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Genetic hypervariability of telomere-related sequences is associated with meiosis in *Plasmodium falciparum*

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ABSTRACT

Sequences related to those near chromosome telomeres in the human malaria parasite, Plasmodium falciparum, were extremely unstable during a genetic cross between two different clonal genotypes. Many progeny of the heterologous cross displayed telomere-homologous restriction fragments found in neither parent. A significant number of the new fragments resulted from rearrangements at chromosome-internal locations which were bounded by more complex tracts of DNA sequence. The same instability was not seen to arise during an inbreeding cross, nor during mitotic replication of parasites. Thus, a form of genetic hypervariablity results from molecular events which occur during meiotic reduction and is apparent only in a cross between heterologous strains of parasite. Since other sequences were entirely stable under the same conditions, it appears that chromosome-internal blocks of telomeric sequences in the P. falciparum genome may designate conditionally unstable chromosomal domains. We discuss some potential implications of these findings for the population biology of P. falciparum.

INTRODUCTION

The mechanisms by which malaria parasites can generate genetic variability at rapid rates are central to some of the epidemiological and biological questions concerning control of the disease. Examples of one kind of variability include the accelerated nucleotide sequence change of the immunodominant portions of the circumsporozoite protein (CS) and S-antigen genes of Plasmodium species [1-4]. Sequence change in the CS and S-antigen genes appears to be rapid only in the central repeat region while the flanking portions of the same gene are highly conserved [2, 4]. Another kind of variability was recently shown in the genome of P. cynomolgi, which is naturally partitioned into a small number of domains which differ in their rates of overall sequence change [5]. This kind of variability has also been seen in vertebrate genomes [6].

These observations suggest the operation of at least two mechanisms to create genetic variability in Plasmodium: one which permits rapid sequence change in a specific part of an otherwise conserved gene and another in which general instability generates a high frequency of genetic polymorphism at susceptible chromosomal regions. We were interested in the biological implications of this second kind of more generalized variability. Here we present the result of molecular genetic studies in Plasmodium which show extreme variabil-

ity associated with telomere-related but chromosome-internal sequences only under certain biological conditions.

METHODS AND MATERIALS

Parasite Preparation and DNA Purification

The origins of P. falciparum lines used in the genetic cross were previously described [7]. Briefly, parent P1 was line HB3A which was cloned from Honduran isolate H1 by micromanipulation. Parent P2 was line 3D7A, which was cloned from Dutch isolate NF54 by limiting dilution. The uncloned inbreeding progeny of HB3A was culture HB3B, and that of 3D7A was 3D7B. Referring to lane designations in Figure 2, the cloned progeny of the cross P1 x P2 were as follows: lane c, X2; lane d, XP5; lane e, XP2; lane f, X6; lane g, XP9; lane h, X8; lane k, XP1; lane l, XP3; lane m, XP4; lane n, XP8; lane o, XP10; lane p, X5. Progeny clones designated "XP" had survived drug selection with pyrimethamine while clones designated "X" were not subjected to drug pressure.

The derivation of the P. knowlesi parasites used to study the mitotic behavior of telomere-related sequences was previously described [8]. A rhesus monkey which had been immunized with the 143/140 kD merozoite surface proteins was inoculated with clone Pk1(A^+). Asexual progeny of this clone were isolated from five subsequent recrudescent peaks during a chronic infection, at time points ranging from 10 to 100 days after inoculation, and were recloned.

Parasites were routinely collected in heparin, purified away from platelets and leukocytes, and freed from intact red blood cells by saponin lysis essentially as described [9]. Purified DNA was the kind gift of Dr. Thomas Wellems and was prepared by treating resuspended parasites with 0.5% SDS/0.1 mg ml^{-1} Proteinase K, followed by CsCl density gradient centrifugation and spooling [9].

Enzymes, Southern Blotting, and Hybridizations

Restriction endonucleases were obtained from Bethesda Research Laboratories Life Technologies Inc. or New England Biolabs and used according to supplier's recommendations. Exonuclease Bal 31 Fast form was obtained from International Biotechnologies Inc. and was used at a concentration of 0.2 unit per μg DNA. Under these conditions, Bal 31 removed about 150 bp per minute from lambda DNA predigested with HindIII and about 300 bp per minute from genomic P. falciparum DNA.

