A CHROMOSOME 9 DELETION IN *PLASMODIUM FALCIPARUM* RESULTS IN LOSS OF CYTOADHERENCE

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Many lines of Plasmodium falciparum *undergo a deletion of the right end of chromosome 9 during in vitro culture accompanied by loss of cytoadherence and gametocytogenesis. Selection of cytoadherent cells from a mixed population co-selects for those with an undeleted chromosome 9 and the selected cells produce gametocytes. The deletion also results in loss of expression of PfEMP1, the putative cytoadherence ligand, suggesting that PfEMP1 or a regulatory gene controlling PfEMP1 expression and gametocytogenesis may be encoded in this region. We have isolated several markers for the deleted region and are currently using a YAC-P.* falciparum *library to investigate this region of the genome in detail.*

Key words: chromosome deletion – cytoadherence – *Plasmodium falciparum* – pathology – PfEMP1

The ability of asexual stages of *Plasmodium falciparum* to cytoadhere to the endothelial lining of capillaries and venules of various tissues is responsible for the severe symptoms of falciparum malaria (Macpherson et al., 1985; Langreth & Peterson, 1985; Aikawa, 1988), including cerebral malaria. Continuous *in vitro* culture of *P. falciparum* frequently results in irreversible loss of cytoadherence (Udeinya et al., 1983) and gametocytogenesis (Alano & Carter, 1990). We wondered whether this irreversible loss might result from subtelomeric chromosomal deletions as these deletions occur frequently during continuous *in vitro* culture of *P. falciparum* and account for the irreversible loss of a number of functional genes (Corcoran et al., 1986; Pologe & Ravetch, 1986; Kemp et al., 1987; Patarapotikul & Langsley,

1988; Biggs et al., 1989; Ravetch, 1989). For example subtelomeric deletions of chromosome 2 result in loss of expression of the gene encoding the knob-associated histidine-rich protein (KAHRP) (Pologe & Ravetch, 1986; Biggs et al., 1989; Ravetch, 1989). Parasites with KAHRP deletions do not produce surface membrane deformations called "knobs" which are possible points of attachment between the parasitized erythrocyte and vascular endothelial cells during asexual cytoadherence. Knobless parasites are generally unable to cytoadhere *in vivo* and *in vitro* although some knobless cloned lines with KAHRP deletions can retain cytoadherence (Udomsangpetch et al., 1989; Biggs et al., 1990) and continue to express the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a putative parasite cytoadherence ligand (Biggs et al., 1990). Consequently loci other than those involved in KAHRP deletions are required for expression of asexual cytoadherence and PfEMP1. The property of gametocytogenesis is also readily lost during *in vitro* culture of *P. falciparum* but to date no subtelomeric deletions have been linked to expression of this phenotype (Alano & Carter 1990). Independent clones and isolates of *P. falciparum* also commonly undergo subtelemeric deletions of chromosome 9 and these have similar breakpoints (Shirley et al., 1990).

We report here that subtelomeric deletions of chromosome 9 of *P. falciparum* are corre-

lated with loss of expression of PfEMP1 and loss of expression of the cytoadherent, gamete-producing phenotype.

MATERIALS AND METHODS

PFGE – Samples were electrophoresed for 70 h at 140V using a pulse time of 225s. Chromosomes were prepared from isolates cultured *in vitro* (Forsyth et al., 1989), harvested when cultures contained 8-10% healthy trophozoites and processed as described by Shirley et al. (1990).

Cytoadherence – At the time of preparation of chromosomes for PFGE, isolates and clones were examined for ability of asexual stages to cytoadhere to C32 melanoma cells as described by Biggs et al. (1990).

^{125}I *Surface labelling* – Suspensions of infected erythrocytes containing trophozoites were ^{125}I radiolabelled using the lactoperoxidase technique Triton X-100 and SDS extracted as previously described (Leech et al., 1984).

