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Extensive turnover of telomeric DNA at a *Plasmodium berghei* chromosomal extremity marked by a rare recombinational event

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ABSTRACT

The dynamics of telomere turnover were studied in *Plasmodium*, whose telomeric structures consist of linear, recognisable sequences of two distinct repeats (TTTAGGG and TTCAGGG). Independent recombinant clones containing a well-defined chromosomal extremity of *Plasmodium berghei*, both before and after a rare insertion event took place, were obtained from clonal parasite populations and analysed. The insertion, which splits the original telomere and causes a significant reduction in the size of the telomeric structure, is shown to consist of an integer number of subtelomeric repeats typical of *P.berghei*, flanked on both sides by telomere-derived motifs. Analysis of the telomeric repeat sequence heterogeneity in the otherwise homogeneous populations examined, is compatible with a model in which diversification of a given telomere is driven by the occurence of breakpoints whose frequency rapidly increases along the telomeric tract when moving in the outward direction. The breakpoints might be due either to terminal deletions followed by random serial addition of the two repeat versions, or to recombination events. The shortening/elongation mechanism is favoured against the recombination hypothesis because of the absence of higher-order patterns in the sequence of telomeric repeats.

INTRODUCTION

Telomeres, the specialised structures that ensure the stability of eukaryotic chromosomes and the complete replication of their DNA (reviewed by Blackburn and Szostak (1) and by Blackburn(2)) consist of arrays of simple repeats (see list in ref. 3) characterised by an intrinsic length heterogeneity. The latter is generally thought to be due to a fluctuation in the number of repeats around an average value which results from a dynamic equilibrium between lengthening and shortening processes.

The first direct evidence of telomere growth (7–10 bp per generation) came from experiments with logarithmically multiplying tripanosomes (4, 5) where telomere growth was balanced by occasional, large deletions. Coordinated and developmentally regulated variations in telomere length were reported to occur in different systems (6–9). The identification of telomerase activity (10–14) provided a plausible mechanism for the addition of species-specific (15) telomeric repeat units, whose template is found in the RNA component of the enzyme (16, 17, 18).

A model involving a dynamic equilibrium between a stepwise lengthening activity (such as that of telomerases)and a telomere shortening process caused by incomplete replication or exonucleolytic degradation was proposed by Shampay, Szostak and Blackburn (15). Its predictions were experimentally verified (19) by studying the length distribution of individual telomeres in clonal yeast populations.

The possibility that recombinational events also play a role in telomere formation received support from the work by Pluta and Zakian (20) and by Wang and Zakian (21). The subject is reviewed by Zakian (22).

In general, telomeric structures are composed of identical repeat units, and telomeres belonging to different chromosomal ends are indistinguishable. This renders a direct demonstration of interchromosomal exchange of telomeric stretches impossible. In order to reveal telomere-telomere transfer (21,22) 'chimaeric' linear plasmids, bearing different termini ($[C_4A_4]_n$ from *Oxytricha* and $[C_4A_2]_n$ from *Tetrahymena*), were exploited.

Telomeres from *Saccharomyces cerevisiae* are not uniform, being composed of repeats which conform to the 'consensus' $C_{2-3}A(CA)_{1-6}$ (23). This allowed Wang and Zakian (23) to analyse the progeny of individual *S.cerevisiae* transformants by comparing the arrays of yeast-specific repeats added onto the ends of the linear plasmids originally carrying *Tetrahymena* and *Oxytricha* termini. Their analysis showed that telomeres cloned from a single transformant differ from each other in their distal portion, while the proximal 120–150 bp are conserved in the clonal population, as if they had been 'protected' from the

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recombination or shortening and elongation events that are probably responsible for the variability of the distal domain.

The experimental situation studied by Wang and Zakian (23) refers to the stabilization in yeast of an exogenous plasmid, presumably by the same mechanism(s) acting on chromosomal telomeres.

Chromosomal telomeres from *Plasmodium* species (24) are formed by two kinds of 7 bp repeats (TTTAGGG and TTCAGGG) (25) irregularly alternating in a tandem array which can be represented as a linear, recognizable sequence of two symbols. We took advantage of a rare insertion event in a telomere of *P.berghei* (26) to study telomere sequence variability at a well-defined chromosomal extremity in the clonal population originated by the parasite in which the event took place.