Electrophoresis was routinely carried out in 0.8% agarose gels which were run and transferred to nitrocellulose filters essentially as described [10]. Double stranded DNA probes were labelled by nick translation or random hexamer priming [11] and were hybridized essentially as described [10]. Oligonucleotides were made using an Applied Biosystems 380B DNA synthesizer. About 0.2 pmole of kinased oligonucleotide probe was used per ml of hybridization solution, which was 6x SSC, 0.2% SDS, 0.1% each BSA, PVP, and Ficoll, 50

μg/ml tRNA, and 0.05% sodium pyrophosphate. Hybridization was overnight at 42°C. Three non-stringent washes for 15 min each at room temperature were followed by a 3 min stringent wash in 2x SSC at a liquid temperature calculated by the function: °C=94-(820/probe length) [12]. Oligonucleotide probes were stripped from blots by washing in 0.1x SSC, 0.1% SDS at 65°C for 15 min. Double-stranded probes were stripped from blots by washing in 0.2 N NaOH, 0.1% SDS at room temperature for 10 min, briefly neutralizing in 0.5 M NaCl, 0.2 M Tris-HCl, pH 7.4, and briefly washing in 0.1x SSC, 0.1% SDS.

RESULTS

Genetic Instability in a Cross Between Unlike Parents

We used whole nick-translated DNA from a variable genomic domain of P. cynomolgi [5] as a probe to screen a library of mung bean nuclease-cleaved P. falciparum genomic DNA [13]. We anticipated that selected clones would be from a genetically-variable domain of the P. falciparum genome, since the probe consisted of genetically variable P. cynomolgi DNA, but would contain phylogenetically conserved sequences, since we were screening with heterologous DNA. We chose the clone with the largest insert, about 3 kb, for further genetic analysis. The primary sequence and structural characteristics of the clone, pPftel.1, are described elsewhere [14]. The cloned fragment originated from a telomere of P. falciparum and consists of about 2 kb of pre-telomeric sequence and the final 1 kb of short tandem repeats related to the telomeric simple repeat sequence, TT(T/C)AGGG (Fig. 1).

In order to analyze genetic behavior of sequences related to pPftel.1, we used material resulting from a genetic cross between two cloned parental lines of P. falciparum, as described recently [7]. Briefly, in the genetic cross gametocytes from both parent lines of parasite were mixed, fed to mosquitoes to allow fertilization in the mosquito midgut and subsequent development to the infective salivary gland sporozoite stage, which material was finally used to infect a primate host. Cloned progeny lines were then re-isolated from among the offspring of the cross in cultures derived from the mammalian host. In parallel, each parental line was allowed to undergo self-fertilization by passage through mosquitoes to a primate host. Parasites were collected both before and after this inbreeding cross and were expanded in culture. Genomic DNA samples were isolated from each parent clone before and after mosquito passage and from each cloned progeny line. Each DNA sample was cleaved with restriction enzyme BstNI, electrophoresed and blotted, and analyzed on Southern blots using various probes.

Figure 2 shows DNA from the two parental lines of the cross, P1 (lane a) and P2 (identical lanes i and q), and from cross progeny lines (lanes c-h and k-p). When blots of parent and progeny DNAs were probed with the telomeric clone pPftel.1, it was seen that cross progeny frequently displayed new restriction fragments found in neither parent (Fig.

