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Identification of a telomeric DNA sequence in *Plasmodium berghei*

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A fragment of *Plasmodium berghei* DNA was cloned using a technique designed to select for telomeric sequences. The cloned fragment recognizes *Bal*31-sensitive bands in *P. berghei* genomic digests. It contains at its distal end at least 70 tandem repeats of the heptanucleotide sequence CCCT$^{A}_{G}$AA. The presence of natural single strand discontinuities in the telomeric regions of *P. berghei* DNA is demonstrated by the selective incorporation of deoxyribonucleoside triphosphates in the absence of DNase. The number of copies of the cloned sequence present in each genome agrees with an estimate of 6–12 chromosomes per nucleus.

Key words: P. berghei DNA/telomere/genome structure

Introduction

Notwithstanding the number of recent studies on the fine structure of several antigen-coding genes in *Plasmodium* (Ozaki *et al.*, 1983; Coppel *et al.*, 1983; Enea *et al.*, 1984; Dame *et al.*, 1984; Koenen *et al.*, 1984), extensive work is still needed to clarify the genome structure of this protozoan which is responsible for one of the major health problems in the world. The structural organization of the plasmodial genome cannot be easily studied using cytological or genetic approaches. In spite of extensive ultrastructural studies (Canning and Sinden, 1973; Lanners and Trager, 1985), condensed chromosomes have never been observed at any stage of the life cycle. Linkage of the limited number of available genetic markers has never been evidenced (Walliker, 1983). Quite recently, however, the novel technique of pulsed-field gradient gel electrophoresis has been applied to *Plasmodium falciparum* by Kemp *et al.* (1985). Their valuable results (which appeared while the present paper was still in preparation) indicate that this genome contains seven distinct fractions of chromosomal DNA ranging between 0.8 and 3 megabases, along with some ill-defined material of lower mol. wt. (minichromosomes or degradation products).

Our purpose in the present study was to test whether the parasite genome is organized in linear molecules whose termini, one expects, would exhibit the peculiar features common to all lower eukaryotes studied so far. The latter include ciliated protozoa, both the holo- and the hypotrichs (Blackburn and Gall, 1978; Katzen *et al.*, 1981; Yao and Yao, 1981; Oka *et al.*, 1980; Klobutcher *et al.*, 1981), slime molds (Johnson, 1980; Emery and Weiner, 1981), flagellates (Blackburn and Challoner, 1984; Van der Ploeg *et al.*, 1984a) and fungi (Shampay *et al.*, 1984; Walmsley *et al.*, 1984).

The above organisms have very different genome organizations [presence of macro- and micro-nucleus in ciliated protozoa; presence of a high number of minichromosomes in *Trypanosoma* (Van der Ploeg *et al.*, 1984b)] yet they share some conserved properties in their telomeric structures. This conservation is notably evidenced by the finding that yeasts can recognize telomeres from the distantly related organism *Tetrahymena* (Szostak and Blackburn, 1982; Shampay *et al.*, 1984).

Among the conserved features (see review by Blackburn and Szostak, 1984) is the presence, at both ends of each chromosome or minichromosome, of a stretch of variable length which results from the tandem reiteration of a basic repeat. In the cases studied, the basic repeat is 6–10 bp long and invariably initiates (5′) with a group of 3–8 cytosine residues. Repeats found in holo- and hypotrichous ciliates, in flagellates and in the slime mold *Physarum* are very similar and all conform to the general formula $C_3(^{T}_{C})A_n$. The telomeric region composed of such repeats is insensitive to known restriction enzymes and contains a number of single-strand discontinuities which follow a pattern related to the basic repeat.

The above properties, along with the known sensitivity to *Bal*31 exonuclease of any free DNA extremity, have been variously used for the molecular cloning and sequencing of telomeric regions from the organisms quoted above. The strategy we adopted for the construction and screening of a DNA sequence library of *Plasmodium berghei* is based on the approaches of Van der Ploeg *et al.* (1984a) and of Blackburn and Challoner (1984). Our results show that, in line with other lower eukaryotes, *P. berghei* possesses natural extremities having the above-mentioned properties and characterized by the basic repeat $^{5'}$CCCTGAA$^{3'}$, where the G is irregularly substituted by an A.