Gametocyte production – Isolates and clones were examined for gametocyte production using standard gametocyte culture conditions. Cultures were initiated at 5% haematocrit and 0.5% ring stage parasitaemia. Cells were cultured for gametocytes in O,Rh$^+$ human erythrocytes and RPMI-HEPES (Forsyth et al., 1989) supplemented with 50µg/ml hypoxanthine and 10% human serum in an atmosphere of 5% CO_2. Gametocytogenesis results are expressed as the number of gametocytes (G) produced per 100 infected red blood cells in a Giemsa-stained blood film taken 20 days after establishing gametocyte cultures.

RESULTS

To determine whether loss of expression of asexual cytoadherence and/or gametocytogenesis related to size changes in chromosome 9 we examined karyotype changes in field isolates from Papua New Guinea (Forsyth et al., 1989) during the first four to six weeks of adaptation *in vitro*. Fig. 1A shows PFGE analysis of chromosome 9 in early passages of the recent field isolate designated 1776 and cloned lines derived from it. The full length chromosome 9 was present in isolate 1776 but this was rapidly overtaken in culture by a population with the smaller form. Hence all the clones

derived from it (B9, E10, G11 and C10) had only the short form. Recent isolates 1934, 1904, 1775, 1933, 1935 and the clone 3D7 showed two populations of parasites differing in size of chromosome 9 (Day et al., unpublished observation). The subtelomeric deletions observed in chromosome 9 occurred frequently among different isolates and parasites with this deletion were rapidly selected during adaptation to culture. Chromosome 9 deletions were much more frequent than chromosome 2 deletions (data not shown).

Recent isolates were screened for expression of gametocytogenesis and cytoadherence phenotypes at the time of preparation of chromosomes for PFGE analysis. The presence of the large form of chromosome 9 in an isolate always correlated with a high number of cytoadherent cells i.e. > 240 infected red blood cells (IRBC)/100 C32 melanoma cells (C32MC) and > 26% conversion to gametocytes. Recent isolates 1934 and 1904, which had predominantly the small form of chromosome 9, had few cytoadherent cells (< 40 IRBC/100 C32MC) and low (< 10 gametocytes (G)/100 IRBC) or zero gametocyte production (Day et al., unpublished observations). The correlation between size of chromosome 9 and expression of cytoadherence and gametocytogenesis was also examined in three cloned lines of *P. falciparum* i.e. 3D7, C10 and B9 (data not shown). Many cytoadherent cells and gametocytes were observed in 3D7 (302 IRBC/100 C32MC) which had a large size chromosome 9. The cloned lines B9 and C10, derived from isolate 1776, had small forms of chromosome 9 (Fig. 1A) and few B9 or no C10 cytoadherent cells and zero gametocyte production.

Previously (Shirley et al., 1990) we showed in a limited number of isolates that the small form of chromosome 9 was generated from the large form by a subtelomeric deletion in the right end of chromosome 9 as well as a much smaller deletion in the left end as shown in Fig. 1B. To verify that similar deletions resulted in the size changes found during adaptation of field isolates to *in vitro* culture, two of these recent isolates, C10 and B9 with the small form of chromosome 9, were mapped. The major deleted region of chromosome 9 in recent isolates was located at the right end of chromosome 9.