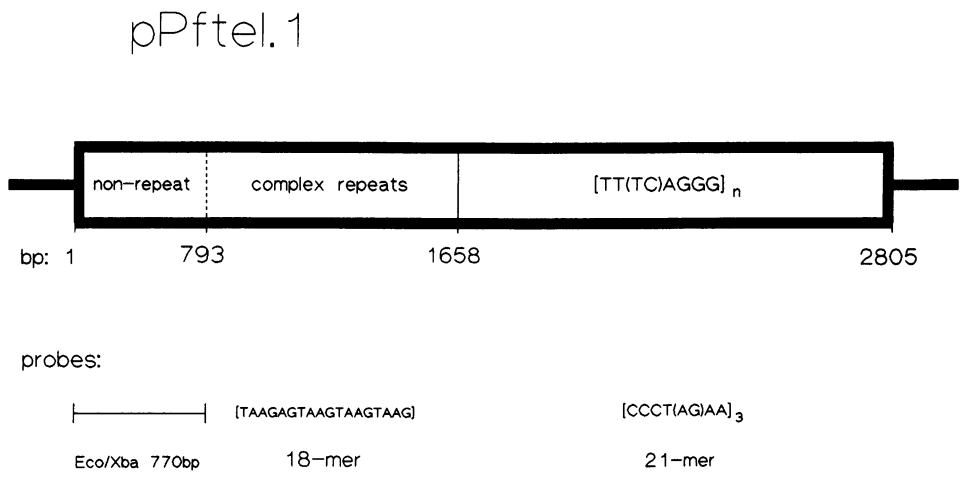

Figure 1. Map of telomeric clone pPftel.1. Probes used in study are shown below the diagram. Structural regions and their boundaries were derived from sequence analysis of the clone [14].

2A, new bands marked by numbered darts). We have been conservative in designating new bands. A variety of autoradiograph exposures were examined for differences between parental and progeny patterns. In each case, only progeny bands distinct and dark enough so that a corresponding parental band would have been visible at the appropriate exposure were considered newly generated fragments. The relatively diffuse appearance of probe signal in Figure 2A results from the heterogeneity in simple repeat number commonly observed at the ends of chromosomes [14, 15]. The standard appearance of telomeres as broad bands bounded on one side by a restriction enzyme site and on the other side by a simple-sequence terminal region essentially barren of restriction sites is particularly evident in Figure 2A, lane g. In contrast, it is important to note that the sharply defined bands in Figure 2A result from probe-homologous sequence which was bounded on both sides by regions which were sufficiently complex in primary sequence to contain restriction sites. Such sharply-defined bands thus are located at chromosome-internal positions which are not directly adjacent to the chromosome end [14]. Genetic variability of chromosome-internal sequences precludes the possibility that the observed variabilty results from addition or loss of simple repeats at the chromosome end.

In contrast to the generation of new restriction fragments seen with the telomeric probe, Figure 2B shows that when the identical blots were hybridized with a probe for the circumsporozoite (CS) protein gene of P. falciparum, the two parental BstNI restriction fragment morphs were transmitted to progeny in an apparently Mendelian fashion (P1 allele found in progeny lanes d,e,f,k,m,o,p; P2 allele in progeny lanes c,g,h,l,n). The data in