Results

Selective radiolabelling by Escherichia coli DNA polymerase I

As shown by Blackburn and Gall (1978) and by Blackburn and Challoner (1984), endogenous single strand gaps in telomeric sequences allow for the incorporation of deoxyribonucleoside triphosphates (dNTPs) when using a nick-translation system in the absence of DNase. For the incorporation to proceed, a suitable combination of nucleoside triphosphates must be present in the mixture. By testing all possible combinations of two or three dNTPs it is, therefore, possible to identify the bases present in the gap-containing region.

We used total *P. berghei* ANKA DNA in nick-translation reactions in the absence of DNase and in the presence of each of the four possible combinations of the three dNTPs (A,T,C; A,T,G;A,C,G;T,C,G). No measurable incorporation had been obtained in tests performed with combinations of two dNTPs (results not shown). The DNA thus labelled was then digested with *Hae*III, a frequently-cutting enzyme, and size fractionated on a 0.8% agarose gel. Figure 1 shows the autoradiography of the dried gel. It can be seen that incorporation of the labelled precursors is higher when the complementary combinations A,T,C and A,T,G are used (lanes 2 and 3), but that some incorporation also occurs in the presence of the other two complementary combinations A,C,G and T,G,C (lanes 4 and 5). In all cases

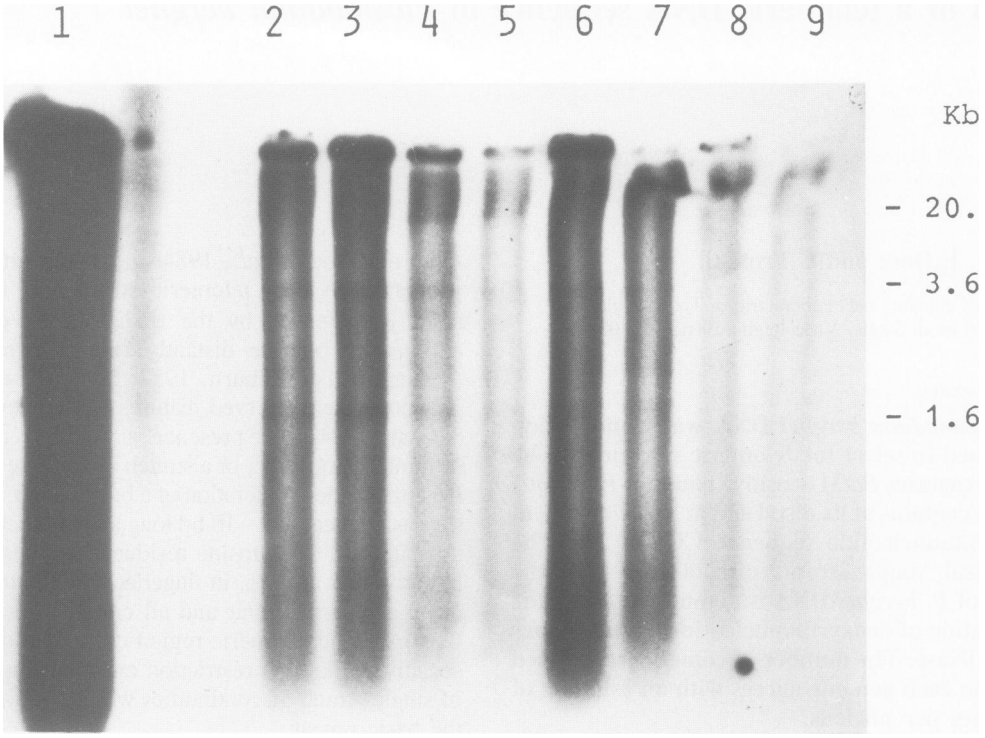

Fig. 1. Selective radiolabelling of *P. berghei* DNA was done by incubating 1 μg of DNA for 30 min at 37°C with 2.5 U of *E. coli* DNA polymerase I, endonuclease free, in the presence of different combinations of [α-^{32}P]deoxyribonucleoside triphosphates (dNTPs) (200 Ci/mmol). Each mixture contained 25 pmol (5 μCi) of each dNTP. After phenol extraction and precipitation with isopropanol, labelled DNA was digested with *Hae*III and fractionated by electrophoresis on 0.8% agarose gel at 20 mA for 16 h. After soaking in 10% acetic acid, the gel was dried in a BioRad Gel Slab Dryer and directly autoradiographed. **Lane 1:** all four dNTPs; **lane 2:** A,T,C; **lane 3:** A,T,G; **lane 4:** A,C,G; **lane 5:** T,C,G; **lanes 6–9:** DNA labelled as in **lane 2** (A,T,C) was treated for increasing times (0, 5, 10, 20 min) at 30°C with 0.1 U of *Bal*31, then digested with *Hae*III and run on the same gel. The main bands discernible over a continuous background are indicated.

a pattern of bands (at 1.6 kb, 3.6 kb and at an unresolved region higher than 20 kb) is discernible over a continuous background. When all the four labelled precursors are simultaneously present in the nick-translation mixture (lane 1), a continuous, heavily labelled smear appears, most likely indicating the occurrence of non-selective incorporation at other nicks, which might be randomly distributed in our DNA preparations. Shorter exposures of the heavily labelled smear revealed the same band pattern observed with the combinations of three dNTPs.

