

DELETION, INSERTION AND TRANSLOCATION OF DNA SEQUENCES CONTRIBUTE TO CHROMOSOME SIZE POLYMORPHISM IN *PLASMODIUM BERGHEI*

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Extensive chromosome size polymorphism arises in Plasmodium berghei during in vivo mitotic multiplication. Size differences between homologous chromosomes mainly involve rearrangements in the subtelomeric regions while internal chromosomal regions are more conserved. Size differences are almost exclusively due to differences in the copy number of a 2.3 kb subtelomeric repeat unit. Not only deletion of 2.3 kb repeats occurs, but addition of new copies of this repeat sometimes results in the formation of enlarged chromosomes. Even chromosomes which originally lack 2.3 kb repeats, can acquire these during mitotic multiplication. In one karyotype mutant, 2.3 kb repeats were inserted within one of the original telomeres of chromosome 4, creating an internal stretch of telomeric repeats. Chromosome translocation can contribute to chromosome size polymorphism as well. We found a karyotype mutant in which chromosome 7 with a size of about 1.4 Mb is translocated to chromosome 13/14 with a size of about 3 Mb, resulting in a rearranged chromosome, which was shown to contain a junction between internal DNA sequences of chromosome 13/14 and subtelomeric 2.3 kb repeats of chromosome 7. In this mutant a new chromosome of 1.4 Mb is present which consists of part of chromosome 13/14.

Key words: *Plasmodium berghei* – mitosis – asexual multiplication – chromosome size polymorphism – deletion – insertion – translocation – subtelomeric repeats

Extensive chromosome size variation is found in several species of malaria parasites. In *Plasmodium falciparum*, for example, chromosome size polymorphism is observed in field isolates and between clones derived from *in vitro* cultures. Molecular events such as unequal crossing-over between homologous chromosomes during meiosis and deletion of subtelomeric DNA sequences in culture appear to play a role in the generation of novel sized chromosomes of *P. falciparum* (Walliker, 1989; Kemp et al., 1990). The exact mechanisms underlying these polymorphisms and the possible biological function of the rearrangements are still unknown.

To obtain more insight in the mechanisms and the function of chromosome size polymorphism, we studied this phenomenon in the rodent malaria parasite *P. berghei* in collaboration with the research group of prof. C. Frontali (Istituto Superiore di Sanita, Rome). *P. berghei* is well suited for this purpose since mutants with novel sized chromosomes (karyotype mu-

tants) arise frequently during asexual multiplication of cloned parasites (Janse et al., 1989, 1991). In this paper we describe three mutants which have been studied in detail and we discuss the mechanisms and possible function of chromosomal rearrangements.

MATERIALS AND METHODS

We studied parasites derived from the ANKA and K173 strain of the rodent malaria parasite *P. berghei*. Clones from these strains were derived by the method of limiting dilution. After cloning the parasites were kept in liquid nitrogen.

Clone 8458 and 8417 (= HP clone) from the ANKA strain were obtained from Dr A. Said (Antwerp, Belgium). Clone PE from the ANKA strain was provided by Dr W. Eling (Nijmegen, The Netherlands) and clone 233 by Dr R.E. Sinden (London). In our laboratory, a large number of parasite lines, showing an altered karyotype have been obtained from clone

8117 after prolonged periods of mechanical transmission (Janse et al., 1989, 1991).

The molecular karyotype of the parasites was established by Field Inversion Gel Electrophoresis (FIGE) (Janse et al., 1989). Specific electrophoresis conditions allow the separation of eleven chromosomal bands, containing all 14 chromosomes of *P. berghei*. In all lines and clones described here chromosomes 13 and 14, and chromosomes 9, 10 and 11 comigrate in FIGE gels.

A large panel of chromosome specific probes have been generated in our laboratory and by Dr M. Ponzi in Rome (unpublished results; Ponzi et al., 1990) to facilitate recognition and characterization of individual chromosomes. Apart from a large number of anonymous probes, we use probes for the actin-I gene, DHFR gene (unpublished results), small subunit RNA genes (Dame & McCutchan, 1983), β -tubulin gene (Van Belkum et al., 1991) and probes for the telomeric and for 2.3 kb subtelomeric repeat sequences (Ponzi et al., 1990).

Particular chromosomes have been studied in detail by recovering the separated chromosomes from FIGE gels followed by digestion with rarecutting enzymes and hybridization with specific probes. In addition, restriction maps and sequence data have been obtained from defined restriction fragments. Detailed description of these procedures is found in Ponzi et al. (1990) and Pace et al. (1990).

