A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes

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Bacterial plasmids introduced into the human malaria parasite *Plasmodium falciparum* replicate well but are poorly segregated during mitosis. In this paper, we screened a random *P.falciparum* genomic library in order to identify sequences that overcome this segregation defect. Using this approach, we selected for parasites that harbor a unique 21 bp repeat sequence known as Rep20. Rep20 is one of six different repeats found in the subtelomeric regions of all *P.falciparum* chromosomes but which is not found in other eukaryotes or in other plasmodia. Using a number of approaches, we demonstrate that Rep20 sequences lead to dramatically improved episomal maintenance by promoting plasmid segregation between daughter merozoites. We show that Rep20⁺, but not Rep20⁻, plasmids co-localize with terminal chromosomal clusters, indicating that Rep20 mediates plasmid tethering to chromosomes, a mechanism that explains the improved segregation phenotype. This study implicates a direct role for Rep20 in the physical association of chromosome ends, which is a process that facilitates the generation of diversity in the terminally located *P.falciparum* virulence genes.

Keywords: plasmid segregation/*Plasmodium falciparum*/Rep20/terminal chromosomal cluster/transfection

Introduction

The protozoan parasite *Plasmodium falciparum* is one of the world's most important pathogens, resulting in ~400 million clinical cases of malaria and more than 1 million deaths each year. During blood-stage growth, the unusually AT-rich *P.falciparum* genome is haploid and consists of 14 linear chromosomes varying in size from 0.7 to 3.4 Mb. Differentially expressed virulence genes in this parasite, most notably the *var* genes (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995), are predom-

antly found at the ends of chromosomes immediately internal to a 15–30 kb stretch of subtelomeric non-coding sequence (Rubio *et al.*, 1996; Hernandez Rivas *et al.*, 1997). This non-coding sequence is comprised of a unique set of six different telomere-associated repeat elements (TAREs) present in a conserved arrangement at the end of each chromosome (Gardner *et al.*, 1998; Bowman *et al.*, 1999; Figueiredo *et al.*, 2000; Scherf *et al.*, 2001). Much remains to be learned about the mechanisms underpinning the control of expression and creation of extensive antigenic diversity in these terminally located virulence genes, although there is evidence to suggest that both mechanisms involve chromosomal modifications (Freitas-Junior *et al.*, 2000; Deitsch *et al.*, 2001). It has been shown, for example, that terminal *var* genes undergo ectopic recombination and that this is facilitated by the formation of terminal chromosomal clusters involving tight associations between heterologous chromosome ends (Freitas-Junior *et al.*, 2000). Hence, it appears likely that potentially unique DNA–protein interactions that govern the architecture and spatial arrangement of specific chromosomal regions are important virulence determinants in this organism.

One way to identify elements involved in chromosomal interactions is to test sequences for their ability to improve plasmid segregation during mitosis. This approach has been used extensively in yeast to identify and characterize telomeric and centromeric sequences that bind nuclear proteins (Longtine *et al.*, 1992; Enomoto *et al.*, 1994; Ansari and Gartenberg, 1997). Bacterial plasmids transfected into *P.falciparum* replicate episomally as head-to-tail concatamers (Crabb *et al.*, 1997b; O'Donnell *et al.*, 2001), but these forms can be integrated into the genome, provided an appropriate targeting sequence is present in the plasmid (Crabb and Cowman, 1996; Wu *et al.*, 1996; Crabb *et al.*, 1997a). It is well established that plasmids maintained episomally in *Plasmodium* parasites are segregated unevenly between daughter merozoites such that some parasites do not receive plasmids during each division (van Dijk *et al.*, 1997; O'Donnell *et al.*, 2001). This explains why transformants grow at a slower rate when under drug selection and why they are rapidly lost when drug pressure is removed. Poor plasmid segregation, along with a low number of parasites receiving plasmid DNA at the time of transfection, is likely to be a key reason for the inefficiency of the *P.falciparum* transfection system.