The terminal localization of incorporated dNTPs is confirmed by the sensitivity of the labelled material to digestion with *Bal*31 as documented in Figure 1, lanes 6–9. Here samples of *P. berghei* DNA, nick-translated in the presence of A,T,C, were treated for variable and increasing lengths of time (0, 5, 10, 20 min) with *Bal*31, digested with *Hae*III and electrophoresed. *Bal*31 digestion was carried out under controlled conditions which allowed us to estimate that 0, 75, 150 and 300 bp, respectively had been removed by the action of the enzyme. It therefore appears that the DNA region labelled in the conditions just described is between 150 and 300 bp long and that its base composition is essentially A,T,C/T,A,G.

Molecular cloning strategy

The presence of a terminal hairpin and the absence of restriction sites, which appear to be characteristic features of telomeric sequences, exclude telomeres from any genomic library which has not been especially designed to include them.

To overcome this difficulty, Van der Ploeg *et al.* (1984a) describe a cloning strategy which consists of the following steps: (i) Digestion of high mol. wt. DNA with *Bal*31, which reduces molecular extremities by a known number of base pairs and creates blunt ends; (ii) ligation of the *Bal*31-digested DNA to *Hind*II-restricted pUR222 plasmid; (iii) digestion of the recombinant molecules (composed of the whole vector and of a long foreign tail) using a different enzyme which finds only one suitable site on the vector and reduces the attached foreign DNA to an acceptable size; (iv) dilution and circularization of the constructed molecules at low concentration; (v) transformation.

The technique we used for *P. berghei* ANKA DNA (see Materials and methods) essentially follows the procedure described above, except for the following modifications. The vector used was pBR322 restricted with *Pvu*II and *Bam*HI. A 1.7-kb fragment is thus removed from pBR322. After blunt-end (*Bal*31 and *Pvu*II) ligation, *P. berghei* DNA is cut with *Bgl*II so that it can be ligated to the *Bam*HI site on the vector without reconstituting a restriction site. This allows a second double digestion to be carried out with *Pvu*II and *Bam*HI in order to eliminate reconstituted plasmids or dimers.

Although the strategy described is specifically designed for cloning telomeres, it is still to be expected that the frequency of telomeric inserts would be low because of the unavoidable presence of double-stranded breaks even in the best DNA preparations. A suitable selection method must therefore, be applied (see following section).