RESULTS AND DISCUSSION

Chromosomes of *P. berghei* – The genome of *P. berghei* comprises 14 chromosomes in the size range of 0.5 to 3.5 Mb. Fig. 1 shows the molecular karyotypes of a number of clones and lines of the ANKA and K173 strain. Fig. 2 lists several genes which are mapped to the different chromosomes. The comparison of karyotypes in Fig. 1 clearly shows the extent of chromosome size polymorphism in *P. berghei*.

Research carried out in the laboratory of Prof. C. Frontali in Rome has shown that the telomeres of the chromosomes of *P. berghei* consist of a least 140 repeats of the heptanucleotide sequence CCCTA(G)AA (Ponzi et al., 1985). In a number of chromosomes the telomeric repeats are directly joined to sub-

telomeric repeats of 2.3 kb (Dore et al., 1990). This particular subtelomeric repeat sequence is conserved within *P. berghei* since several, independently isolated, strains contain 2.3 kb repeats. No sequence homology, however, has been found with repeat elements in other malaria parasites.

Between clones there is a large difference in the total number of repeats per haploid genome. We found that some clones of the ANKA strain contain up to three hundred copies of the 2.3 kb repeat, comprising 3–4% of the total genome size, while clones of the K173 strain contain less than 50 copies (Ponzi et al., 1990; unpublished results). Also between individual chromosomes differences exist between the number of repeats. They may be present at one end of a chromosome, at both ends or sometimes they can be totally lacking.

Differences in the number of subtelomeric repeats contribute to chromosome size polymorphism – We studied the relative abundance of 2.3 kb repeats in the chromosomes of several clones and lines of *P. berghei* (Ponzi et al., 1990). A comparison was made between the hybridization intensities of size variants of several chromosomes after hybridization with a probe specific for the 2.3 kb repeat sequence. Large differences exist between the size variants, the smaller version of the chromosome always showing a weaker hybridization signal. We also observed that chromosomes which lack the subtelomeric repeats are more stable in size during mitosis than chromosomes which contain these repeats. These results suggest that a significant part of size polymorphisms in *P. berghei* is due to variation in the number of 2.3 kb repeats. Several observations indicate that internal regions of the chromosomes are more conserved in size than the telomeric regions. For example, we observed no restriction fragment length polymorphism in digested genomic DNA after hybridization with a number of chromosome specific unique probes. On the other hand, the subtelomeric 2.3 kb probe revealed extensive restriction fragment length polymorphism. In addition, internal Apa-I restriction fragments of purified chromosomes have the same size in all size variants, but the two telomeric Apa-I fragments differ greatly. See for example figure 3 where the sizes of Apa-I restriction fragments are shown for four variants of chromosome 7. The two telomeric fragments of a large variant of chromosome 7 (clone 8417) hybridize strongly with the 2.3 kb