To this aim a number of recombinant clones spanning the distal junction of the insertion and part of the telomeric structure were obtained from the clonal population marked by the insertion event. Other telomeric clones were derived from the original population, which is also clonal (29).

Sequencing of the distal junction confirmed previous evidence concerning the involvement of telomere-derived motifs in the mechanism of insertion.

Intra-clonal sequence heterogeneity (under stationary conditions concerning telomere length) indicated that events responsible for telomere turnover are distributed over a large portion of the telomere, their frequency steadily increasing in the outward direction.

MATERIALS AND METHODS

Strains

Clone 8417HP was obtained at the Institut de Medicine Tropicale 'Prince Leopold' in Antwerp, by limiting dilution, from an ANKA strain of *P.berghei*. This ANKA strain had been maintained by alternating blood passages and mosquito transmission in the laboratory. Clone 8417HP, derived at the 28th blood passage after the last mosquito transmission, was characterised as a high-producer (HP) of gametocytes, (ref. 29 and A.Saïd, Thesis, Vrije Universiteit Brussels, 1987). Through the courtesy of M.Wery and A.Saïd, the 8417HP clone was made available to us and to other laboratories. In the Parasitology Laboratory of the University of Leiden, the 8417HP clone was followed during weekly mechanical passages in mice (by intraperitoneal injection of 10^6 parasites) in several independent experiments (30). HPA is one of these serially passaged lines. HPA62 and HPA87 are samples frozen at the 62nd and 87th mechanical passage, respectively. We are indebted to B.Mons and C.J.Janse for these samples. The molecular karyotypes of 8417HP and HPA62 are given in ref.30. Polymorphism affecting chromosome 4 is described in ref.26.

Chromosome 4 separation, identification and purification

Contour-clamped Hexagonal Field (CHEF) separation of the four smallest chromosomes in 8417HP and HPA62 is described in ref.26. Probe 4.1 (30) was used for the identification of chromosome 4 on Southern blots of CHEF gels. SEAKEM-GTG agarose is used whenever CHEF separation is to be followed by restriction digestions (performed directly on the excised agarose block containing the band of interest) or by recovery of the chromosomal band through extraction by phenol, saturated with 0.5 M Tris, 0.1 M NaCl, 0.01 M EDTA.

Molecular cloning strategy

Recombinant clones spanning the proximal junction of the insertion and containing about 1.5 kb of a unique sequence specific for chromosome 4 are described in ref.26. The centromere-proximal (696 bp) EcoRI-TaqI fragment of one of these clones (AH1, see Figure 1d) was subcloned to obtain a chromosome-4 subtelomeric probe (clone ET3, see Figure 1b and d). The latter was used to select the desired extremity from an especially designed 8417HP genomic library.

To generate this library, high molecular weight, total DNA from 8417HP blood-stage parasites (average fragment size was estimated to be 350 Kb by CHEF separation in the LKB Pulsaphor apparatus using 250 volts, 12 sec. pulses) was first made blunt-ended by Bal31 digestion (60 bp removed by a 10 minute treatment at 30°C with 0.3 Units per microgram of DNA) and then ligated to SmaI-EcoRI digested pUC8. After ligation, the mixture was re-digested with EcoRI. The large, linear recombinant molecules thus obtained were re-ligated after dilution to a concentration of 2 ng/ml (so as to favour circularization) and used to transform *E.coli* DH5-α cells. The four clones selected from this library by the ET3 probe (indicated as HP.1-HP.4 in Figure 1b) reacted positively to the purely telomeric probe pTB4.1 (30).

A different strategy was adopted to clone the modified extremity of chromosome 4 from HPA62. The problem here was that the actual telomere is preceded by 2.3 Kb repeats present also in many other chromosomal ends, while the sequence recognised by the chromosome-specific probe ET3 lies ~50 Kb upstream. To circumvent this problem, we exploited the fact that 2.3 Kb repeats are present only at one end of the modified version of chromosome 4 (26), and utilised a 2.3 Kb probe to select the clones containing the desired extremity from a HPA62-chromosome 4 library. The library was constructed as follows: DNA of the enlarged version of chromosome 4 was extracted from SEAKEM agarose blocks as decribed above, after having been restricted by ClaI digestion (ClaI cuts once within the 2.3 Kb unit, see Figure 1c). The recovered fragments were exposed first to Bal31 (0.3 U/μg, 10 min. at 30°C), then to HindIII digestion, and finally ligated to SmaI-HindIII digested pUC8. Among the ~1000 transformant colonies thus obtained, 7 were selected by the 2.3 Kb probe and shown to be positive also to the purely telomeric probe pTB4.1 (30). They are indicated as 62.2−62.8 in Figure 1f).