The absence of recombinant colonies incorporating host DNA fragments, which might contaminate the original DNA preparation (but escape observation with an analytical ultracentrifuge, see Materials and methods), was verified by means of colony

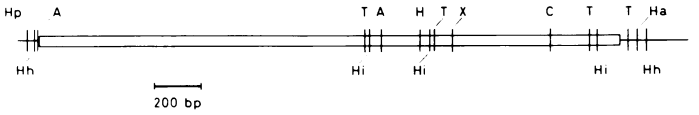

Fig. 2. Restriction map of the insert of the selected clone deduced from digestion analysis of the recombinant plasmid with 22 restriction enzymes (*Alu*I, *Ava*II, *Bal*I, *Bam*HI, *Bgl*I, *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, *Hha*I, *Hind*III, *Hinf*I, *Hpa*I, *Hpa*II, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sma*I, *Taq*I, *Xba*I, *Xho*I). The boxed region indicates the *Plasmodium* insert. Hp=*Hpa*II, Hh=*Hha*I; A=*Alu*I; T=*Taq*I; Hi=*Hinf*I; H=*Hind*III; X=*Xba*I; C=*Cla*I; Ha=*Hae*III.

hybridization to total nick-translated DNA from rat lymphocytes.

Selection of telomeric recombinants

Our first attempt to use total *P. berghei* ANKA DNA which had been selectively radiolabelled at the natural ends with A,T and C (lane 2 in Figure 1) as a screening probe produced an insufficient signal. A higher activity is obtained when total DNA is labelled under the same conditions with the four dNTPs (lane 1 in Figure 1), most probably at the expense of specificity. Due to single strand breaks, possibly introduced during extraction, this probe may in effect recognize internal as well as telomeric sequences.

To circumvent this difficulty, the filters, to which recombinant colonies had been transferred, were pre-hybridized with an excess of cold total DNA which had been digested with *Bal*31 so as to remove ~300 bp from each natural or artificial extremity. Due to the random distribution of artificial breaks, all the internal sequences are likely to be adequately represented in this material while true telomeres might be expected to lack their terminal portion. As a result, saturation of the filters with this cold DNA presumably leaves only those colonies which contain true telomeres available for subsequent hybridization.

After this pre-hybridization step, the library (~1600 colonies) was screened with the probe, which had been previously digested with *Hae*III in order to reduce its mol. wt. A few colonies yielded a faint signal, and only one colony yielded a fairly strong hybridization spot. The latter was selected for subsequent characterization.

Characterization of the insert

The *Bam*HI and *Pvu*II sites of pBR322 used in molecular cloning are no longer present in the final construct. The delimitation and orientation of the insert in the selected clone are therefore deduced from the known restriction sites present in the adjacent regions of the plasmid.

Figure 2 presents data concerning the physical length (2.5 kb) as well as the restriction map of the plasmodial DNA insert. The cleavage site distribution reveals a long region (1.4 kb) which is devoid of restriction sites and occupies more than one half of the total insert length, starting from the distal part of the chromosome.

That the insert actually contains a true telomeric sequence is demonstrated by the following experiment. Southern blots of total *P. berghei* ANKA DNA, digested to various extents with *Bal*31 and reduced to a measurable size by *Hae*III digestion, were probed with the recombinant plasmid which had been labelled with ^{32}P using conventional nick-translation. The first lane in Figure 3 which refers to zero time *Bal*31 digestion, shows about seven hybridization bands whose approximate mol. wt. can be inferred from the position of the markers on either side of the gel. No corresponding bands are revealed by the ethidium bromide staining of the gel. The heterogeneity in mol. wt. typical of telomeric sequences is particularly evident, as can be expected,