In this paper we describe the use of random genomic library screening to identify *P.falciparum* sequences that improve plasmid maintenance in transfected *P.falciparum* parasites. This approach selected for parasites that harbor a plasmid containing a 1.4 kb stretch of Rep20 repeats. Rep20 is a novel 21 bp degenerate repeat and is the longest of the six TAREs referred to above (Aslund *et al.*, 1985;

Fig. 1. Screening of a random genomic library for improved episomal maintenance in *P. falciparum* selects for a Rep20-containing plasmid. The plasmid library pHH/DRlib was constructed by ligation of random 0.5–2.0 kb *P. falciparum* inserts into the *EcoRV* (E) site of pHH1 (Reed *et al.*, 2000). pHH/DRlib plasmids contain a marker to confer WR99210 resistance to parasites (Fidock and Wellems, 1997) (hDHFR cassette) and a partial fragment of the *P. berghei* DHFR-TS 3' UTR (Pb). DNA from the same pHH/DRlib preparation was transfected directly into *E. coli* and into *P. falciparum* on two separate occasions (Pf #1 and Pf #2). Following the establishment of drug-resistant populations, plasmids from Pf #1 and Pf #2 transformants were recovered in *E. coli*. In all three instances (indicated by arrows), plasmid DNA was extracted from 18 randomly chosen *E. coli* colonies and digested with a combination of *Bam*HI (Ba) and *Not*I (N) to release inserts. Plasmids profiles were examined on ethidium bromide-stained gels (upper panels) that were subsequently transferred to nitrocellulose for hybridization with a Rep20 probe (bottom panels). DNA markers loaded in the left hand lane of each stained gel are indicated in kb. Released fragments of >1.2 kb represent the presence of a *P. falciparum* insert. The asterisk indicates the Rep20⁺ plasmid used in further studies, termed pHH/DR1.4.

Oquendo *et al.*, 1986; Gardner *et al.*, 1998; Bowman *et al.*, 1999; Figueiredo *et al.*, 2000; Scherf *et al.*, 2001). Interestingly, Rep20 appears to be unique to *P. falciparum* (Figueiredo *et al.*, 2000). Although no function has been assigned to this sequence, it is likely that Rep20 is not essential to replication since the complete subtelomeric array is readily lost from some chromosome ends in *in vitro* cultured parasites, without obvious impairment to blood-stage viability (Van der Ploeg *et al.*, 1985; Corcoran *et al.*, 1986; Corcoran *et al.*, 1988; Patarapotikul and Langsley, 1988; Pologe and Ravetch, 1988). Here, we show that Rep20-containing plasmids are more efficiently segregated between daughter merozoites and that this property leads to the rapid establishment of drug-resistant populations post-transfection. We demonstrate that the improved segregation is due to the physical tethering of these plasmids to terminal chromosome clusters, implicating a role for Rep20 in the formation and/or stabilization of these clusters.

Results

A genomic library screen in *P. falciparum* selects for plasmids containing Rep20

A random *P. falciparum* (D10 line) genomic library, termed pHH/DRlib, was screened in *P. falciparum* to select for sequences that allowed improved episomal plasmid maintenance (Figure 1). By direct transfection into *Escherichia coli*, it was evident that >80% of plasmids in the pHH/DRlib library possessed *P. falciparum* inserts of 0.5–2.0 kb. The same plasmid preparation was transfected

into *P. falciparum* (D10 line) on two separate occasions and subjected to selection with the antifolate WR99210. Plasmids recovered from the first transfection (Pf #1) contained inserts of varying sizes, indicating that no specific sequence had been selected (Figure 1). In contrast, most plasmids (~90%) recovered from the second transfection (Pf #2) released a 2.6 kb *Bam*HI–*Not*I fragment which indicated the presence of a 1.4 kb *P. falciparum* insert. These plasmids were recovered from genomic DNA prepared from parasites that had been cultured for 4 weeks after transfection. Plasmids recovered from later cultures of Pf #2 all contained the Rep20 insert (data not shown). Nucleotide sequencing of the insert released from one of these plasmids (termed pHH/DR1.4) revealed that it was entirely comprised of Rep20 repeats with the following consensus TAAGACCTA(T/A)(G/A)TTAGT(G/T)A(A/T)(A/C/G)(G/T). Southern blot analysis of these gels confirmed that all plasmids recovered from the Pf #2 population possessed the same 1.4 kb Rep20 insert (Figure 1). In contrast, pHH/DRlib plasmids recovered directly in *E. coli* and those recovered from the Pf #1 parasite population did not hybridize with the Rep20 probe, indicating that the Rep20⁺ plasmid was not a dominant representative of the parent library (Figure 1, lower left and centre panels).