To demonstrate that the subtelomeric deletion in the right end of chromosome 9 was
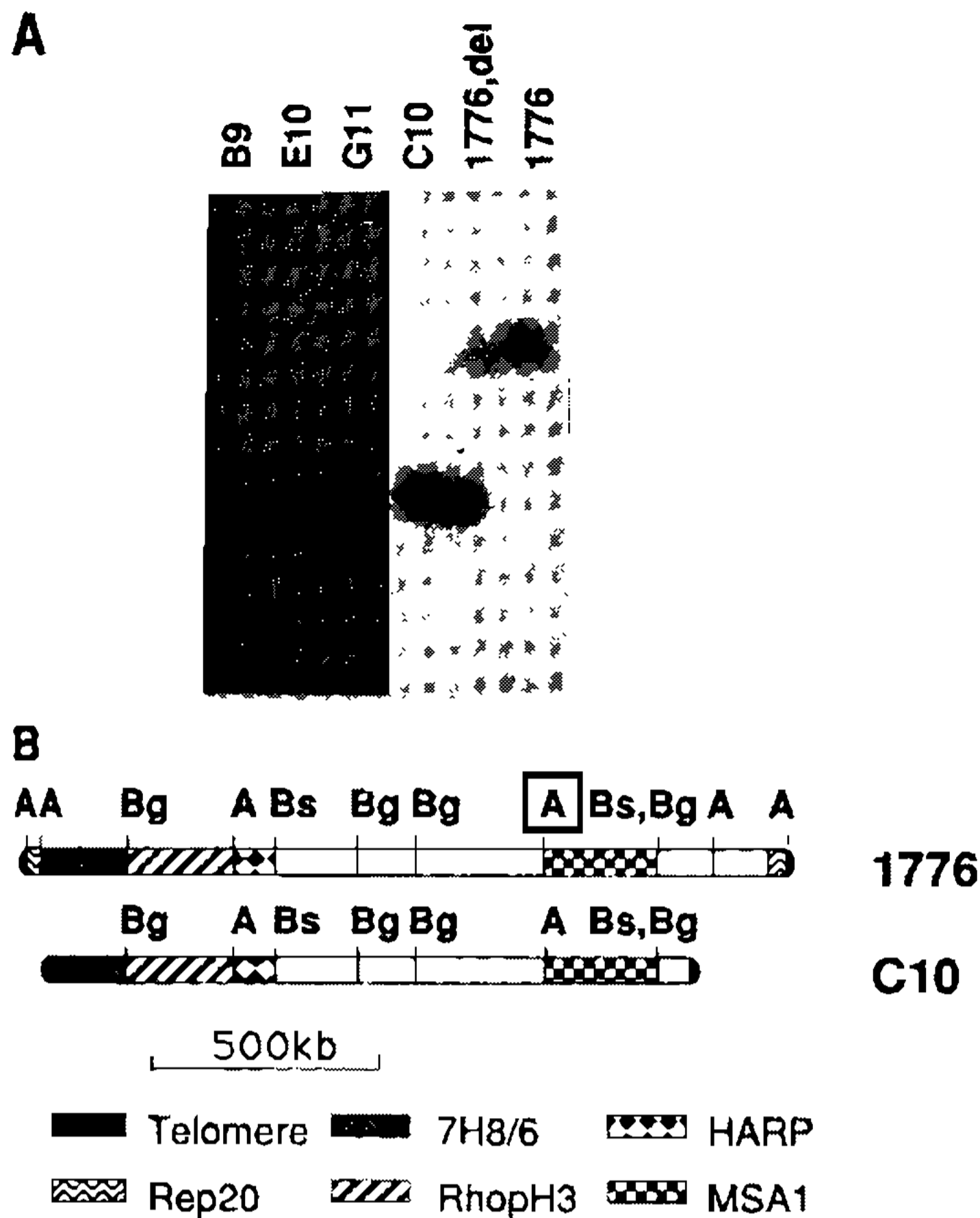
A

