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

Rebecca A.O’Donnell1,2, Lucio H.Freitas-Junior3, Peter R.Preiser4, Donald H.Williamson4, Manoj Duraisingham1, Terry F.McElwain1,2,6, Artur Scherf7, Alan F.Cowman1 and Brendan S.Crabbe1,6

1The Walter & Eliza Hall Institute of Medical Research, Victoria 3050, 2Department of Microbiology & Immunology and the Cooperative Research Centre for Vaccine Technology, The University of Melbourne, Victoria 3010, Australia, 3Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, F-75724 Paris Cedex 15, France, 4National Institute of Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK and 5Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA

Corresponding author
E-mail: crabbe@wehi.edu.au

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 plasmid. 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 usually AT-rich P.falciparum genome is haploid and consists of 14 linear chromosomes varying in size from 0.7 to 3.4 Mbp. 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 predominately 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;
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 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 pHHDRIlib, 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 pHHDRIlib 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 (*Pa* #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 (*Pa* #2) released a 2.6 kb BamHI–NotI 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 *Pa* #2 all contained the Rep20 insert (data not shown). Nucleotide sequencing of the insert released from one of these plasmids (termed pHHDRIlib) 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 *Pa* #2 population possessed the same 1.4 kb Rep20 insert (Figure 1). In contrast, pHHDRIlib plasmids recovered directly in *E. coli* and those recovered from the *Pa* #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 pHHDRIlib suggested that parasites
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% parasitemia. 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 transfected into the Norl 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 pHMC*, a slightly modified version of pHHC* that possesses a minimized HSP86 5' region, to derive pHMC*/DR0.5. 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.

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; Ives 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 ± 7.3%) than in Rep20− transformants (32 ± 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. 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 1–4 × 10⁸ parasites). We estimate that <1/200 of the plasmids in our library contains a Rep20 insert, therefore it is not surprising that a Rep20⁰ 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 Rep20⁰ 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 Rep20⁰ and Rep20⁰ 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 Rep20⁰ and Rep20⁰ transformants, we showed by two different approaches that the efficient maintenance of Rep20⁰ plasmids in transfected parasites is due to an improved ability of plasmids to segregate during mitosis.

Furthermore, we show that Rep20⁰ plasmids co-localize with telomere clusters whereas Rep20⁰ 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 monomorphic 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 pPH1 (Reed et al., 2000). *EcoRV* digestion of pPH1 removes a fragment that encodes 476 bp of the 5’ end of *Plasmodium berghei* DHFR-TS 3’ UTR and the entire HISP6 5’ UTR and 192 bp of the 5’ end of calmodulin 5’ UTR from pPH1. 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 pPH1. The ligation was electroporated into *E. coli* and ~7000 colonies were plated and cultured overnight before CsCl purification of DNA to generate the library pPH/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 pPH/DRlib. pHH/DR0.82 was constructed by BgII 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 BgII and *AflII* digestion of pPH/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’-AGATTCGGCGCCGATTTACCTTCTGATATCGACC-3’; and libR, 5’-AGATCGCGCGCCGAGATATATTTTTTCTTATATTGATATC-3’. The product was ligated into pGEM®-T Easy (Promega), released with NotI and inserted into pHHC*. The vector plasmid is pHHC* with an enzymatically inactive chloramphenicol acetyltransferase (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 (pPHMC*/3R0.5) included 509 bp of Rep20 sequence from chromosome 3 of *P. falciparum* strain 3D7 amplified with REP20F (5’-GGACAGATCTTCTAAATTAGTTGAGGCTGCT-3’) and REP20R (5’-GGACCCGGGCTGTATATTGGTATATAAGACCA-3’) inserted between the *SacII* and *BglII* sites of the vector pHMC*, 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 Wellems, 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 (Sambrueck et al., 1990). DNA was labeled with [α-32P]dATP via the Bresatec DNA Labelling Kit as per the manufacturer’s instructions. Unincorporated nucleotides were removed using ProQuant™ G-50 Micro Columns (Amersham). For plasmid
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