Rep20 leads to improved episomal maintenance by promoting efficient plasmid segregation between daughter merozoites

The predominant recovery of a Rep20⁺ plasmid following transfection with pHH/DRlib suggested that parasites

Fig. 2. Rep20 allows rapid establishment of transfected *P.falciparum* lines. **(A)** Comparison of the ability of Rep20⁺ and parental Rep20⁻ plasmids (pHHΔE and pHH/DRlib) to establish drug-resistant populations. Rep20⁺ plasmids included derivatives of pHH/DR1.4 with the Rep20 sequence truncated as shown. Within each experiment an identical number of parasites from the same parasite population were transfected and the individual transformed populations were cultured under identical conditions. The number of days required for the cultures to reach 1% parasitaemia post-transfection is indicated. **(B)** Verification of individual transformed lines by recovery in *E.coli* of plasmids in *DpnI*-treated DNA. The restriction profile of plasmid DNA extracted from three randomly chosen *E.coli* colonies (designated 1, 2 and 3) was compared to that of the originally transformed plasmid (P). DNA markers in kb (M) are indicated on the left. ND, not determined. **(C)** Additional Rep20⁺ plasmids were derived by transfer of Rep20 sequences into the *NotI* (N) site of pHHC* to derive pHHC*/DR1.4 and pHHC*/DR0.28. Another Rep20⁺ plasmid was derived by insertion of a 0.5 kb Rep20 sequence from a different *P.falciparum* line (3D7) into pHHMC* to derive pHHMC*/3R0.5. These Rep20⁺ plasmids and their parental Rep20⁻ controls were analyzed for their ability to establish drug-resistant parasite lines as described above. **(D)** Verification of individual transfected lines derived by plasmid recovery in *E.coli* as described above.

transformed with this plasmid had a survival advantage under drug selection that was mediated by Rep20. This was investigated further by directly comparing the time taken for drug-resistant parasite populations to be established following transfection with various plasmids, as defined by the time taken to reach 1% parasitaemia. Parasites transfected in parallel with pHH/DR1.4, or with derivatives of this plasmid that contained truncated Rep20 sequences, consistently established drug-resistant populations 13–15 days after transfection (Figure 2A and B). In contrast, parasites possessing parental Rep20⁻ plasmids were slower to establish drug-resistant populations at 20–30 days. This approximates the normal rate at which transformants are derived in *P.falciparum* (Crabb and Cowman, 1996; Wu *et al.*, 1996; Crabb *et al.*, 1997b).

In order to determine whether Rep20 alone was responsible for the rapid establishment of drug-resistant lines, full-length (1.4 kb) and truncated (0.28 kb) forms of the originally selected Rep20 sequence were amplified by PCR and transferred into the *NotI* site of the plasmid pHHC*, a derivative of pHH1 (Reed *et al.*, 2000) containing the bacterial CAT gene, to derive pHHC*/DR1.4 and pHHC*/DR0.28, respectively. These Rep20⁺ plasmids also conferred a similar growth advantage to

P.falciparum parasites with the establishment of drug-resistant populations occurring 7–10 days before parasites transformed with the parental Rep20⁻ plasmid (Figure 2C and D).

To investigate whether other Rep20 sequences are able to confer this property, a specific 509 bp Rep20 sequence was amplified from chromosome 3 of the 3D7 parasite line and inserted between the two expression cassettes of the plasmid pHHMC*, a slightly modified version of pHHC* that possesses a minimized *HSP86* 5' region, to derive pHHMC*/3R0.5. Once again, this Rep20⁺ plasmid conferred a growth advantage relative to the Rep20⁻ control, following transformation and drug selection (Figure 2C and D). Taken together, these results indicate that it is the presence of Rep20 sequence in plasmids *per se* that allows rapid establishment of drug-resistant populations, and that as few as 13 copies of the 21 bp repeat are adequate to confer this property. It is of interest that drug-resistant parasites transfected with Rep20⁺ plasmids were consistently observed by thin blood smears as early as 7–9 days post-transfection, generally 1–2 weeks before such parasites were observed with Rep20⁻ plasmids.