B

Fig. 1: PFGE of recent Papua New Guinean isolate of *Plasmodium falciparum* designated 1776 and uncloned (1766 del, a line in which most cells have undergone the chromosome 9 deletions) and cloned lines B9, E10, G11 and C10 derived from this. A: hybridization with a chromosome 9 specific probe demonstrated size variation in chromosome 9. B: maps of chromosome 9 from isolate 1776 and the clone C10 derived from this. The maps from Shirley et al. (1990) have been revised. The presence of additional Bgl or Bssh sites near the telomeres cannot be excluded. Apal (A), Bgl (B) and Bssh2 (Bs) are indicated together with the mankers shown. The presence of the 7H8/6 sequences is inferred from studies on other isolates.
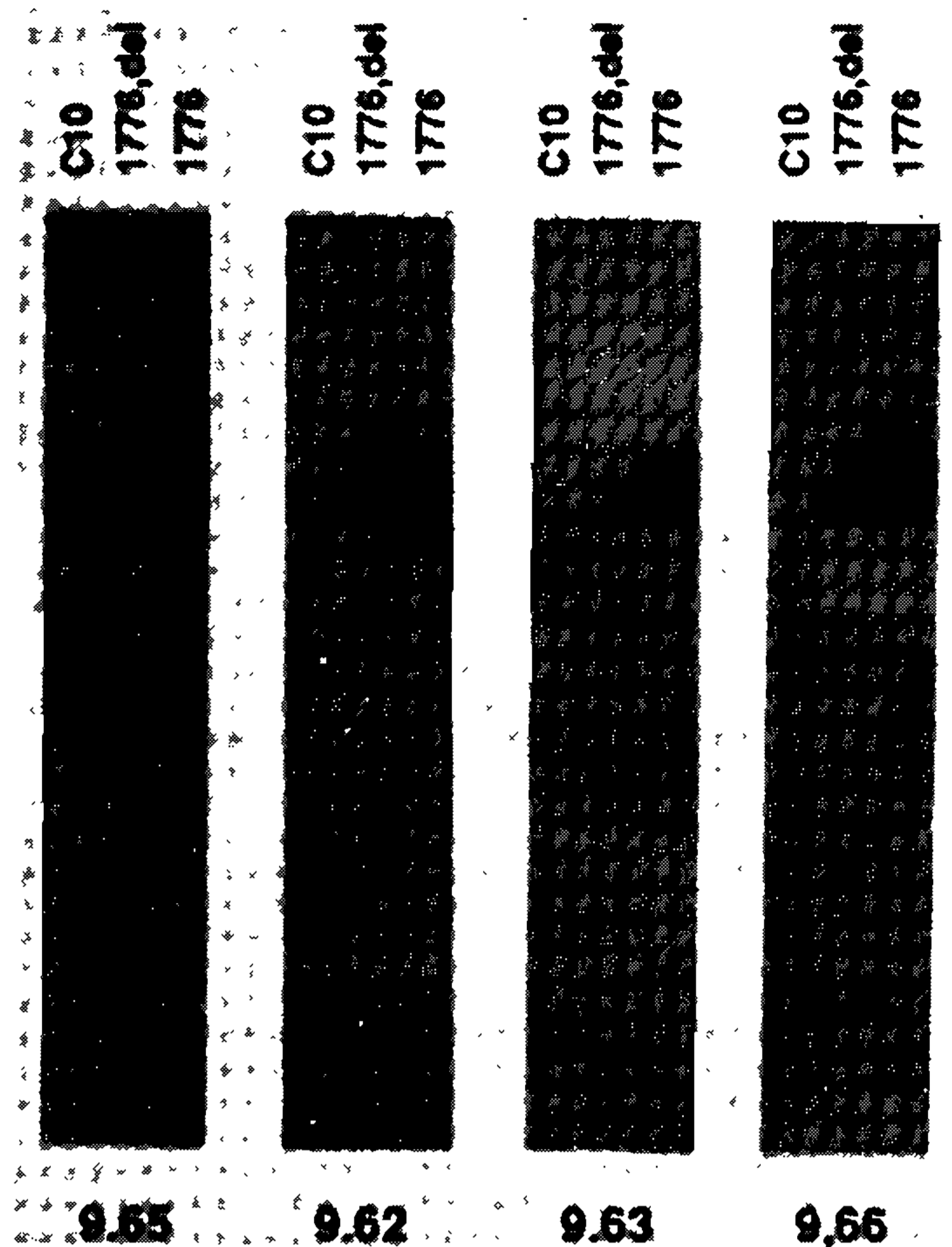

Fig. 3: markers for the deletion on chromosome 9. Chromosomes from clone C10 (deleted at both ends of chromosome 9), 1776 del and 1776 (full length chromosome 9) were fractioneted by PFG, transferred to a Hybond filter and hybridized with the cloned fragments shown (9.62-9.66). Fragment 9.65 hybridized to both the small and large forms of chromosome 9, while the other fragments hybridized only to the large forms, in both 1776 del and 1776.