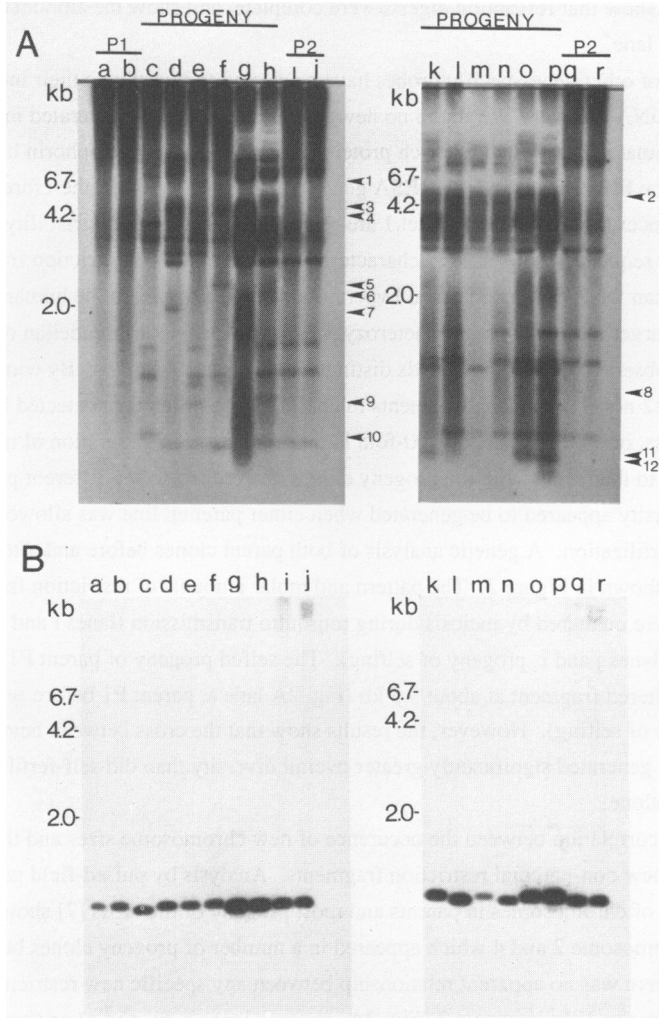

Figure 2. Analysis of telomere-related sequences during sexual replication. Two cloned lines of P. falciparum parasites were either inbred or crossed to each other, and progeny parasites were isolated. Genomic DNA from parents and progeny was cleaved with BstNI and Southern blotted. Lanes a, i, and q: original cloned parental lines P1 and P2; lanes b, j, and r: uncloned inbreeding progeny resulting from parental self-cross; lanes c-h and k-p: cloned outbreeding progeny resulting from the cross P1 x P2. **A)** Both blots in upper panel probed with telomeric clone pPftel.1. Numbered darts indicate positions of new restriction fragments found in progeny but not present in either parent. Numbered bands correspond to progeny lanes as follows: band 1, lane g; band 2, lane o; band 3, lane h; band 4, lane f; band 5, lane f; band 6, lane h; band 7, lane d; band 8, lane p; band 9, lane h; band 10, lane c; band 11, lane o; band 12, lane p. **B)** Identical blots reprobed with single-copy circumsporozoite protein gene from P. falciparum. P1 displays the slow RFLP and P2 the fast RFLP.

Figure 2B also show that restriction digests were complete, and show the amount of DNA present in each lane.

A number of other gene-specific probes have been used to investigate their inheritance in these same DNA samples. We found no new restriction fragments generated in progeny for the 7 ribosomal genes, 3 histidine rich protein (HRP) genes, the glycophorin binding antigen gene, the FIRA gene, and the RESA gene (not shown). We feel, therefore, that genomic sequences homologous to pPftel.1 are regions of genetic hypervariability.

A repeated sequence probe used to characterize "fingerprints" of restriction fragment patterns in human DNA has been termed hypervariable [16]. Although the human loci harboring the target repeats are highly heterozygous, only 1 new non-Mendelian restriction fragment was observed of 240 total bands distinguished in family studies. By comparison, we find about 12 novel restriction fragments in about 24 total fragments detected in the P. falciparum cross, or approximately a 100-fold higher frequency of generation of new alleles.

In contrast to the results with the progeny clones derived from two different parents, much less diversity appeared to be generated when either parental line was allowed to undergo self-fertilization. A genetic analysis of both parent clones before and after self-fertilization is shown in Figure 2. The pattern and molar amounts of restriction fragments of parent P2 were unaltered by meiosis during mosquito transmission (lanes i and q, P2 before selfing; lanes j and r, progeny of selfing). The selfed progeny of parent P1 may have had one altered fragment at about 3.5 kb (Fig. 2A lane a, parent P1 before selfing; lane b, progeny of selfing). However, the results show that the cross between heterologous parental strains generated significantly greater overall diversity than did self-fertilization of each parent alone.

We see no correlation between the occurence of new chromosome sizes and the appearance of new non-parental restriction fragments. Analysis by pulsed-field gel electrophoresis of chromosomes in parents and most progeny of the cross [7] showed variants of chromosome 2 and 4 which appeared in a number of progeny clones but not in the parents. There was no apparent relationship between any specific new restriction bands and a new-sized chromosome 2 (Fig. 2A, progeny lanes e, m, and p) or chromosome 4 (Fig. 2A, progeny lanes c, d, e, and g). Moreover, the parasite clones that were described [7] as maintaining the parental complement of chromosomes (Fig. 2A, progeny lanes h, f, and k) all contain new non-parental restriction fragments. This result is not unexpected, as a direct association between new chromosomes and new restriction fragments would imply very precise sites of genetic change. It is therefore not possible to say at this stage whether rearrangement of telomere-like sequences is involved in the generation of chromosome polymorphism in P. falciparum.

Meiotically Unstable Sequences Are Not Mobilized During Mitosis

We have directly investigated the behavior of pPftel.1-related sequences during prolonged asexual propagation of parasites under selective immune pressure in the natural

host. The immune response of the host is one of the most important selective pressures faced by field populations of malaria parasites, as distinct from the artificial pressures placed on parasites grown in in vitro cultures. Thus, in understanding the natural mitotic behavior of the telomere-related sequences, it was especially important to determine whether the sequences were unstable during asexual expansion under such severe pressure. In addition to general instability, mechanisms such as transpositions of genes into telomeric areas, as occur in trypanosomes, would probably be detected in this fashion.