Using a limiting dilution based efficiency assay we calculated that transfection efficiencies of the original

library pHH/DRlib and of the Rep20⁺ plasmid pHH/DR1.4 were very similar at 0.8×10^{-6} and 1.3×10^{-6} , respectively. Despite little difference in overall transfection efficiency, cultures in the individual microtiter plate wells containing parasites transfected with pHH/DR1.4 reached 1–5% parasitemia from a single transformed parasite in ~15 days, 1–2 weeks before the corresponding wells containing pHH/DRlib transfectants. Hence, although transfection efficiency was not markedly different between Rep20⁺ and Rep20⁻ plasmids, parasites containing Rep20 have an apparent growth advantage in the presence of the selection agent.

We have shown previously, using sensitivity to γ -irradiation and other approaches, that transfected plasmids are replicated as circular concatamers, primarily as double-stranded 3mers, multiple copies of which are present in each parasite (O'Donnell *et al.*, 2001). At least some of these concatamers appear to be linked by material that includes single-stranded DNA and are most likely the product of replication by a rolling circle mechanism. It was evident that both parasite-replicated Rep20⁺ (pHHC*/DR1.4) and Rep20⁻ (pHHC*) plasmids have an identical sensitivity to increasing doses of γ -irradiation, consistent with the presence of the concatameric structures described above (data not shown). Hence, the inclusion of Rep20 in a plasmid does not appear to have altered the mechanism by which it is replicated in parasites.

To determine whether Rep20⁺ (pHHC*/DR1.4) and Rep20⁻ (pHHC*) plasmids are segregated differently, the rate of plasmid loss following removal of the selection agent WR99210 was measured in transformants containing each plasmid form. For this experiment, highly synchronized trophozoite forms of the parasites were first washed to remove residual drug from the cultures and then incubated in medium free of WR99210. DNA was prepared from these lines at different time points following removal of the drug and restricted with combinations of enzymes designed to generate plasmid-derived and endogenous fragments that can be quantitated following Southern blotting (Figure 3, inset). This process allows an estimation of relative plasmid copy number per parasite (Crabb *et al.*, 1997b; O'Donnell *et al.*, 2001). On the day of drug removal (day 0), both Rep20⁺ and Rep20⁻ plasmids were present in very similar copy numbers (Figure 3, inset). This was estimated to be ~10 copies/parasite genome. Following removal of drug, plasmid copies per parasite were reduced by half in 9 and 5 days for Rep20⁺ and Rep20⁻ transfectants, respectively (Figure 3). Further-more, after 8 days in the absence of drug, Rep20⁺ transfectants retained ~60% plasmid copies while plasmids were undetectable in Rep20⁻ transfectants at this time (Figure 3). A repeat copy number quantitation experiment from an independent transfection with these same plasmids revealed an almost identical delay in the rate of loss of Rep20⁺ plasmids (e.g. plasmid copies per parasite were reduced by half in 9 and 6 days for Rep20⁺ and Rep20⁻ transfectants, respectively, in this experiment) (data not shown). Hence, Rep20⁺ plasmids were retained far more effectively than Rep20⁻ plasmids in the absence of selection, consistent with these plasmids having an improved ability to segregate between daughter merozoites.

Fig. 3. Rep20⁺ plasmids show improved retention compared with Rep20⁻ plasmids in transfected *P.falciparum* parasites. Drug pressure was removed from synchronous parallel cultures of pHHC* (open squares) and pHHC*/DR1.4 (open circles) transfected lines at day 0. DNA was extracted at different time points from these cultures, treated overnight with *DpnI* before digestion with various enzyme combinations for Southern blot analysis. Relative plasmid copy number was determined at each time point by phosphorimager analysis of plasmid (P) and endogenous (E) hybridization signals on Southern blots from three different enzyme/probe combinations. The error bars represent the standard deviations from these three calculations. The inset shows a representative blot of *BglIII*-*NsiI*-*EcoRI*-restricted genomic DNA from day 0 and 21 cultures hybridized to a combined pGEM/MSP-1 probe. This illustrates the complete loss of plasmids from both transfected lines after 21 days of culture in the absence of drug.