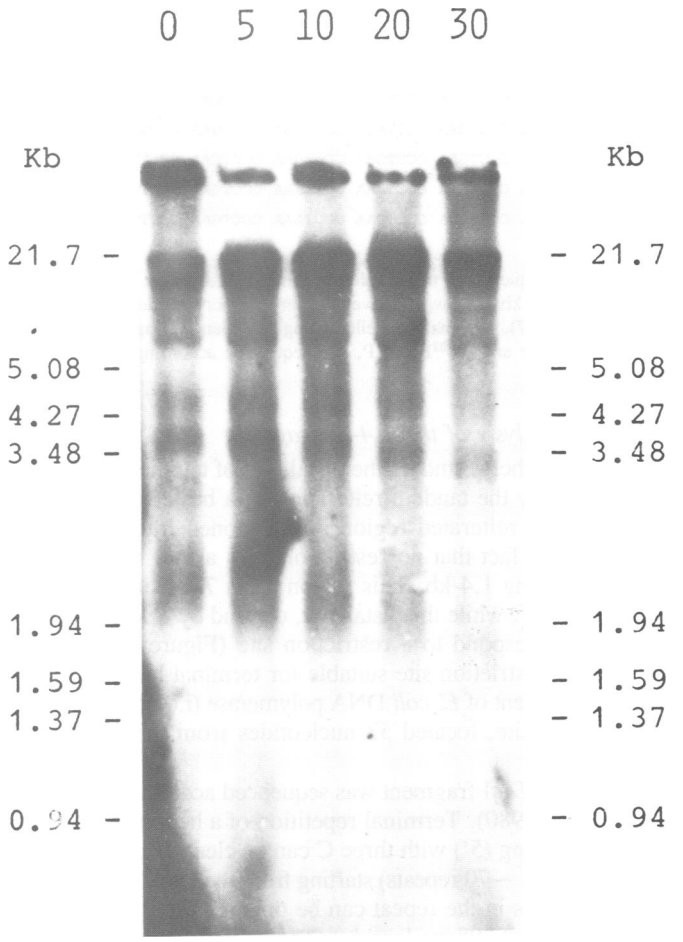

Fig. 3. Southern hybridization of plasmodium DNA, digested with *Bal*31, with labelled recombinant plasmid. Total *P. berghei* DNA was digested for different times (0, 5, 10, 20, 30 min) with *Bal*31; after digestion with *Hae*III the samples were fractionated in 1% agarose gel electrophoresis at 20 mA for 16 h in Tris-borate buffer. Southern blots (Southern, 1975) pre-hybridized 4 h at 65°C in 5 × Denhardt's solution, 10 × SSC, 0.1% SDS, were hybridized overnight at 65°C in the same solution with the recombinant plasmid α-^{32}P-labelled by conventional nick-translation. Post hybridization washes were carried out at 65°C at a moderate stringency (5 ×, 2 ×, 1 × SSC, two changes each). The band position of the mol. wt. marker (λ DNA digested with *Eco*RI and *Hind*III) run on the same gel at the left and right margin is indicated.

in the bands of lower mol. wt. (2.2 kb and 3.7 kb). With increasing times of *Bal*31 digestion these bands become less intense and migrate faster. Both effects are typical of chromosome termini, whereas artificial ends also exposed to *Bal*31 action, insofar as they are randomly distributed throughout the genome, cannot yield definite bands in the digestion pattern. On the other hand, *Bal*31 treatment does not modify the hybridization pattern of a non-telomeric probe such as the ribosomal DNA probe pPFrib2 of Langsley *et al.* (1983) (data not shown).

The 1.4-kb fragment recovered after plasmid digestion with *Alu*I and *Taq*I and ^{32}P-labelled by conventional nick-translation, was used in excess in the dot spot hybridization (see Materials and methods). Densitometric comparisons of hybridization intensities indicate that the copy number per genome of the sequence recognized by this probe is between 12 and 25 (dispersion interval of several independent determinations). When hybridized to Southern blots of *Bal*31-*Hae*III digests, as in Figure 3, this probe yields exactly the same hybridization pattern as the whole plasmid.