Transformation

E.coli DH5-α cells were electroporated in the presence of the appropriate recombinant plasmid using a Gene-Pulser Bio-Rad apparatus under the conditions indicated by the manufacturer.

Sequencing by primer extension

The distal portions of telomeric clones HP.1-.4 and 62.2-.8 were sequenced in the telomere → centromere direction using M13 primers (in the first case the M13 sequencing primer, in the second case the reverse M13 sequencing primer). The complementary strand was sequenced in the centromere → telomere direction, using as primers suitable synthetic oligonucleotides for each set of clones. In the case of HP.1-.4, the synthetic oligonucleotide reproduced the sequence TGAGTTAATAAGAAAATGGT between nucleotides 1199 and 1180 of the subtelomeric, chromosome 4-specific portion of clone T1 (26) (see Figure 1d). In the case of 62.2-.8, the synthetic

primer corresponded to the sequence AATATTGTATCAATG-ACT between nucleotides 623 and 640 of the 2.3 Kb element (subfragment α, adjacent to the telomeric structure in these clones, Figure 1f). The complete sequence of the 2.3 Kb element is given in ref.27.

RESULTS

Figure 1 describes an event which occurred occasionally during mitotic multiplication of *P.berghei* ANKA clone 8417HP. The parasite population recovered after 62 mechanical passages, and referred to as HPA62 (30), exhibited a modified molecular karyotype, as revealed by pulse field electrophoresis. In particular, a major fraction of the passaged parasite population possessed an enlarged version of chromosome 4.

The modification affecting chromosome 4 was observed only once in the course of several parallel propagation experiments (30), indicating that the corresponding event must be sufficiently rare. It was demonstrated (26) that the extra-DNA (~50 Kb) present in the enlarged version corresponds to the tandem insertion of a number of 2.3 Kb repeats, typical of *P.berghei* subtelomeric regions, into the telomere of a chromosome (chromosome 4) which completely lacks this repeated structure in the original parasite population.

The 2.3 Kb unit (whose restriction map is given for comparison in Figure 1c) contains a stretch (160 bp long) of telomere-related sequences (27, 28). By sequencing a cloned 2.3 kb element, it was shown (27) that this stretch contains three directly repeated elements (27 bp long), each of which is formed by two non-canonical telomeric repeats flanked by canonical copies of the two repeats (TTTAGGG and TTCAGGG) present in plasmodial telomeres (25). Four instead of three of these 27 bp elements are present at the proximal junction of the insertion (26).

In the present study we analyse four independent clones (HP.1-Hp.4, Figure 1b) containing the desired extremity of chromosome 4 in its original form and seven independent clones (62.2−62.8, Figure 1f) of the chromosome's enlarged version. Results relating to the structure of the insertion and to telomere sequence variability are described in the following sections.

Structure of the insertion

In order to analyse the distal junction, linking the outermost 2.3 Kb unit to the actual telomere of chromosome 4 HPA62, the relevant part of clones 62.2−62.8 was sequenced by primer extension. Results are presented in Figure 2a).

Alignment with the sequence (27) of the 2.3 Kb repeat (first line in Figure 2a) reveals an almost perfect identity between the proximal part of the seven sequenced clones and the corresponding portion of the 2.3 kb unit (subfragment α), up to the group of three 27mers. In all except one of the clones, a short duplication (possibly due to a slippage event) modifies the innermost 27mer. In all seven clones, a fourth, almost perfect 27mer is present. Note that the few changes with respect to the α sequence (surprisingly, all G additions. See asterisks in Figure 2a) and a single base substitution in the fourth 27mer are identical in the seven clones. This confirms their common origin from a single ancestor.