In an alternative approach to investigate the efficiency of plasmid segregation, multinucleated schizont-stage parasites from Rep20⁺ and Rep20⁻ transformants were analyzed in parallel by fluorescence *in situ* hybridization (FISH) analysis for the presence of plasmid DNA. Almost all schizonts from Rep20⁺ transformants (101/104) possessed some detectable plasmid DNA, unlike schizonts from Rep20⁻ transformants where less than one-third (10/35) were plasmid positive. Representative examples of plasmid-positive schizonts are shown in Figure 4A. In these parasites, plasmid signal was detected far more often in the multiple individual nuclei of Rep20⁺ transformants (65%) than in nuclei of Rep20⁻ transformants (18.4%) (Figure 4B). It is unlikely that all plasmids are detected by FISH, as higher copy number plasmid multimers may be required to produce a signal. However, as there is no apparent difference in plasmid structure or copy number between Rep20⁺ and Rep20⁻ transformants, these data are consistent with the substantially improved ability of Rep20⁺ plasmids to segregate evenly during mitosis.

The tethering of Rep20⁺ plasmids to telomeric clusters explains their improved ability to segregate and reveals a function for Rep20

Physical tethering to chromosomes is one way in which efficient plasmid segregation is achieved by viral plasmids in mammalian cells (Harris *et al.*, 1985; Kirchmaier and Sugden, 1995; Lehman and Botchan, 1998; Ilves *et al.*, 1999) and by bacterial plasmids transformed into yeast (Longtine *et al.*, 1992; Enomoto *et al.*, 1994; Ansari and Gartenberg, 1997). It has been shown that *P.falciparum* chromosome ends are found in clusters at the nuclear periphery (Freitas-Junior *et al.*, 2000). As Rep20 is one of

six subtelomeric repeat sequences found in this region, we hypothesized that these arrays may mediate telomeric clustering and hence that Rep20⁺ plasmids may tether to these clusters via this same Rep20-mediated interaction. Using FISH analysis, we show that plasmid DNA, which generally appears as a single fluorescent spot in parasite nuclei, co-localizes with telomeric clusters (Figure 5). Forty-five nuclei from pHHC*/DR1.4 (Rep20⁺) transfectants and 50 nuclei from pHHC* (Rep20⁻) transfectants were analyzed for co-localization by two independent

slide readers. Plasmid–telomeric cluster co-localization was observed significantly more frequently in Rep20⁺ transformants ($73.3 \pm 7.3\%$) than in Rep20⁻ transformants ($32 \pm 2\%$). The relatively low level of plasmid–telomeric cluster co-localization observed with Rep20⁻ transformants is probably mostly due to random signal overlap, given that the signal for telomeric clusters has been calculated as occupying ~25% of the parasite nucleus. The appearance of the plasmid signal as a single spot is explained by the covalent association of the multiple plasmid copies present in each transfected parasite (O'Donnell *et al.*, 2001). These FISH slides were prepared from the same trophozoite-stage parasite population used to derive the day 0 DNA analyzed in Figure 3, confirming that the plasmids were maintained episomally and discounting the possibility that the plasmid–telomeric cluster co-localization is the result of plasmid integration in a telomeric location. The ability of Rep20⁺ plasmids to tether to chromosomes explains their improved ability to segregate between daughter merozoites, and provides insight into the biological function of Rep20 as shown diagrammatically in Figure 6.

Discussion

In this paper we have screened a genomic library in *P.falciparum* for sequences that improve the maintenance of plasmids in parasites. A plasmid containing a 1.4 kb fragment comprised entirely of Rep20 repeats was isolated in this screen and was subsequently shown to confer a selective advantage to transfected parasites maintained under drug selection. A Rep20⁺ plasmid was only isolated from one of the two selection experiments. It is likely that the low efficiency of the system (approximately one out of

Fig. 4. FISH analysis confirms that Rep20⁺ plasmids are segregated more efficiently than Rep20⁻ plasmids in transfected *P.falciparum* parasites. (A) Representative multinucleated schizonts from pHHC*/DR1.4 (Rep20⁺) and pHHC* (Rep20⁻) transfected populations by hybridization to a plasmid probe. (B) Quantitation of the number of plasmid-positive nuclei in schizonts that possessed at least one plasmid signal. The number of nuclei counted in each population is shown.

Fig. 5. Rep20⁺ plasmids physically associate with *P.falciparum* telomeric clusters. Smears of trophozoite-stage parasites from Rep20⁻ pHHC* (A) and Rep20⁺ pHHC*/DR1.4 (B) transfected populations, prepared from day 0 cultures shown in Figure 3, were analyzed by DAPI staining and by FISH using TARE 3 (Figueiredo *et al.*, 2000), and plasmid backbone probes used to detect telomeric clusters and transfected plasmid, respectively. The two rows in each panel show two different representative fields.