In this experiment, we used the monkey malaria parasite P. knowlesi in its natural host, since the corresponding experiment using the human parasite P. falciparum in its natural host would not be possible. The sequence of the telomeric repeats is identical for these two species, and there is a similar organization of telomere-related sequences internal to chromosome ends (not shown). We consider, therefore, that the biological behavior of telomere-related sequences in the two species is probably comparable.

The P. knowlesi parasites studied were those described by Klotz et al. [8]. Briefly, monkeys were vaccinated with the 143/140 kD merozoite surface proteins and were then challenged with cloned blood-stage parasites. Immunity to the 143/140 kD proteins conferred partial protection to two of the four treated monkeys. Recrudescent parasites, which escaped anti-143/140 kD immunity and established sequential peaks of parasitemia, were cloned by dilution. An analysis of parasites from several peaks of recrudescent parasitemia showed that in all samples, the 143/140 kD proteins were either altered or missing, indicating that biological changes had occurred as a result of the immune pressure [8].

This represents a situation that is parallel to the analysis of material from the genetic cross. The central difference between the two experiments was that, in this case, the cloned parasites only underwent asexual replication. We expected that the biological selection imposed by immune pressure would, if anything, expedite the appearance of rearrangements in the telomere-related sequences.

In fact, Figure 3 shows no detectable differences between the cloned parent line (lane a) and the recrudescent mitotic progeny digested with EcoRI (lanes b-f), or between the same cloned parent (lane g) and its mitotic progeny digested with HindIII (lanes h-l). Each of these restriction enzymes produce more numerous internal fragments containing probe-homologous sequence, and terminal broad bands which are less pronounced than BstNI does in Figure 2. Thus, asexual blood stage growth of parasites did not seem to generate a significant frequency of rearrangement of pPftel.1-related sequences, even when parasites were subjected to immune selection. Meiosis, therefore, appears to create much greater diversity in these sequences than many generations of mitotic events.

Rearrangements of Telomere-Related Hypervariable Sequences Often Occur At Locations Internal to Chromosome Ends

We wished to identify the specific sequences within pPftel.1 responsible for hypervari-

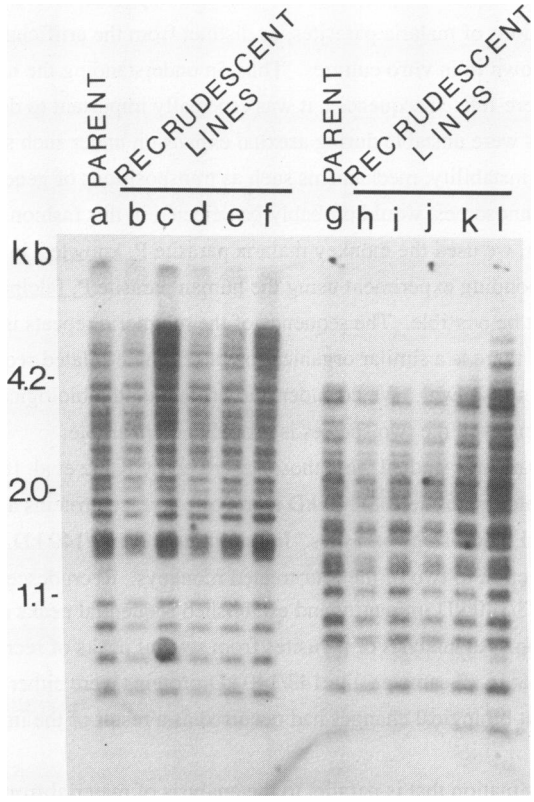

Figure 3. Analysis of telomere-related sequences during mitotic replication. Cloned bloodstage P. knowlesi parasites were used to innoculate their natural monkey host, which had been vaccinated with the 143/140 kD parasite surface protein. Recrudescent parasites were isolated from two peaks of parasitemia during chronic infection, and were recloned. Genomic DNA was purified from parents and asexual progeny, digested with a restriction enzyme, electrophoresed and Southern blotted. The Southern blot was probed with telomeric DNA clone pPftel.1. Lane a, cloned asexual parasites used for innoculation of rhesus monkeys, cleaved with EcoRI; lane g, the same cloned parasite line cleaved with HindIII; lanes b-f, recloned asexual progeny of cloned parent after recrudescence, cleaved with EcoRI; lanes h-l, the same recloned recrudescent asexual progeny, cleaved with HindIII.