Together with the results previously obtained for the proximal junction (26) (also summarised in Figure 1d) these results indicate that the insertion contains an integer number of 2.3 kb units but

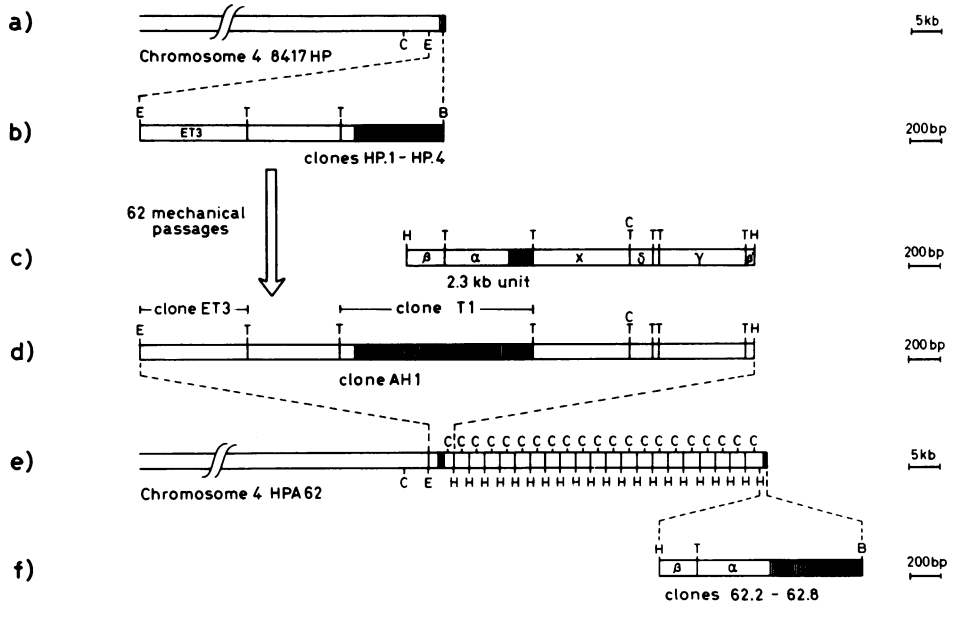

Figure 1. Schematic representation of the events modifying one of the extremities of chromosome 4. The original extremity, shown in a), does not contain the ubiquitous 2.3 kb repeats. The telomeric array is flanked by sequences specific for chromosome 4, as shown by the structure of the four independent telomeric clones HP.1−HP.4, presented in b) on an enlarged scale. After 62 mechanical passages, most of the passaged population (HPA62) possesses an enlarged version of chromosome 4 (shown in e) on the same scale as in a)) containing an insertion which splits the original telomere. The insertion consists of tandemly arranged copies of the 2.3 kb repeat unit. The restriction map of the 2.3 kb unit is drawn, for comparison, in c). The proximal junction of the insertion had been previously cloned (clone AH1, described in ref.26). Its structure is presented, on an enlarged scale, under d). In the present work the distal junction and the adjacent telomeric tract have been characterized by studying and sequencing 7 independent clones (62.2−62.8), indicated under f). ET3 and T1 are subclones of AH1; α,β,β′,γ,δ,x are TaqI subfragments of the 2.3 kb unit. E = EcoRI; C = ClaI; T = TaqI; H = HindIII; B = Bal31.