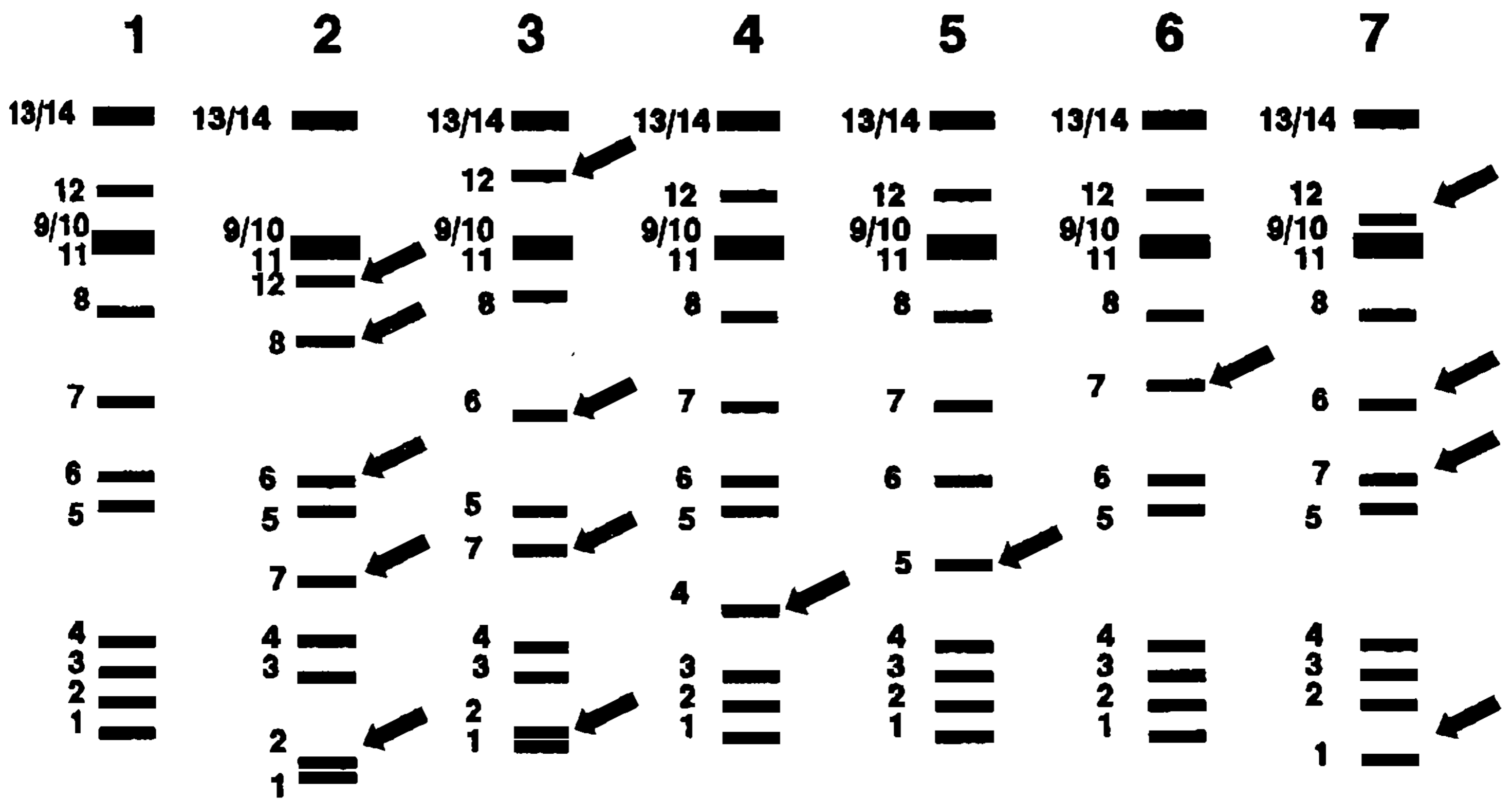


Fig. 1: molecular karyotypes of clones and lines of *Plasmodium berghei*. Chromosomes were separated by Field Inversion Gel Electrophoresis. Clone 8417 (lane 1) is our reference clone for the numbering of the chromosomes. The homologous chromosomes in the other lines and clones have been identified using at least two chromosome specific probes for each chromosome. Arrows show the major size variation of the chromosomes compared to the reference clone. Lane 1: clone 8417; lane 2: clone 1 (K173 strain); lane 3: clone 8458. Lane 4-6: lines derived from clone 8417 after prolonged periods of mechanical multiplication. Lane 4: line HPA62; Lane 5: line HPEm53; lane 6: line CL15clm105; lane 7: clone 233.