able rearrangement. The probes chosen from the clone pPftel.1 for this experiment, shown in Figure 1, were a 770 bp restriction fragment from the centomere-proximal end of pPftel.1; an 18-mer oligonucleotide which appeared on structural criteria [14] to be potentially recombinogenic; and a 21-mer oligonucleotide consisting of 3 copies of the simple telomeric repeats. Figure 4 shows the blot from Figure 2 (right side) hybridized to each of three probes. The telomeric simple repeat 21-mer was found to hybridize to all of

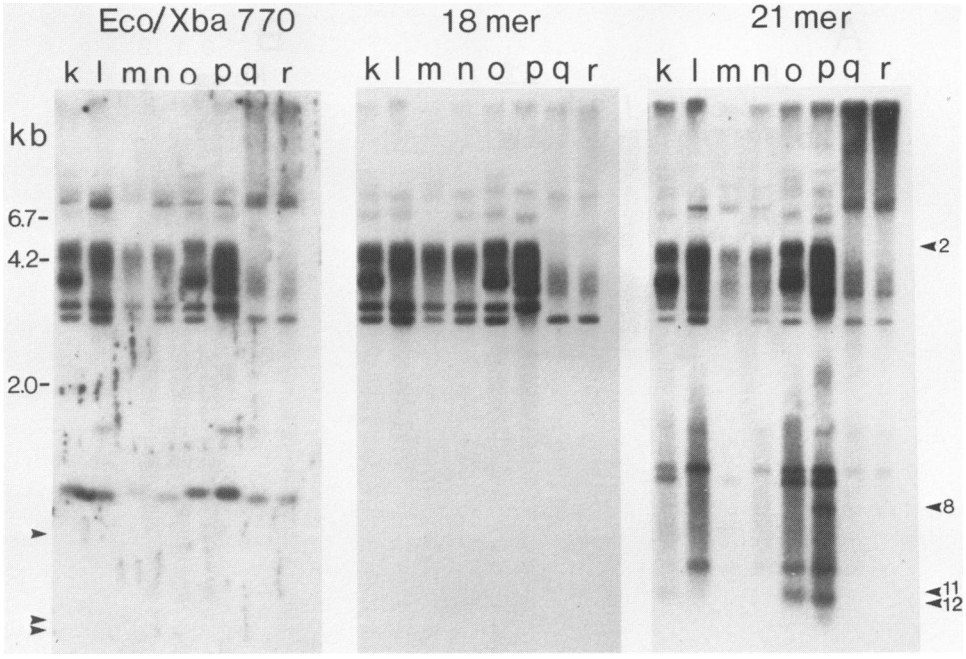

Figure 4. Detailed analysis of the telomere-related sequences associated with genetic hypervariability. The Southern blot in the right panel of Figure 2 was reprobed with shorter sequences taken from within telomeric DNA clone pPftel.1. Numbered darts corresponding to those in Figure 2 identify the new restriction fragments found in progeny, which hybridized to any of the 3 probes used. Probes were: a 770 bp EcoRI-XbaI restriction fragment from the most complex sequence region of the clone; an 18-mer oligonucleotide from the region of intermediate complexity; and a 21-mer oligonucleotide consisting of 3 tandem copies of the simple telomeric repeats. Identification of lanes is identical to Figure 2, right panel.

the restriction bands originally identified as hypervariable ones in Figure 2A (numbered darts). Interestingly, hypervariable band 2, which was hybridized by the 21-mer in Figure 4 (lane o), was also hybridized by the other two internal probes. This indicates that although meiotic hypervarability was associated with the telomeric simple repeats, the extent of the resulting chromosomal rearrangements was not physically limited to the simple repeat region. The results of reprobing the left-hand blot from Figure 2A were similar in that all of the original hypervariable bands were also hybridized by the simple repeat 21-mer (not shown). Additionally, hypervariable bands 3 and 4 in Figure 2 (lanes h and f, respectively) were also visualized when the same blot was re-probed with the 18-mer probe (shown in Fig. 5a) and the Eco/Xba 770 bp probe (not shown). Another band, marked by the starred dart in Figure 5a, was identified by all three probes as well and may be absent from both parents, although we did not feel it could be designated a