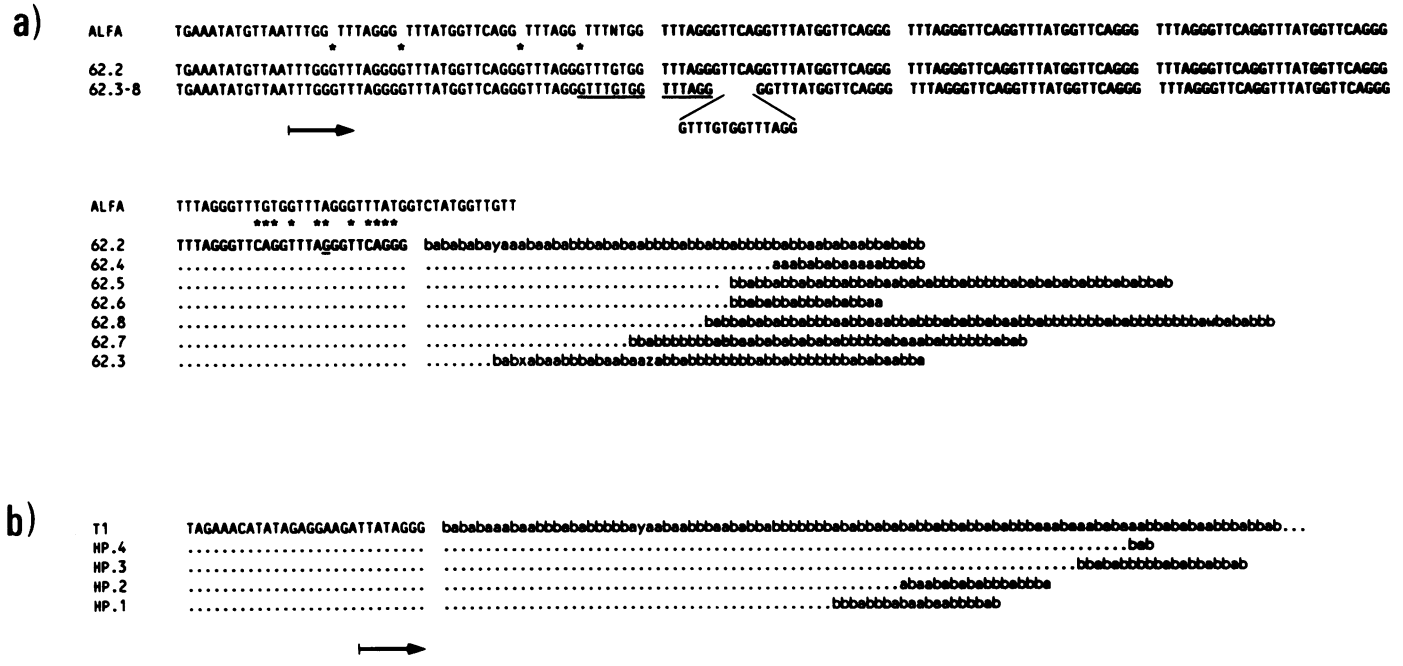

Figure 2. a) Sequences of clones 62.2−62.8 are aligned with the portion of the α-subfragment containing three 27 bp elements (nucleotide blocks in bold characters). Differences are marked by asterisks. The 14 bp insertion duplicates the immediately preceding group of nucleotides (underlined). Distally to the three 27 bp elements, the sequence of the α-subfragment continues with apparently degenerate telomeric repeats, while the seven clones bear a fourth 27 bp element (in bold characters) carrying one base substitution (underlined) with respect to the standard 27-mer. b) Sequences of clones HP.1−HP.4 are aligned with the corresponding portion of clone T1 (see Figure 1d). Telomeric repeat variants are indicated by the symbols: a:TTTAGGG, b:TTCAGGG, x:TGTAGG, y:TTCAGGT, z:TTTCAGGG, w:TTTAGC. Arrows indicate the presumed start of the telomeric structure.

is flanked on both sides by a group of four 27mers. This fact is reminiscent of a duplication of an insertion site, but it is difficult to further speculate on this point for two different reasons: on the one hand, because groups of four 27mers — though present in the insertion only at its boundaries — can be found in a small number of copies in the genome of the original strain 8417HP (unpublished results); on the other hand, because no possibility exists to determine the sequence of the empty insertion site, given the high degree of intraclonal telomere variability, especially in the distal region (see below).

Telomere sequence variability

Directly joined to the fourth 27mer, the array of telomeric repeats presents a further identical stretch of 8 repeats (56 bp) in all the seven sibling clones obtained from chromosome 4 HPA62 (Figure 2a). Intraclonal variability appears to progressively increase thereafter. The longest identical series of repeats is found in clones 2 and 4, which share a common series of 41 repeats (~290 bp) after the last 27mer.

Figure 2b shows the results obtained on four clones containing the same end of chromosome 4 as found in the parasite population (85417HP) before the insertion event took place. The telomeric stretch they contain is compared with the corresponding part of the internal, 1 Kb telomeric sequence present in the enlarged version of the chromosome (first line in Figure 2b). An identical initial sequence of at least 47 repeats (~330 bp) confirms the homogeneity of the initial population. Variability in the sequence of telomeric repeats appears to steadily increase thereafter, so that from the 84th repeat on (i.e. ~590 bp) each of the four telomeres continues with a different sequence.