Fig. 6. Proposed model demonstrating a mechanism by which Rep20 mediates plasmid tethering and plays a role in telomeric cluster formation. The subtelomeric region of *P.falciparum* chromosomes extends ~60 kb from the telomere and is comprised of six different non-coding TAREs and a coding region containing virulence-associated genes that are organized in a conserved arrangement on different chromosomes. The physical association of Rep20-containing plasmids (which exist as covalently linked concatamers; O'Donnell *et al.*, 2001) with telomeric clusters demonstrated in this paper presumably occurs via an interaction with Rep20-binding proteins. These proteins, and perhaps others that bind to different TAREs (question marks), would cross-link the subtelomeric regions of *P.falciparum* chromosomes and promote the formation/stabilization of the cluster. This alignment of chromosome ends favors ectopic recombination and hence the generation of diversity in the neighboring virulence genes, most particularly *var* (Freitas-Junior *et al.*, 2000). Truncated chromosomes that have spontaneously lost their subtelomeric sequences, but not their telomere tracts, which are in fact amplified, do not associate with telomeric clusters, consistent with a role for TAREs in cluster stabilization (Scherf *et al.*, 2001; Figueiredo *et al.*, 2002). These truncated chromosomes remain anchored to the nuclear membrane (NM) by a different mechanism that probably resembles peripheral nuclear membrane tethering seen in yeast (Tham and Zakian, 2000; Scherf *et al.*, 2001).

a million parasites is stably transformed) only allows for 100–400 independent transformation events per transfection (which is usually of $1\text{--}4 \times 10^8$ parasites). We estimate that $<1/200$ of the plasmids in our library contains a Rep20 insert, therefore it is not surprising that a Rep20⁺ plasmid was only isolated in one of the two library screens.

Evidence that Rep20 alone confers this property of improved episomal maintenance was obtained by the introduction of different Rep20 fragments, including one from a different parasite line (3D7) to that used in the transfection (D10), into various transfection plasmids. In each case, parasites containing Rep20⁺ plasmids were maintained far more efficiently than their parental controls. Hence, the inclusion of Rep20 sequences in plasmids provides a powerful new transfection tool that leads to the rapid establishment of transformed *P.falciparum* populations. The lack of a robust transfection system is currently a severe impediment to the functional analysis of *P.falciparum* genes, the sequences of most of which have recently been determined via the Malaria Genome Project (Gardner *et al.*, 1998; Bowman *et al.*, 1999; <http://plasmodb.org/PlasmoDB.shtml>). Such a tool should facilitate functional genomic studies, particularly those involving complementation and transgene expression. It remains to be determined whether the inclusion of Rep20

sequences in transfection plasmids can be used to facilitate gene targeting. Because of the considerable degeneracy of Rep20 repeats, it is unlikely that integration into subtelomeric Rep20 regions will occur if a Rep20 element from a different parasite line to that used for transformation is used in the transfection vector. However, it is possible that Rep20-mediated plasmid tethering will interfere with homologous integration.

Interestingly, the advantage conferred by Rep20 is not related to a dramatic improvement in the efficiency by which parasites are stably transformed. We measured the efficiency of transfection with Rep20[−] and Rep20⁺ plasmids to be 0.8×10^{-6} and 1.3×10^{-6} , respectively. This is the first time that stable (drug-resistant) transformation efficiency has been formally calculated in malaria parasites, data that confirmed the prevailing view from indirect evidence of the very low efficiency of the system. While the number of parasites transfected was unaltered in Rep20⁺ and Rep20[−] transformants, we showed by two different approaches that the efficient maintenance of Rep20⁺ plasmids in transfected parasites is due to an improved ability of plasmids to segregate during mitosis.