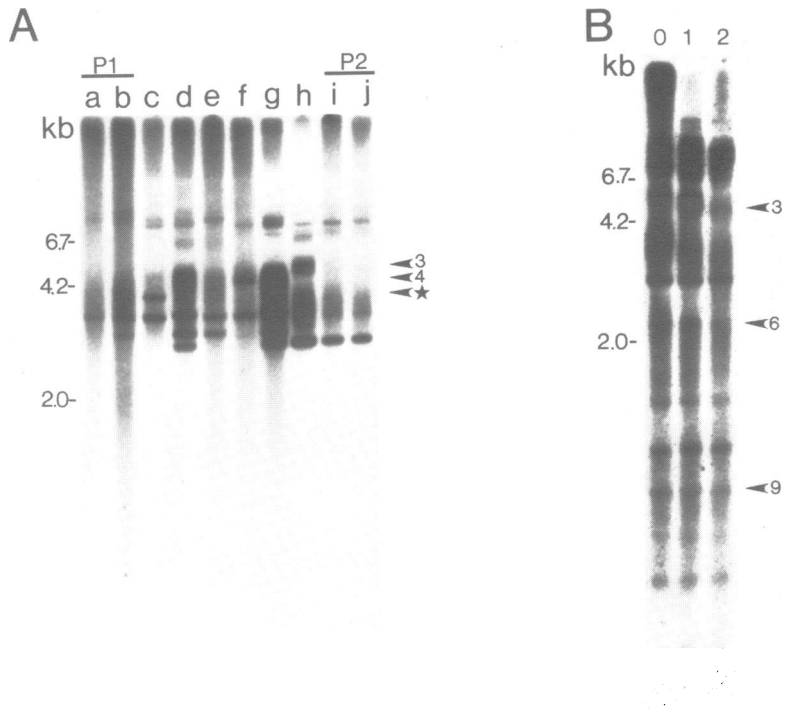

Figure 5. Identification of genetically hypervariable restriction fragments in chromosome-internal locations. A) Southern blot from left panel of Figure 2 reprobed with the 18-mer oligonucleotide shown in Figure 1 map, to visualize the new restriction fragments in cross progeny. New fragments are identified by numbered darts identical to Figure 2, except for the starred dart which is described in Results as a probable new band in lane c. Lanes are as designated in Figure 2 legend. **B)** Genomic DNA from the progeny clone in lane h (Figs. 2 and 5a) was treated first with nuclease Bal 31 and then BstNI followed by electrophoresis and Southern blotting. The blot was probed with the telomeric DNA clone pPftel.1 . Lanes contain aliquots treated for 0, 1, and 2 min with Bal 31, at a rate of about 300 bp/min. New restriction fragments in cross progeny are identified by numbered darts, which correspond to those in the previous figures. Band 3 decreased in mobility with Bal 31 treatment while bands 6 and 9 remained stationary.

hypervariable band with certainty due to the comigrating smear in both parental lanes (Fig. 2). Thus, at least two of 12 newly-generated fragments represented meiotic rearrangements extending some distance internal to the chromosome end. The other 10 newly-generated fragments may likewise result from internal changes, but in chromosomes other than the one for which we have subtelomeric probes. Alternatively, the other changes may be associated with the terminal simple repeats.

A possible explanation for the observation that the hypervariable restriction fragments can hybridize to both the telomeric simple repeat probe and the internal probes could be

that the hypervariable bands we describe represent chromosome-internal copies of telomeric sequences. This hypothesis is supported by the resolution of many of the hypervariable restriction fragments as sharp bands rather than as the broad smear characteristic of actual chromosome ends (for example, Fig. 4, bands 8, 11, and 12). If the hypothesis were true, the hypervariable bands seen in progeny DNA should be resistant to treatment with Bal 31 exonuclease. In fact, analysis of DNA from two progeny lines showed that four of the original five hypervariable bands in both progeny were resistant to Bal 31 treatment under conditions in which digestion proceeded about 600 bp internal to DNA fragment ends.