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5' AA  CCCTAAA CCCTGAA CCCTGAA CCCTGAA CCCTAAA CCCTGAA CCCTAAA
       CCCTGAA CCCTGAA CCCTAAA CCCTGAA CCCTAAA CCCTGAA CCCTAAA
       CCCTGAA CCCTAAA CCCTGAA CCCTGAA CCCTGAA CCCTAAA CCCTGAA
       CCCTAAA CCCTGAA CCCTGAA CCCTAAA CCCTGAA CCCTAAA CCCTAAA
       CCCTGAA CCCTGAA CCCTAAA CCCTGAA CCCTGAA CCCTAAA CCCTGAA
       CCCTGAA CCCTGAA CCCTGAA CCCTGAA CCCTGAA CCCTGAA CCCTGAA
       CCCTGAA CCCTAAA CCCTGAA CCCTAAA CCCTGAA CCCTGAA CCCTGAA
       CCCTGAA CCCTAAA CCCTGAA CCCTAAA CCCTGAA CCCTGAA ..... 3'
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Fig. 4. DNA sequence of the distal part of the cloned insert. *Hpa*II-*Taq*I subfragment 1.4 kb long was recovered from polyacrylamide gel (Maxam and Gilbert, 1977), terminally labelled using the Klenow fragment of *E. coli* DNA polymerase and [α-^{32}P]dCTP, and sequenced according to Maxam and Gilbert (1980).

Sequence analysis of the 1.4-kb fragment

In all cases hitherto studied the distal part of telomeric sequences is occupied by the tandem reiteration of a basic sequence. The presence of a reiterated region in our cloned fragment is suggested by the fact that no restriction sites are to be found in a region spanning 1.4-kb. This region has a *Taq*I site at its proximal extremity, while the distal end, obtaind by *Bal*31 digestion, does not correspond to a restriction site (Figure 2).

The next restriction site suitable for terminal labelling by the Klenow fragment of *E. coli* DNA polymerase (i.e., 5′ protruding) is the *Hpa*II site, located 53 nucleotides from the distal insert extremity.

The *Hpa*II-*Taq*I fragment was sequenced according to Maxam and Gilbert (1980). Terminal repetition of a heptanucleotide sequence initiating (5′) with three C can be clearly seen for ~500 nucleotides (or ~70 repeats) starting from the distal end, although the other bases in the repeat can be unambiguously determined only for the first 308 nucleotides. The complete sequence of this stretch is shown in Figure 4. The basic repeat is CCCT$^{G}_{A}$AA, the two purines A and G in the fifth position being irregularly distributed. The ~400 bp sequenced on the complementary strand starting from the other extreme (*Taq*I) do not contain any regular patterns (data not shown). Where the reiterated sequence terminates is not easily determined since the remaining, centrally located 0.5 kb are scarcely accessible by sequencing methods.

The sequence in Figure 4 represents the first example of a reiterated telomeric sequence involving all the four nucleotides. This fact should account, at least in part, for the higher incorporation obtained in the presence of the four dNTPs and in the absence of DNase (lane 1 in Figure 1).

Discussion

A number of conserved features for telomeric sequences from several simple eukaryotes have been described by various authors and reviewed by Blackburn (1984) and by Blackburn and Szostak (1984). Some of these features (e.g., the presence of a terminal hairpin or of single strand discontinuities) have been ascertained for telomeres in their original chromosomal context and are not present in the cloned versions of telomeric fragments used for sequencing studies.