In both groups of sequenced telomeres there is very little similarity between sequences of repeats once they have started to diverge, i.e. there are breakpoints after which a completely different telomeric stretch replaces the original sequence. Note that for the same reason it is impossible to identify sequence changes accumulating distally to the first breakpoint.

Average telomere length in the '*in vivo*' experimental situation

We next investigated whether the newly formed telomere, after the drastic shortening of the telomeric structure caused by the insertion, gradually returns to the original size. As shown in Figure 3, further mitotic propagation of line HPA62 did not result in an increase of the average length of the telomere concerned. The enlarged chromosome 4 recovered from passage 87 (each passage involving 6−8 schizogonic replication cycles), after ClaI digestion and hybridization to the telomeric probe, still exhibits the same three bands present at passage 62 (26). In particular, the size of the lowest, smeared band (2.15 ± 0.15 Kb) is identical in the two populations (Figure 3). If one takes into account the 1.5 Kb belonging to the last 2.3 Kb repeat (see Figure 1e), this figure corresponds to a telomeric structure with an average length 0.65 Kb (~95 repeats) including the 160 bp region rich in non-canonical repeats. It thus appears that, by the time passage 62 is reached, the clonal population — stemming from the individual parasite which had suffered the insertion at some point in the course of the first 62 passages — is already in a situation of equilibrium as far as shortening and lengthening processes are concerned, at least at the extremity involved in the event described. Similarly, the length of the original telomere in the 8417HP population can be judged from the size of terminal EcoRI

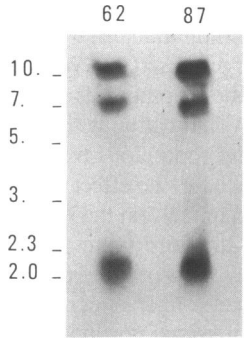

Figure 3. Chromosome-4 DNA, purified from HPA62 and HPA87 (the 62nd and 87th passages, respectively, of the mechanically passaged line HPA) restricted with ClaI and hybridized to the telomeric probe pTB4.1 (30). Of the three bands recognised by the telomeric probe, the highest one corresponds to the extremity of chromosome 4 not interested by the insertion event. The intermediate and the lower bands correspond to the ClaI fragments containing respectively the internal and outer portions of the interrupted telomere.

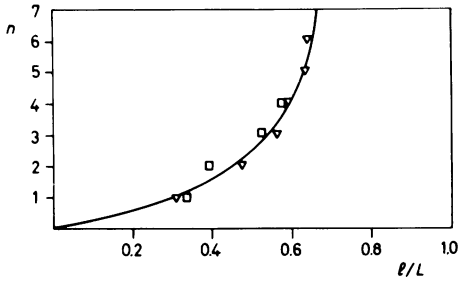

Figure 4. Linear cumulative distribution of initial breakpoints. n is the total number of breakpoints occurring within a distance *l* from the telomere start (arrows in Figure 2) in the examined samples. L is the average telomere length. (▽) clones 62.2−62.8 (L = 0.65 kb); (□) clones HP.1−HP.4 (L = 1 kb).

and ClaI restriction bands (paper in preparation) to have an average size of about 1.0 Kb (or about 140 telomeric repeats). The same figure holds for the subpopulation of HPA62 carrying an unmodified chromosome 4. Also for chromosome 3 (paper in preparation) the length of the telomeric EcoRI fragments does not change during the first 62 passages.

It thus appears that during the vegetative, intra-erythrocytic stage, the average length of plasmodial telomeres is constant. It also appears that a telomere whose length has been occasionally reduced to 0.65 Kb is still able to perform its specific functions and does not require elongation during this life stage.

Intraclonal telomere variability under equilibrium conditions

The linear distribution of the breakpoints along the telomeric structure can provide some clues as to the dynamics of telomere variation in a situation in which, as shown in the preceding section, average telomere length remains constant over many generations.