Figure 5b shows the results of Bal 31 treatment of DNA from the progeny line shown in lane h of Figure 2, which was then probed with clone pPftel.1. The three hypervariable bands originally found in this progeny clone are marked by numbered darts. Band 9 and probably band 6 in Figure 5b did not decrease in size as would be expected if the sequence occurred at a chromosome end, and so were therefore bounded on both sides by restriction sites. In contrast, hypervariable band 3 decreased in size as expected for terminal sequences treated with a processive exonuclease. The three hypervariable bands were also visualized using the 21-mer simple repeat probe with the same blot (not shown). Thus, it is clear that blocks of telomeric simple repeats and other telomere-associated sequences can be bounded by restriction sites at locations internal to the actual ends of chromosomes. Our data suggest that internal telomere-related sequence blocks are often associated with genetic hypervaribility. We do not know how far inside chromosomes the hypervariable regions extend, although the original organization of the probes in clone pPftel.1 would suggest that this distance is probably at least 3 kb.

DISCUSSION

We have shown that chromosomal DNA sequences related to sequences found in the vicinity of telomeres changed so rapidly during a genetic cross between two genetically distinct clones of P. falciparum that a large percentage of the progeny contained restriction fragments seen in neither parent. We did not see variability when analyzing the same DNA with a number of other probes, which suggests that the mechanism involved is related to the behavior of telomere-related sequences. However, many of the genetically variable telomere-related sequences were located some distance internal to the actual ends of chromosomes. We do not yet know whether the chromosomal rearrangements resulted from genetic recombination or meiotic transposition, although in either case they occurred at a very high frequency, and were related to a specific sequence.

The telomere-related sequences appeared stable over the course of repeated rounds of asexual replication of the monkey malaria parasite P. knowlesi in its natural host, even under immune selective pressure. This is consistent with the observation in yeast that certain telomere-associated sequences can be meiotically unstable but remain stable during mitotic expansion [17]. Further, parasites resulting from selfing of either parental geno-

type did not generate the same diversity as that seen in the cross progeny. Hence, genetic hypervariability for these sequences appears to be associated with the meiotic divisions following cross-fertilization but not self-fertilization. Since, as mentioned above, many other sequences were genetically stable under the same conditions, this work suggests that the Plasmodium genome is compartmentalized into stable and conditionally unstable domains.

Such conditional instability would have direct phenotypic consequences if it generated heightened genetic polymorphism among a particular subset of genes, while sparing the remainder of the genome the effects of rapid sequence change. For this to be so, it would probably be necessary to fulfill two main conditions. First, genes in which rapid sequence change or polymorphism of expression can be beneficial would need to be specifically located in regions which are accessible to chromosomal rearrangement involving telomere-related sequences. These could, for instance, be genes for which a population mosaic enhances group fitness, or genes which respond to variable kinds of selective pressure such as host immune response. In yeast, chromosomal rearrangements involving telomeric sequences are associated with phenotypic polymorphism for at least two genes known to be located in regions which are accessible to the effects of terminal rearrangement, the genes for sucrose-fermentation, SUC [18], and maltose fermentation, MAL [19]. In P. falciparum, two nonessential genes which affect parasite growth rate and the ability to evade host response to it by sequestering in deep tissues are both localized in subtelomeric regions [20, 21]. Further, the chromosome size polymorphisms in P. falciparum, which apparently arise meiotically but not mitotically, can affect coding sequences and thus lead to phenotypic polymorphism [22, 23]. Second, successful parasite lines should be spared the effects of a high rate of sequence change. A genetic mechanism such as described here, where self-mating produced little effective change while a cross between genetically different parents generated heightened variability in a certain component of the genome, would accomplish this.

The structure of populations of Plasmodium species remains largely unknown, although such questions have potentially interesting epidemiological and biological implications. For example, certain environmental conditions may promote a large diversity of genetic forms within a population while other conditions, such as occur during malaria epidemics, may promote the expansion of a single or a few clonal types. Certainly a local population comprised of relatively few genetic forms would undergo self-fertilization more often than would a genetically complex population. As we show here, the ratio of self-fertilization to cross-fertilization in the population appears to regulate the induction of conditional instability in a component of the genome. It is tempting to speculate that the conditional genetic instability shown here may operate to regulate and compartmentalize the generation of variability in Plasmodium populations in a way that is sensitive to the environmental context of the population. We think that the description of this sequence-related condi-

tional genetic instability will prove useful in gaining an understanding of population-level behavior of Plasmodium.

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