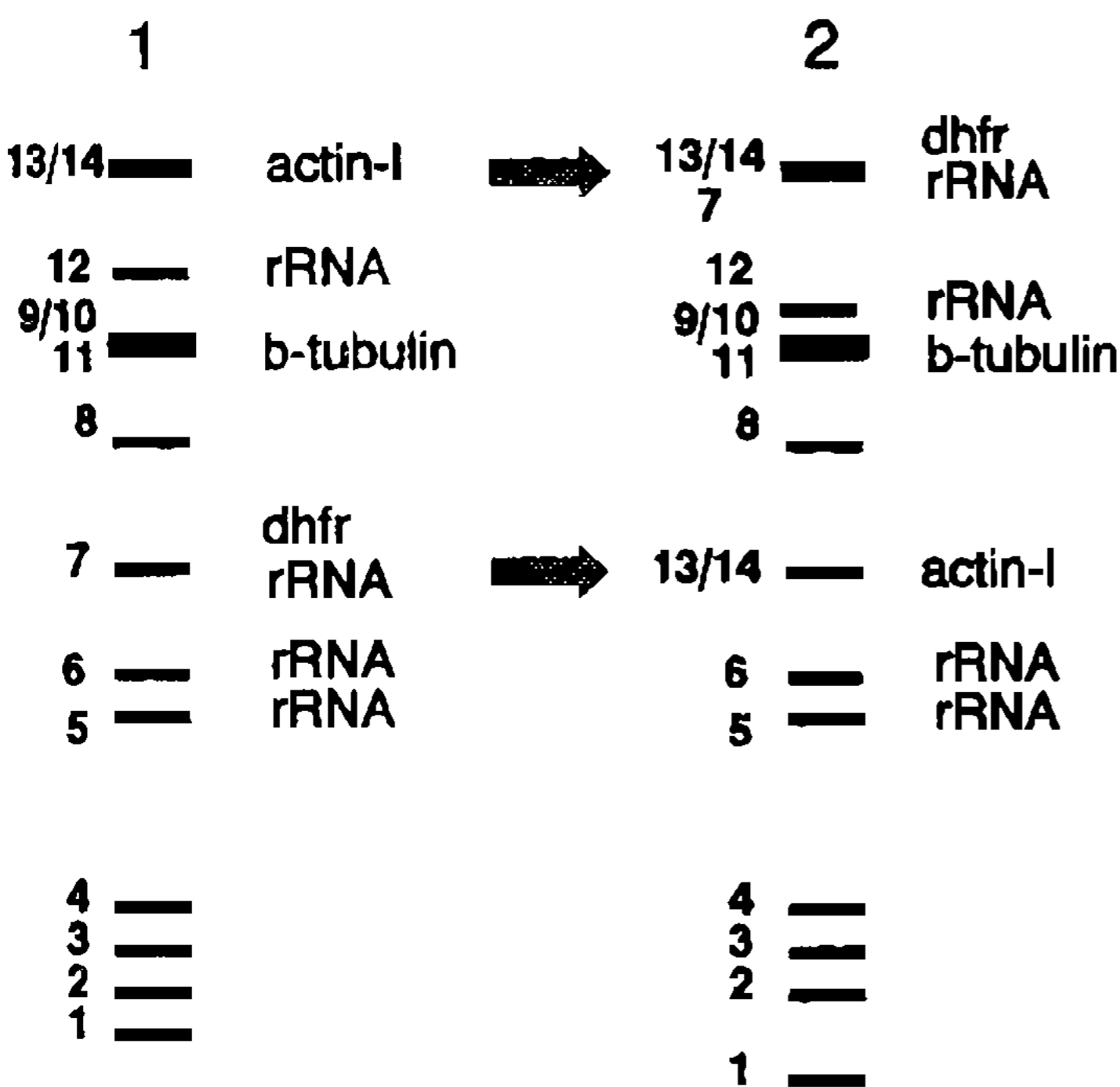


Fig. 2: location of several genes on the chromosomes of *Plasmodium berghei*. 1. Karyotype of the reference clone 8417 of the ANKA strain. 2. Karyotype of mutant EP of the ANKA strain in which chromosome 7 is translocated to chromosome 13/14 and a new chromosome is present which comprises chromosome 13/14 sequences.

probe and we estimated that about 100 copies of this repeat were present. The smallest variant (K173, clone 1) completely lacks these

repeats, while the telomeric fragments of an intermediate variant (clone 8458) shows a weak hybridization with the 2.3 probe. Thus the size differences of up to 0.5 Mb between variants of chromosome 7 appeared to be confined to the two telomeric fragments, and to be largely accounted for by a reduction in the number of subtelomeric 2.3 kb repeats.

The mechanism by which the 2.3 kb repeats can vary in copy number is presently under study in the group of Prof. Frontali. Double stranded breaks may occur in a (sub)telomeric region, resulting in the loss of DNA sequences after which telomere repeats are added at the site of breakage to heal the chromosomes. These processes appear to play a role in the generation of chromosome size polymorphisms in *P. falciparum* (Pologe et al., 1990). Unequal crossing-over events between homologous chromosomes during meiosis in the mosquito may also result in differences in the relative abundance of repeat sequences.

Another possibility is that illegitimate recombination events during mitosis results in novel-sized chromosomes. Below we will

present evidence for involvement of the latter mechanism.

Addition of 2.3 kb subtelomeric repeats during mitosis contributes to chromosome size polymorphism – During mechanical transmission of cloned parasites, not only karyotype mutants arise with chromosomes which have reduced their size but also mutants appear with enlarged chromosomes. For example, we found karyotype mutants in which either chromosome 7, 6 or 4 had increased in size (Janse et al., 1989, 1991; Pace et al., 1991). This demonstrated that size polymorphisms observed during mitotic multiplication do not only arise as a result of deletion of DNA sequences as a result of double stranded breaks but that other mechanisms may be involved as well.

We found evidence in one selected clone that an increase in size of 100 kb of chromosome 7 (line CL15clm105; Fig. 3) was the result of the increase of the number of 2.3 kb repeats at one end of the chromosome (unpublished results). The group of Prof. C. Frontali studied the size difference of 50 kb of two variants of chromosome 4 in detail (Pace et al., 1990). They also found that the increase in size is due to a larger number of 2.3 kb repeats. Interestingly, the original chromosome of the parent clone lacked 2.3 kb repeats at both ends while the mutant contained these repeats at one end of its chromosome 4. Restriction mapping and sequencing of parts of the (sub)telomeric regions of chromosome 4 showed that the 2.3 kb repeats were inserted within the telomere structure resulting in an internal stretch of about 1 kb of telomeric sequence (Fig. 4).