The data in Figure 2 indicate that, during the vegetative multiplication of the parasite, abrupt changes occur in the sequence of telomeric repeats. Their frequency appears to increase when moving toward the tip of the telomere. This trend is more evident in the linear distribution presented in Figure 4, where the total number of observed breakpoints occurring within a distance *l* from the start of telomeric structure (arrows in Figure 2) is plotted as a function of the ratio *l*/L, L being the average telomere length. This reduced coordinate is easily calculated in the case of the unmodified telomere (Figure 2b), proximally joined to a unique sequence specific for chromosome 4. In the case of the modified extremity, some ambiguity exists as to the precise origin of the telomeric structure, since the telomere-related stretch of the outermost 2.3 kb unit forms a continuum with the telomere. Given its high degree of relatedness to telomeric sequence, this region has been considered as part of the actual telomere in calculating distances and in estimating the average telomere size (0.65 kb, or about 95 telomeric repeats).

Our telomeric sequence data thus appear to indicate that the events leading to intraclonal sequence variability occur with rapidly increasing frequency, over a large portion of the telomere. Actually by $l/L > 0.66$ all of the sequenced clones show at least one breakpoint, while no breakpoints has thus far been observed for $l/L < 0.3$. Although this latter figure agrees with the relative size of the protected region postulated by Wang and Zakian (23) it might as well be, in both cases, the result of limited statistics. In effect a continuous curve can easily be fitted through the experimental points in Figure 4, by assuming, e.g., that the probability of a breakpoint varies with some inverse power of the distance from the telomere end. A large amount of cloning and sequencing work would then be required in order to increase the size of the examined sample sufficiently enough to include some clone diverging in the low probability region.

DISCUSSION

Subtelomeric regions, often harbouring repeated structures, are known to be preferential sites for genomic rearrangements in different lower eukaryotes such as *S.cerevisiae* (31) and *Trypanosome* (32). In *Plasmodium*, the extensive chromosome size variability observed in PFGE karyotypes has, in many cases, been shown to originate from polymorphisms in subtelomeric regions (reviewed in 33).

In *P.berghei* an exclusively subtelomeric repeat family is present in varying copy number at several, but not all, chromosomal ends (27, 30). The repeat unit, 2268 bp in length, is usually referred to as the 2.3 Kb repeat. The subtelomeric repeated structure could also be described (28) as a periodic array in which 2108 bp units are regularly intercalated with 160 bp stretches of a telomere-related sequence, in a pattern similar to that described for *S.cerevisiae* (34−36). The telomere-related stretch contains a series of canonical and non-canonical telomeric repeats, most of which are organised in a higher-order repeat structure formed by three perfect, tandemly arranged elements 27 bp in length (TTTAGGG TTCAGG TTTATGG TTCAGGG).

Variations in the copy number of the subtelomeric 2.3 Kb repeats were shown to be related to chromosome size polymorphisms arising in an initially homogenous population of *P.berghei* ANKA (clone 8417HP) during asexual multiplication '*in vivo*' (30). In the case of chromosome 4, which completely lacks 2.3 Kb repeats in the original *P.berghei* clone (8417HP), the actual transfer of 2.3 Kb units, possibly from a non-homologous chromosome, was demonstrated by the occasional appearance of an enlarged version of the chromosome bearing more than 20 copies of the 2.3 Kb repeats as an insertion which splits one of the original telomeres.

The portion (~1 Kb) of the original telomere remaining on the proximal side of the insertion, and its junction to the first

2.3 Kb unit had already been sequenced (26). It had been demonstrated that the insertion starts in correspondance with the 27 bp, telomere-related elements. On the basis of this observation, we suggested (26) that the latter elements might have a role in mediating the recombinational event which resulted in the insertion.

This view is further substantiated in the present work, in which it is shown that also the distal end of the insert coincides with these elements, while the insertion comprises an integer number of 2.3 Kb repeats. It thus appears that 2.3 kb repeats can be mobilised as whole units through some recombination mechanism specifically involving the 27 bp, telomere-derived elements.

As already discussed in ref.28, *P.berghei* subtelomeric 2.3 kb repeats bear some resemblance to the Y′ elements of *S.cerevisiae*, which can be transferred to exogenous plasmids (37) and be integrated in (or excised from) subtelomeric chromosomal regions most probably via a recombination event between telomeric sequences present at the Y′ junctions and at telomeres (38).