Compared with cases previously studied, the fragment cloned by us from *P. berghei* ANKA DNA exhibits the following sequence characteristics: (i) The distal portion of our cloned insert consists of the reiteration of an heptanucleotide sequence for at least 500 nucleotides (or ~70 repeats), to which should probably be added the 75 bp (or 11 repeats) removed by *Bal*31 digestion from actual chromosome extremities so as to achieve molecular cloning. A repeat number >50 is found in *Trypanosoma brucei* (Van der Ploeg *et al.*, 1984a); 20–70 is the range reported for ribosomal DNA from *Tetrahymena* macronuclei (Blackburn *et al.*, 1983), while macronuclear DNA from hypotrichs has a much lower number of repeats (Klobutcher *et al.*, 1981). (ii) The basic repeat CCCT$^{G}_{A}$AA we found in *Plasmodium* is different from the various repeats thus far reported (Blackburn, 1984) and represents the only known case in which all the four nucleotides are present. However it has the initial (5′) cytosine cluster and its size is comparable with those of other known repeats, normally comprised between 6 and 10 bp. (iii) The barren region, 1.4 kb long, extends beyond the reiterated portion. This feature corresponds to what has been observed (Van der Ploeg *et al.*, 1984a) in the case of trypanosomes, in which, however, the barren region is much longer (5–35 kb).

The above properties, together with the fact that our recombinant plasmid recognizes restriction bands sensitive to the action of *Bal*31 exonuclease in total genomic DNA, leave little doubt that the cloned fragment contains a true telomeric sequence.

With regard to those features which characterize telomeres at their original location, our results show that nicks or gaps generally found at chromosome termini are in effect also present in *P. berghei* DNA and allow for *Bal*31-sensitive, selective incorporation of radiolabelled deoxyribonucleoside triphosphates. The fact that selective incorporation is highest with the combinations A,T,C/T,A,G lends support to the idea that the gaps are located in a sequence of the same type as that found in the cloned fragment. Relevant to this point is the observation that an enhancement of labelling efficiency in the presence of the four nucleoside triphosphate is to be expected on the basis of the sequence revealed for the cloned fragment. Such an enhancement may explain why total DNA radiolabelled with the four dNTPs was a better screening probe than the same DNA labelled in the presence of A,T and C.

The main question one would like to be able to answer in a clear way is the following: does our cloned fragment contain at least one sequence common to all chromosomal termini in *P. berghei* DNA? The pulsed electrophoresis technique (Kemp *et al.*, 1985; Schwartz and Cantor, 1984) will provide this answer. What we know from the present work is that our cloned insert recognizes at least seven different restriction fragments in a genomic digest, and that it is represented 12–25 times in a complete genome. Since in other cases (Walmsley *et al.*, 1983; Dunn *et al.*, 1984; Van der Ploeg *et al.*, 1984a) the same reiterated sequences have been found in sub-telomeric and internal regions as well as in telomeric regions, it is impossible for us to draw exact quantitative conclusions as to the number of chromosomes recognizing the cloned fragment. Our results are, however, fully compatible with the estimate of seven chromosomes obtained for *P. falciparum* by Kemp *et al.* (1985) and rule out the possible existence in our parasite population of a large number of minichromosomes possessing the same telomeres.

Studies are under way to assess the cross-hybridization capability of the cloned *P. berghei* telomere with the DNA from other *Plasmodium* species. We have also begun to explore the adjacent regions, which interest us as putative sites for genomic rearrangements.

Materials and methods

Parasite lines – DNA extraction and characterization

P. berghei line ANKA was a kind gift by Dr.Walliker. Purification of parasites and extraction of DNA was performed as already described (Birago *et al.*, 1982). Care was used to avoid shear stresses in DNA extraction. Each DNA preparation was tested in the analytical ultracentrifuge to check purity from possible con-

tamination by host DNA. The mol. wt. range of extracted DNA, estimated from gel electrophoresis of undigested DNA, is >30 kb.

Selective radiolabelling by E. coli DNA polymerase I

Incorporation of [α-^{32}P]deoxyribonucleoside triphosphates (dNTPs) at endogenous single strand nicks or gaps was performed as described by Blackburn and Gall (1978) and by Blackburn and Challoner (1984).

Digestion with Bal31 nuclease (BioLabs)

Digestion times were calibrated following the size reduction of the bands of the mol. wt. marker III from Boehringer (*Eco*RI + *Hind*III-digested λ DNA). The reaction was standardized at 15 nucleotides removed per min, under the following incubation conditions: 0.1 U of *Bal*31 per μg of DNA, 30°C, buffer indicated by the manufacturer.