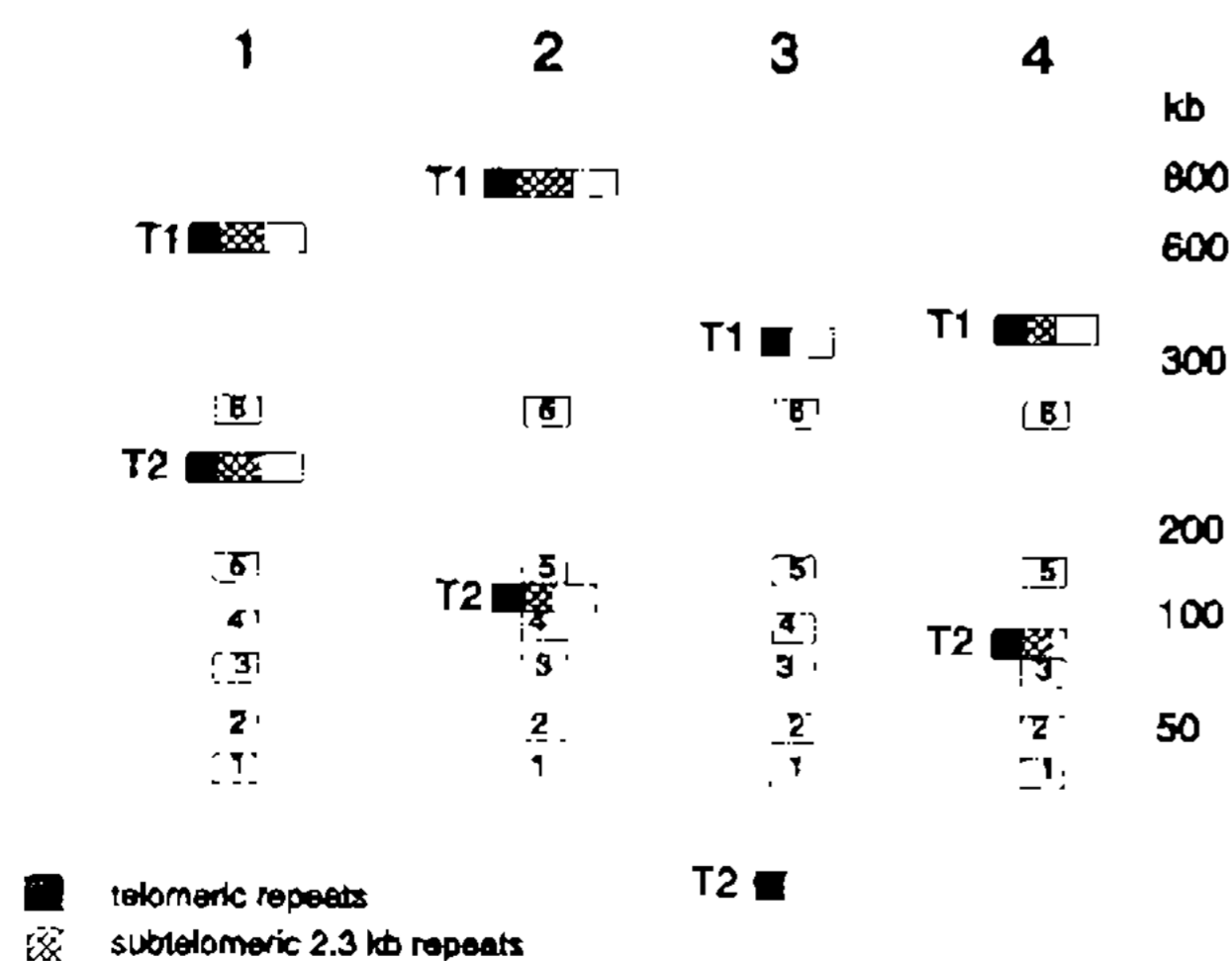


Fig. 3: size variation in Apa-I fragments of chromosome 7 of several clones/lines of *Plasmodium berghei*. The Apa-I fragments were separated by Field Inversion Gel Electrophoresis (FIGE) after digestion of purified chromosomes from FIGE gels. 1: clone 8417; 2: line CL15clm105; 3: clone 1 (K173 strain); 4: clone 8415.

The 2.3 kb unit contains 160 base pairs of telomeric sequence, including some perfect tandem repeats of the 27 bp motif CCCTGAA CCATAAA CCTGAA CCCTAAA heptanucleotides typical of telomeres. The presence of these telomere-related, 160 bp-stretches within the 2.3 kb repeats, regularly spaced in an organization similar to that described for $(C_{1-3}A)_n$ repeats in *Saccharomyces cerevisiae*, suggested that these sequences might play a role in recombinational events which lead to the maintenance and dispersal of subtelomeric repeat structures (Pace et al., 1990). The observation that in the enlarged version of chromosome 4 the 2.3 kb repeats were joined to the internal telomeric region through this 27 bp repeat region supports this hypothesis. The exact mechanism by which 2.3 kb negative chromosomes can acquire these sequences from non-homologous chromosomes and the biological function of 2.3 kb repeats remain to be studied.