Furthermore, we show that Rep20⁺ plasmids co-localize with telomere clusters whereas Rep20[−] plasmids do not. Hence, it appears that Rep20 mediates an interaction with

the telomere cluster and that this chromosomal tethering facilitates segregation between daughter merozoites (Figure 6). This finding implicates a direct role for Rep20 in the formation and/or stabilization of chromosomal telomere clusters. This process of telomeric clustering is thought to promote recombination between the virulence genes, most particularly the *var* genes, located on the ends of heterologous chromosomes (Freitas-Junior *et al.*, 2000). Telomere clustering is distinct from nuclear periphery tethering, a process that does occur in *P.falciparum* (Scherf *et al.*, 2001) and which is well described in yeast (Figure 6). Our data support and extend a recent study from the Scherf laboratory that examined the localization and arrangement of naturally truncated chromosome ends in *P.falciparum*. These authors concluded that subtelomeric sequences are crucial to telomere cluster formation but are not involved in nuclear peripheral tethering (Figueiredo *et al.*, 2002) (Figure 6). While our data highlight a role for Rep20 in cluster formation, they do not preclude a similar role for other TAREs. However, it is significant that plasmids containing other TAREs were not isolated in either of the two genetic screens performed here. Moreover, we have explored the potential of one of these elements, TARE 3 (also known as R-CG7), to mediate enhanced plasmid segregation via chromosomal tethering. One or two copies of this 700 bp element in transfection plasmids did not improve transfection efficiency over the parental control plasmid (data not shown). Hence, it appears unlikely that this sequence on its own mediates chromosomal tethering.

The minimum number of Rep20 repeats required to confer the improved segregation phenotype was not defined here; however, we do show that Rep20 fragments of between 0.28 and 1.4 kb (encompassing 13–66 individual repeats) were sufficient in this regard. Although this appears to be somewhat less than the number of Rep20 repeats found normally in the subtelomeric region of *P.falciparum* chromosomes (estimated to be 250–1000 per chromosome end), it should be recognized that this is not necessarily an accurate estimate of the number of repeats required for chromosomal interaction. This is because plasmids replicate in *P.falciparum* as large concatamers and hence the actual number of plasmid-encoded Rep20 repeats available for interaction with the chromosomes is considerably higher than that present in the monomeric plasmid.

The observation that a subtelomeric sequence alone can enhance plasmid segregation and mediate chromosomal interactions is a novel finding. Although it has been shown that telomere (and centromere) repeats enhance plasmid segregation in yeast (something that would be interesting to test in *P.falciparum* transfection vectors) and that the addition of subtelomeric sequences to telomere-containing plasmids can enhance this property (Longtine *et al.*, 1992; Enomoto *et al.*, 1994; Ansari and Gartenberg, 1997), it has not been demonstrated previously in any system that subtelomeric elements alone can mediate this effect. Considering also that Rep20 is a unique repeat element not present in yeast or identified in any other eukaryote, it is likely that the mechanism of Rep20-mediated plasmid segregation and telomere cluster formation involves distinct molecular interactions.

It is now of interest to identify and characterize the Rep20-binding protein(s) that presumably mediate this process. Such studies are likely to provide considerable insight into the mechanisms that underpin the generation of diverse antigenic and adhesive phenotypes in *P.falciparum*; events that are central to the pathogenesis of *falciparum* malaria (Chen *et al.*, 2000; Craig and Scherf, 2001). It remains possible that the process of Rep20-mediated telomere cluster formation is of a unique type even in the plasmodia, and is a key reason why *P.falciparum* is more virulent than other human malaria parasites.

Materials and methods

Genomic library and plasmid construction

Plasmodium falciparum strain D10 gDNA was digested with *RsaI* and fragments (generally 0.5–3.0 kb) were purified using the BANDPURE™ DNA purification kit (Progen) and ligated into the *EcoRV* site of pHH1 (Reed *et al.*, 2000). *EcoRV* digestion of pHH1 removes a fragment that encodes 476 bp of the 5′ end of *Plasmodium berghei* DHFR-TS 3′ UTR, the entire HSP86 5′ UTR and 192 bp of the 5′ end of calmodulin 5′ UTR from pHH1. A functional hDHFR selection cassette driven by a shortened but still fully functional calmodulin promoter (Crabb and Cowman, 1996) and a small fragment (230 bp) of the *P.berghei* DHFR-TS 3′ UTR remain in the *EcoRV*-digested pHH1. The ligation was electroporated into *E.coli* and ~7000 colonies were pooled and cultured overnight before CsCl purification of DNA to generate the library pHH/DRlib.

