchan, A. Szarfman, W. T. London, L. M. Corcoran, T. R. Burkot, and R. Carter.** 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science **236**:1661–1666.
18. **Walmsley, R. W., C. S. M. Chan, B. K. Tye, and T. D. Petes.** 1984. Unusual DNA sequences associated with the ends of yeast chromosomes. Nature (London) **310**:157–160.
19. **Wellems, T. E., D. Walliker, C. L. Smith, V. E. do Rosario, W. L. Maloy, R. J. Howard, R. Carter, and T. F. McCutchan.** 1987. A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *P. falciparum*. Cell **49**:633–642.