Molecular cloning of telomeric sequences

Several restriction enzymes suited for molecular cloning in pBR322 were tested on *P. berghei* DNA and *Bgl*II, which fractionates the parasite DNA in a broad range of fragments, was chosen. *P. berghei* DNA (0.5 μg) pre-treated with *Bal*31 nuclease for 5 min (75 nucleotides removed) was ligated to pBR322 (0.5 μg) double digested with *Pvu*II and *Bam*HI. Blunt-end ligation (*Bal*31 and *Pvu*II) was performed overnight at room temperature, by adding 1 U of T4 DNA ligase. The final volume was 35 μl. The ligation mixture was then digested with *Bgl*II, which has no sites in pBR322 but generates in *Plasmodium* DNA sticky ends complementary to those generated by *Bam*HI in the plasmid. A second double digestion with *Pvu*II and *Bam*HI allowed us to reduce the background represented by dimers or reconstituted plasmids. After heat inactivation of the restriction enzymes, the mixture was diluted to a plasmid concentration of 2 μg/ml to favour circularization of the recombinant molecules. Sticky end ligation was performed overnight at 4°C, by adding 1 U of T4 DNA ligase. Transformation of *E. coli* HB101 cells was performed using the $CaCl_2$ procedure described by Maniatis *et al.* (1982). 90% of the ampicillin-resistant colonies obtained were sensitive to tetracycline, which is the phenotype expected for cells harboring recombinant plasmid.

Selection of recombinant colonies containing telomeric sequences

HB101 colonies plated on a medium containing ampicillin (50 μg/ml) were transferred to nitrocellulose filters, denatured (Maniatis *et al.*, 1982) and hybridized overnight at 65°C with 2 μg of cold total *P. berghei* DNA digested with *Bal*31 for 20 min (300 bp removed from every extremity). Filters were then washed in 2 × SSC (30 min) and hybridized to 1 μg of total *P. berghei* DNA labelled as described in the legend to Figure 1, in the presence of the four dNTPs, 50 μCi each. Before hybridization, the labelled probe was digested with *Hae*III to reduce the average fragment size. The colony selected was grown on a medium containing ampicillin (50 μg/ml). Plasmid extraction was performed according to Godson and Vapnek (1973).

Restriction endonuclease digestion – restriction map

Restriction endonucleases (Boehringer) were used as specified by the manufacturer. Enzmes cutting only once inside the insert (*Cla*I, *Hind*III, *Xba*I, *Alu*I) were mapped by complete digestion. Enzymes cutting the insert more frequently (*Taq*I and *Hinf*I) were mapped by partial digestion of end labelled DNA as described by Smith and Birnstiel (1976). End labelling was carried out using the Klenow fragment of *E. coli* polymerase (Boehringer).

Dot spots

The procedure described by Kafatos *et al.* (1979) was followed. Nitrocellulose (Schleicher and Schüll) filters washed in 1 M NH_4-acetate were placed in the Minifold apparatus (Schleicher and Schüll) over a 3 MM (Whatman) paper soaked in the same solvent. Samples, denatured in 0.2 M NaOH (10 min at 37°C, final volume 100 μl) and neutralized by the addition of an equal volume of 2 M NH_4acetate were applied under water vacuum. Samples contained 0.05 – 0.4 μg of total *P. berghei* DNA (corresponding to $3 \times 10^6 - 2.4 \times 10^7$ copies of whole genome, 1.5×10^7 bp in complexity) or 0.12 – 8 ng of the recombinant plamid (corresponding to $2.2 \times 10^7 - 1.5 \times 10^9$ plasmid copies). Filters washed in 4 X SSC were then dried for 2 h at 80°C and then hybridized with >500-fold excess of the 1.4-kb fragment, labelled by conventional nick-translation. After hybridization the filters were washed at high stringency (5 × to 0.1 × SSC, in steps, at 65°C) and exposed at −80°C using Fuji X-ray films.

Acknowledgements

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