The Rep20 sequence isolated from the stable genomic library contained restriction enzyme sites utilized in reducing the size of this element. pHH/DR1.4 is the parent 1.4 kb insert isolated from parasites transfected with the genomic library pHH/DRlib. pHH/DR0.82 was constructed by *BglII* digestion and re-ligation of pHH/DR1.4, releasing 588 bp of Rep20 sequence from within the insert and leaving 820 bp. pHH/DR0.28 involved *BglII* and *AflIII* digestion of pHH/DR0.82 to release a further 542 bp, followed by treatment with Klenow DNA polymerase and re-ligation, resulting in a 283 bp Rep20 sequence. To insert both the full-length 1.4 kb and shortened 283 bp Rep20 sequences into a second transfection vector, the sequence was amplified from both pHH/DR1.4 and pHH/DR0.28 with primers flanking the insert sequence: libF, 5′-AGATCTGCGGCCGCTCATTTACCTGCTAATACGATTGC-3′; and libR, 5′-AGATCTGCGGCCGCAAGTATATATTTTGTTC-ATAAATTGATATC-3′. The product was ligated into pGEM®-T Easy (Promega), released with *NotI* and inserted into pHHC*. The vector pHHC* is pHH1 with an enzymatically inactive chloramphenicol acetyl transferase (CAT) gene in the *XhoI* site. The resultant plasmids have been designated pHHC*/DR1.4 and pHHC*/DR0.28. The *P.falciparum* 3D7 Rep20 plasmid (pHHMC*/3R0.5) included 509 bp of Rep20 sequence from chromosome 3 of *P.falciparum* strain 3D7 amplified with REP20F (5′-GGACCAGATCTCCTAACATTAGTATAGTCTG-3′) and REP20R (5′-GGACCCCGCGTCTATTTGTTAAATTAAGACCA-3′) inserted between the *SacII* and *BglIII* sites of the vector pHHC*, i.e. between the two expression cassettes.

Parasite culture and transfection procedures

Plasmodium falciparum line D10 was cultivated and synchronized as per standard procedures (Trager and Jensen, 1976; Lambros and Vanderberg, 1979). Ring-stage parasites (~5% parasitemia) were transfected with 80 µg of CsCl-purified plasmid DNA unless specified otherwise, as described previously (Crabb and Cowman, 1996) using modified electroporation conditions (Fidock and Welles, 1997). Parasites were cultured in a 5 cm dish for 48 h, prior to the addition of 2.5 nM WR99210. Fresh media and WR99210 were added to cultures for a further 48 h, then every 4 days until parasite establishment.

Nucleic acid analysis

gDNA was extracted from mixed trophozoite/schizont stage parasites as described (Coppel *et al.*, 1987). Manipulation of recombinant DNA and analysis of nucleic acids by Southern blot hybridization were carried out using standard procedures (Sambrook *et al.*, 1989). DNA was labeled with [α -³²P]dATP via the Bresatec DNA Labelling Kit as per the manufacturer's instructions. Unincorporated nucleotides were removed using ProbeQuant™ G-50 Micro Columns (Amersham). For plasmid

recovery from transfected parasites, gDNA extracted from transfected parasites was treated with *DpnI* overnight to destroy any of the originally transfected *E.coli*-derived plasmid that may still be in the preparation. Following transformation into *E.coli*, DNA was prepared from individual colonies for restriction analysis and electrophoresis.

Transfection efficiency assay

pHH/DRlib and pHH/DR1.4 plasmid DNA (100 µg each) were transfected in parallel into 2×10^8 ring-stage parasites that had been synchronized twice 4 h apart. Following 24 h culture in a 5 cm dish, transfectants were plated out into 96-well trays at 1/10, 1/100 and 1/1000 dilution in a 2% hematocrit. At 48 h post-transfection, 2.5 nM WR99210 was added to all wells and cultures were fed fresh media each day for the next 3 days, and every 5 days thereafter. Positive wells first detected via a color change in the media (Kirkman *et al.*, 1996) were confirmed by thin blood smear. Plates that contained <30% positive wells were used to calculate transformation efficiencies.

FISH

FISH analysis was performed essentially as described previously (Freitas-Junior *et al.*, 2000) using the biotinylated telomeric repeat TARE3 (Figueiredo *et al.*, 2000) and fluoresceinated plasmid pHH2 (Waller *et al.*, 2000) as probes.

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