Chromosome translocation contributes to chromosome size polymorphism – From our results it is clear that most of the chromosome size polymorphism observed in *P. berghei* is the result of the loss or addition of subtelomeric repeats. However we recently found a karyotype mutant of the ANKA strain in which size variation could not be explained by subtelomeric rearrangements. In this mutant (named EP) all probes specific for chromosome 7, hybridized to chromosome 13/14. In addition a 'new' chromosome of about 1.4 Mb was present. This chromosome was only recognized

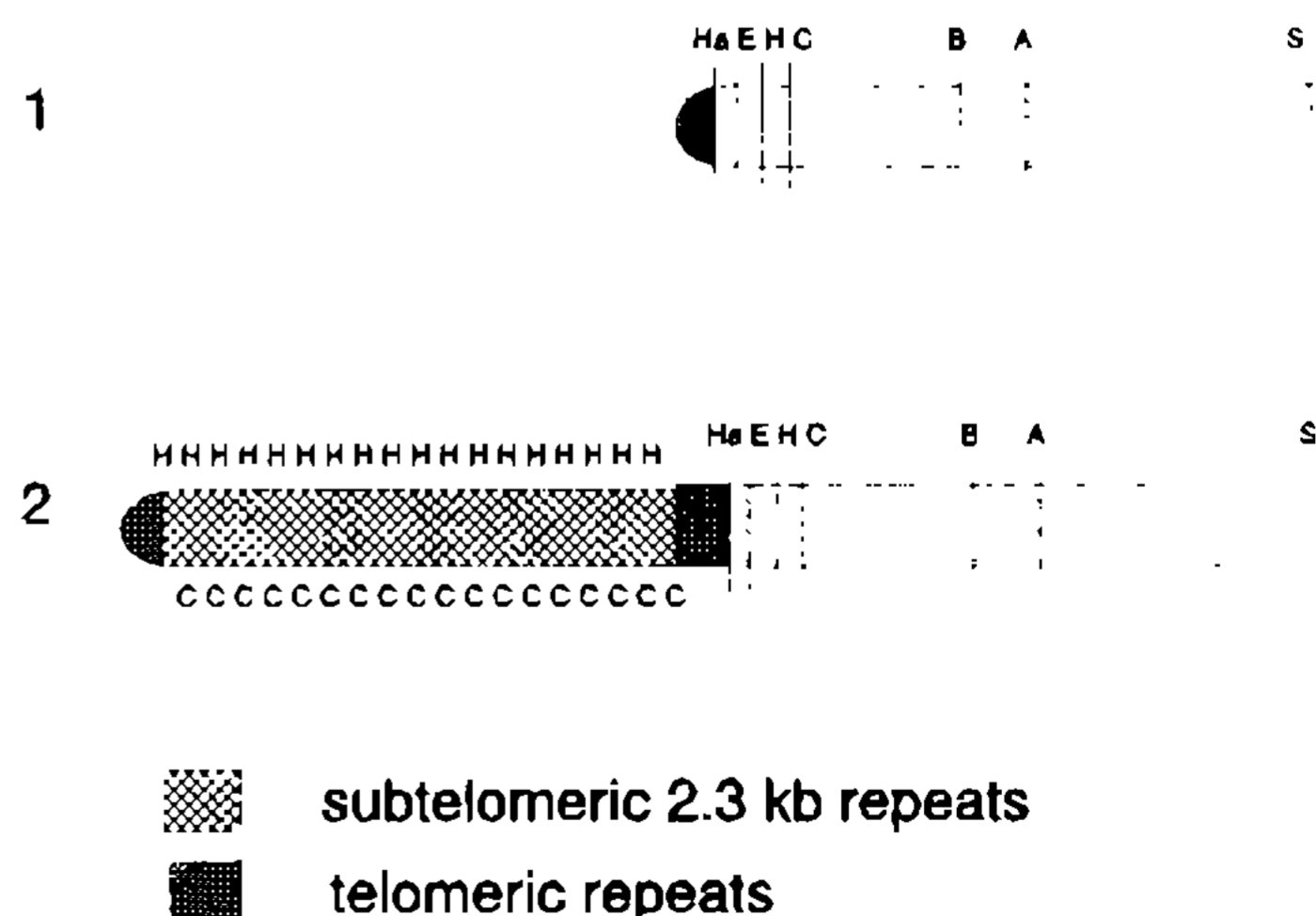


Fig. 4: restriction maps of one end of two size variants of chromosome 4 (from: Pace et al., 1990). 1. Unmodified extremity of chromosome 4 of clone 8417, not containing 2.3 kb repeats. 2. The end of chromosome 4 of parasites of line HPAm62 in which 2.3 kb repeats are present within the telomere structure. These parasites arose during asexual multiplication of parasites of clone 8417.

by various DNA sequences which are normally located on chromosome 13/14, a doublet band containing the two largest chromosomes of *P. berghei*, which have a size of more than 3 Mb.