Fig. 2: immunoprecipitation analysis of surface radioiodinated proteins of HB3 and HB3-Sel4 reacted with hyperimmune sera (Ab1 and Ab2) from adult Papua New Guineans resident in a malaria endemic area and with normal human serum (NHS). Molecular weight markers are shown in the track labelled M and the SDS extract of the Triton X-100 insoluble pellet is shown in the track labelled SE.

linked to expression of cytoadherence and gametocytogenesis we repeatedly selected for cytoadherence cells by binding to C32MC. After each selection the population of binding cells was expanded, karyotyped by PFGE and phenotyped. Passages of the line HB3 and the isolate 1934 which had predominantly small forms of chromosome 9 and were low in expression of both cytoadherence (< 20 IRBC/ 100 C32MC) and gametocytogenesis (< 10 G/ 100 IRBC) were used for this selection experiment. In addition the clone of parent isolate 1776 designated C10 which did not cytoadhere nor produce gametocytes and had a small form of chromosome 9 was also selected.

Cytoadherent cells were successfully selected from HB3 and 1934 indicating that these passages originally contained mixed populations of binding and non-binding cells. In the case of HB3 selection of cytoadherent cells greatly enriched for populations of cells with a large form of chromosome 9 (Day et al., unpublished observations). HB3 and the lines selected from it all showed the same unique polymorphism of the ApaI site boxed in Fig. 1 and so are clearly derived from the same parental clone. Hence we can be confident that these selected lines were not simply contaminated with another cell line during selection. Expression of both the cytoadherence phenotype and an isolate-specific agglutinogen have been associated with the presence of a trypsin sensitive high molecular weight antigen on the surface of trophozoite infected cells (Marsh & Howard, 1986). This molecule designated PfEMP1, which can be radioiodinated and immunoprecipitated by hyperimmune sera, was found to be present on the surface of trophozoite-infected cells of parasites bearing the large form of chromosome 9 (HB3-se14 and 1776) but not those with the deleted form of chromosome 9 (HB3 and C10) (Fig. 2).

The original and selected lines were also examined for gametocyte production (data not shown). HB3-se14 showed 51% conversion of infected cells to gametocytes after 20 days in culture under conditions known to induce gametocytogenesis whereas unselected HB3 produced very few gametocytes under identical culture conditions.

Isolation of YAC clones corresponding to the deletion on chromosome 9 – In order to isolate large DNA segments corresponding to the region deleted from chromosome 9 we screened a Yeast Artificial Chromosome (YAC)

library of *P. falciparum* DNA segments (Triglia & Kemp, 1991) with anonymous DNA probes (J.T., unpublished observations) corresponding to the deletion. Fig. 3 shows that probes derived from the anonymous clones hybridized to the undeleted form of chromosome 9 in isolate 1776 and to the large form of chromosome 9 in isolate 1776, del, but not to the deleted form of chromosome 9 in clone C10 derived from 1776. Several positive YACs corresponding to these probes were obtained. We conclude that we have isolated YAC clones corresponding to the deleted region.

DISCUSSION

From the above selection experiment we conclude that a subtelomeric deletion in the right end of chromosome 9 correlated with loss of gametocytogenesis, loss of ability of mature asexual blood stages to cytoadhere to C32MC, and loss of expression of the putative cytoadherence ligand PfEMP1. The deletion described on the right end of chromosome 9 (Shirley et al., 1990) may encode structural genes involved in expression of cytoadherence and of gametocytogenesis. Alternatively, this region of chromosome 9 may encode products such as transcription factors regulating expression of both these phenotypes. PfMEP1 can undergo antigenic variation (Biggs et al., 1991) so it is possible that this region encodes an expression site for PfEMP1. Duplicative transposition for the variant surface glycoproteins of trypanosomes has been documented to be due to site specific recombination occurring at given breakpoints (Borst & Cross, 1982). We postulate that similar breakpoints involved in the deletions in chromosome 9 of independent clones and isolates of *P. falciparum* may result in a similar mechanism of antigenic variation. If such events were frequently incomplete in *P. falciparum* they might explain the observed breakage. On the other hand, the breakpoint might simply reflect the presence of an essential gene near the breakpoint and a gene that is deleterious to growth in culture on the region deleted. As we have now cloned this region in yeast artificial chromosomes it can now be studied in detail.

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