Taking advantage of the presence of two different basic repeats in plasmodial telomeres (TTTAGGG and TTCAGGG), we proceeded to compare the sequences present in the seven recombinant clones extracted from the progeny of the molecule which received the insertion. Intraclonal variability was then compared with the situation existing at the same chromosomal extremity before the insertion took place i.e. in a completely physiological condition. To this aim, four clones containing the terminal part of chromosome 4 and the adjacent proximal portion of the telomeric structure were obtained from the original *P.berghei* ANKA clone 8417HP. Their sequences were compared with the corresponding region of the enlarged chromosomal version.

In either case the initial sequence of telomeric repeats is perfectly conserved among the sibling clones. Even non-canonical repeats are found in exactly corresponding positions. Sequence divergence, in both groups, starts at a definite breakpoint in each clone. There is very little similarity in the repeat sequence between any two sibling clones in the region after the breakpoint. Such breakpoints might be the result either of occasional terminal deletions of different sizes, followed by the random addition of plasmodial telomeric repeats (two telomerases with different RNA templates were suggested (18) to be active in Plasmodia), or of recombination (reciprocal crossover or telomere conversion) with other chromosomal extremities present in the cell.

By checking average telomere lengths over many generations in the course of serial passages, it became apparent that the examined population is in a steady state situation, where the individual telomeres are continually changing, but there is no net growth or decrease in average length.

The linear breakpoint distribution observed in these conditions does not indicate a clearcut separation between a 'protected' and a variable region. The data obtained from the limited sample of sequenced clones are, rather, compatible with a continuous gradient of probability for the events (whatever their nature) which result in a change of the distal sequence of telomeric repeats.

It is noteworthy to observe that the breakpoints positions observed in either set of clones can be brought to coincide when normalised with respect to the average telomere length (which is different for the examined chromosomal extremity in its modified and unmodified versions). In both sets of data, the conserved portion appears to correspond to the proximal third of the telomeric structure.

Two alternative interpretations of the experimental data can be formulated. According to the first one (only slightly modified with respect to that proposed by Wang and Zakian (23)) the proximal third of the telomere is effectively shielded from variation, possibly by interacting with some 'protective' protein. In this hypothesis, the continuous trend observed over the next portion might be viewed as the effect of a decreasing 'protection' gradient. The second model, on the other hand, assumes that all along the telomeric structure there is a continuous increase in the probability of diversification events (e.g. shorter deletions being more likely than longer ones). In this case, the apparent conservation of the proximal portion would simply be the effect of limited statistics. Work is in progress to collect more telomeric sequence data which would enable us to discriminate between the two models.

In the 11 sequenced telomeric tracts, there are a number of non-canonical repeats. Most of these fall within the proximal third of the terminal array. This might suggest that modified repeats (probably randomly arising by point mutation or slipped replication) are faithfully inherited when residing in the proximal region, being rarely subject to the correction activity implicit in the mechanism of loss and re-addition by the putative telomerase(s). (See also ref.18 for tolerance of modified telomeric repeats when 'locked' into the proximal part of the telomere).

Also in other *P.berghei* chromosomes, non-canonical repeats present at the start of the telomeric structure are organised in 27-bp motifs (28). Apart from these motifs, no higher-order periodicity or pattern is observed when inspecting sequences of plasmodial telomeres reported in this and in other papers (25, 28, 39). This fact (also reported for the yeast telomeres (23)), does not correspond to what is easily evidenced in subtelomeric repeated regions of widely different organism such as man (7), yeast (40) and plasmodium (41), as well as in plasmodial intragenic repeats (42, 43). Telomere-derived tandemly repeated motifs are present also in the innermost part of *Trypanosoma brucei* minichromosomes (44), where six tandem copies of a 29 bp suprarepeat, containing canonical and non canonical telomeric repeats, immediately precede the regular array of T_2AG_3 repeats.

Formation of hierarchical supraperiodicities (41, 43, 45) as well as homogenisation of repeat variants (41, 46, 47) is to be easily expected as a result of unequal crossover events between arrays of tandem repeats (48). Gene conversion (42) and, especially in the case of relatively short repeats, slipped replication are other possible mechanisms leading to the same result (49, 50).

Why should arrays of simple repeats escape the action of mechanisms leading to the formation of higher-order periodicities when they are distally located and not when they are internally or subtelomerically located? Although of a speculative nature, this argument strongly supports the concept of a high rate of turnover of telomeric sequences, and argues against a purely recombinational model of telomere generation.

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