We compared the Apa-I restriction fragments of chromosome 7 and 13/14 from *P. berghei* and of chromosome 13/14 and the new chromosome ("EP7") of mutant EP, using a large number of probes which are specific for chromosome 13/14 or for chromosome 7 (Janse et al., 1992, in press). We found that in this mutant chromosome 7 had been translocated to chromosome 13/14. The result was a rearranged chromosome containing a junction between internal DNA sequences of chromosome 13/14 and subtelomeric 2.3 kb repeats of chromosome 7 (Fig. 5). The new chromosome, 1.4 Mb in size, comprises sequences of chromosome 13/14 and contains one telomeric region of these chromosome, while at the other end telomeric repeats are added to internal sequences of chromosome 13/14 (Fig. 5). These results show that in addition to rearrangements in the subtelomeric regions, chromosomes can vary in size due to translocation events. As a result of translocation new linkage groups can be generated in *Plasmodium* by joining of two large frag-

ments derived from non-homologous chromosomes. In the mutant described here we found that the genes coding for DHFR and for rRNA were translocated from chromosome 7 to 13/14 and the Actin-I gene from 13/14 to "EP7" (Fig. 2).

The molecular events underlying the described translocation are unknown. Translocation events require breakage and rejoining of the DNA molecules involved. These processes could take place at random sites by illegitimate, interchromosomal recombination or specific sites in the DNA may be predisposed to recombine in certain cells under certain conditions. In *P. falciparum* there is evidence for the introduction of double-stranded breaks in the DNA at specific sites in a chromosome. Healing of the chromosome can then occur by the enzymatic addition of telomere repeats (Pologe et al., 1990). This mechanism appears to have also played a role in the translocation event described here. Breakage of chromosome 13 or 14 in two parts, followed by telomere addition at the site of breakage has resulted in the formation of the new 1.4 Mb chromosome. We also found preliminary evidence that 2.3 kb repeats of the original chromosome 7 are directly joined to chromosome 13/14 at the site of breakage, which may imply that these repeats have played a role in the recombination event resulting in the translocation. Evidence for the involvement of the 2.3 kb repeats in recombinational events has been presented above. However, more detailed sequence information on the junction between sequences from chromosome 7 and from chromosome 13/14 is needed for an insight in the mechanism by which the translocation occurred.

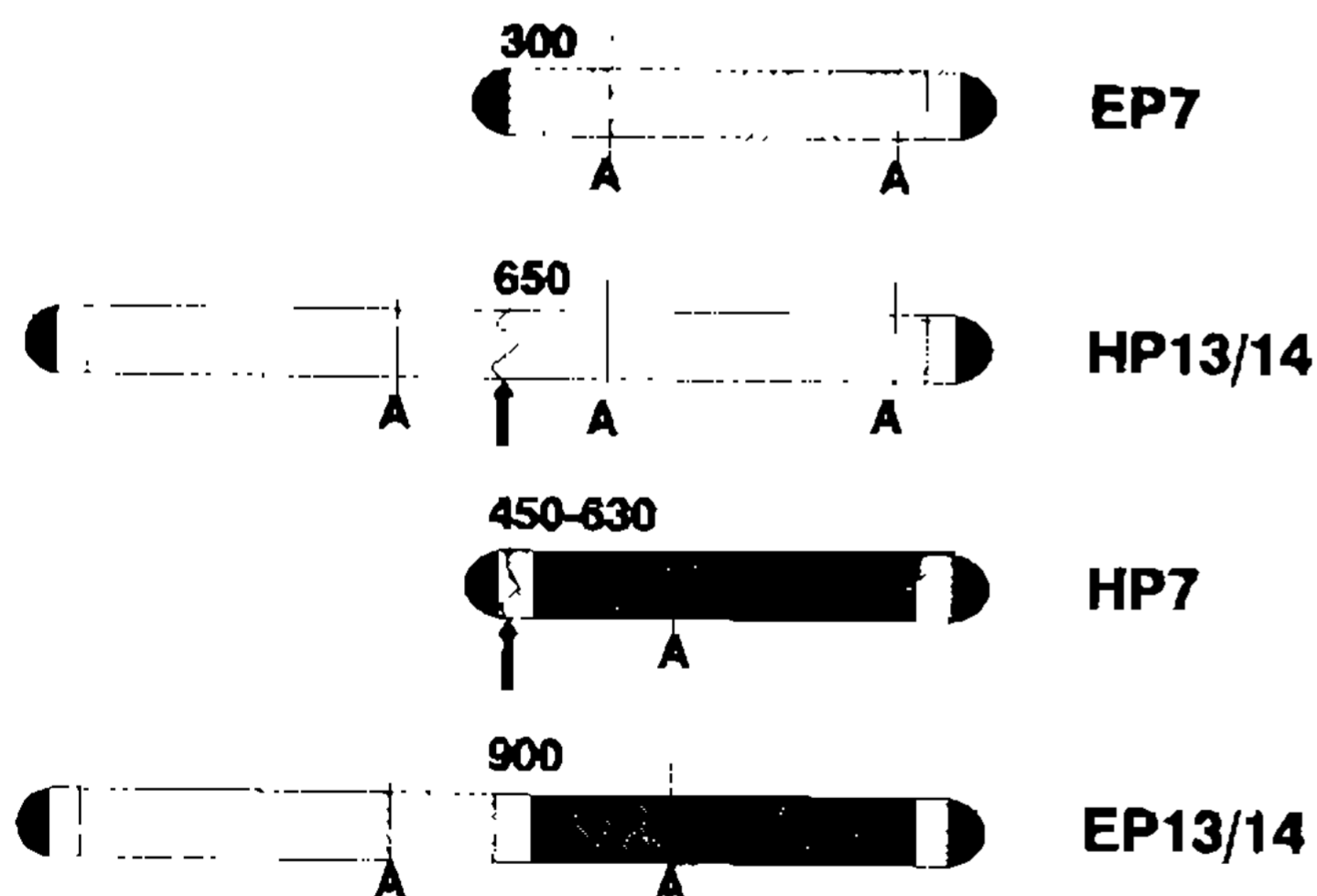


Fig. 5: schematic representation of chromosomes 7 and 13/14 of clone 8417 (= HP clone) and of the recombinant chromosomes EP7 and EP 13/14 from mutant EP after a translocation event. This scheme is based on the comparison of the size of Apa-I restriction fragments of the chromosomes and the hybridization of a large number of specific probes to these fragments (Janse et al., manuscript in prep.). The size of several Apa-I (A) restriction fragments is shown. The arrows and crooked lines indicate the supposed breakage sites, which underly the translocation event. Regions containing islands of the 2.3 kb repeat are indicated by the closely dotted patterns and the main body of the two HP chromosomes are shaded differently to emphasise the exchange that has occurred in mutant EP.

A variety of rearrangements in chromosomes of eukaryotes appear to be produced by illegitimate recombination events during mitosis (Meuth, 1989). Despite the extensive genetic characterization of many kinds of recombination events, few specific functions have been ascribed to mitotic recombination. Some rearrangements in eukaryotes, however, have obvious biological implications, for example immunoglobulin and T-cell receptor rearrangements and rearrangements resulting in mating type switches in yeast and antigenic variation of trypanosome. In malaria parasites the possible function of rearrangements in which subtelomeric repeat sequences are involved, is unknown. It has been suggested that subtelomeric rearrangements in *P. falciparum* are in-

volved in repositioning genes in the vicinity of chromosome ends and may be functioning in gene activation (Ravetch, 1989). Conclusive evidence for this latter hypothesis is, however, lacking. In addition to these subtelomeric rearrangements, we found that chromosome translocations occur in malaria parasites. Since it is the first time that such an event was detected in *Plasmodium*, it seems that chromosome translocation does not occur frequently with a specific biological function. Indeed, in the mutant described here we did not observe phenotypic changes apparently associated with the translocation. However, it shows that specific sequences, for example 2.3 kb repeats, allow illegitimate recombination events to take place, which may suggest that these sequences are also involved in other, unknown, recombination events with specific biological functions. *P. berghei* is a vivax-type parasite and PFGE patterns from this group are not extensively studied. More detailed studies of *P. vivax* are underway and those may generate more information on possible recombination sequences and translocation events in this human parasite. The fact that the 2.3 kb sequence is universally present and conserved in the *P. berghei* species, means that, at least in sexually intact lines, it has an essential biological function, because the maintenance of such a large repeat unit has significant energetic consequences. In the (asexual) K173 clones the number of repeat units is largely reduced after many years of pure mechanical passage. We therefore assume that the function of the repeat is more essential during meiotic than during mitotic processes.

ACKNOWLEDGEMENTS

The work was carried out during a collaborative research programme with the group of Prof. Frontali in the Laboratorio di Biologia Cellulare, Istituto Superiore di Sanita, Rome, financed by the EEC in the framework of the Program 'Science and Technology for Development'.

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