Gene encoding erythrocyte binding ligand linked to blood stage multiplication rate phenotype in *Plasmodium yoelii yoelii*

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Variation in the multiplication rate of blood stage malaria parasites is often positively correlated with the severity of the disease they cause. The rodent malaria parasite *Plasmodium yoelii yoelii* has strains with marked differences in multiplication rate and pathogenicity in the blood. We have used genetic analysis by linkage group selection (LGS) to identify genes that determine differences in multiplication rate. Genetic crosses were generated between genetically unrelated, fast- (17XYM) and slowly multiplying (33XC) clones of *P. y. yoelii.* The uncloned progenies of these crosses were placed under multiplication rate selection in blood infections in mice. The selected progenies were screened for reduction in intensity of quantitative genetic markers of the slowly multiplying parent. A small number of strongly selected markers formed a linkage group on *P. y. yoelii* chromosome 13. Of these, that most strongly selected marked the gene encoding the *P. yoelii* erythrocyte binding ligand (*pyebl*), which has been independently identified by Otsuki and colleagues [Otsuki H, et al. (2009) *Proc Natl Acad Sci USA* 106:10.1073/pnas.0811313106] as a major determinant of virulence in these parasites. In an analysis of a previous genetic cross in *P. y. yoelii*, *pyebl* alleles of fast- and slowly multiplying parents segregated with the fast and slow multiplication rate phenotype in the cloned recombinant progeny, implying the involvement of the *pyebl* locus in determining the multiplication rate. Our genome-wide LGS analysis also indicated effects of at least 1 other locus on multiplication rate, as did the findings of Otsuki and colleagues on virulence in *P. y. yoelii.*

erythrocyte binding ligand protein | rodent malaria | linkage group selection | virulence

Under clinical or laboratory conditions, different lines or strains of malaria parasites can be shown to differ in the severity of the disease that they cause in their hosts (1–3). As these differences in pathogenicity are, by and large, stable properties of the individual parasite lines, it can be assumed that they have a genetic basis. There are many phenotypic, and genetically or epigenetically determined, differences among lines of malaria parasite that could influence their pathogenic potential. These include capacities for rosetting (4), cytoadherence (5), RBC selectivity (6), and multiplication rate (7) of the parasites in the blood of a host. Of these, a feature that tends to associate strongly with the severity of a malarial infection is the rate of multiplication of the blood stage parasites (8).

The rodent malaria parasite, *Plasmodium yoelii yoelli*, provides a striking example of lines of parasite that have major differences in their pathogenicity and in their overall blood stage multiplication rate during an infection (8). Most lines of *P. y. yoelii* exhibit a slow multiplication rate phenotype. They are characterized by a marked invasion preference for reticulocytes (very young RBCs). Parasites of this phenotype achieve parasitemias of no greater than 20% after 2–3 weeks of infection; they are usually cleared within 3–4 weeks and are rarely fatal (9). In sharp contrast are several cloned lines of the 17X isolate of *P. y. yoelii* including the commonly used 17XYM and 17XL lines (10, 11). These parasites lose their preference for reticulocytes very early in an infection and, thereafter, invade equally RBCs of any age. The infections reach parasitemias that approach 100% within 6–7 days and are often lethal to their hosts (10, 11). These fast-multiplying lines of *P. y. yoelii*, were originally, and are still commonly, referred to as "virulent" lines (3). They appeared spontaneously from within one particular slow multiplying line of 17X, represented by 17X(NIMR), with which both of the known fast-multiplying lines, 17XYM and 17XL, are congenic, as shown by our previous AFLP genotyping analysis (8).

Not long after its discovery, analysis of a genetic cross between the fast-multiplying (17XYM) and a slow-multiplying (A/C) wild-type line of *P. y. yoelii* showed that the fast- and slow-multiplying phenotypes were inherited and segregated in cloned lines of recombinant cross progeny, as a simple Mendelian trait consistent with the involvement of a single controlling genetic locus (10). However, the genetic mutations involved in this phenotypic change were not located or identified.

In the present study, we have used linkage group selection (LGS) analysis in an attempt to locate genes controlling fast or slow multiplication in these lines of *P. y. yoelii*. LGS is a genetic method recently developed for the rapid location of genes controlling specific phenotypes (12, 13). Our initial LGS analyses of genetic crosses between the fast- (17XYM) and slowly multiplying (33XC) lines of *P. y. yoelii* indicated that a region in the parasite's genome, spanning about half a megabase on *P. y. yoelii* chromosome 13, contained a major gene or genes that control fast or slow multiplication of the parasites in the blood. While this work was in progress we learned from Otsuki and colleagues (14) that replacement transfection of the *P. y. yoelii* 17XYM gene encoding the erythrocyte binding ligand (*pyebl*) conferred the fast multiplication phenotype on a wild-type line of *P. y. yoelii*. Following this communication, we located *pyebl* in the *P. y. yoelii* genome within the region on chromosome 13 identified by LGS analysis to determine multiplication rate. Further detailed LGS analysis of this chromosomal region showed that the *pyebl* alleles of the fast and slowly multiplying parental lines of *P. y. yoelii* were those which most closely marked the major locus determining

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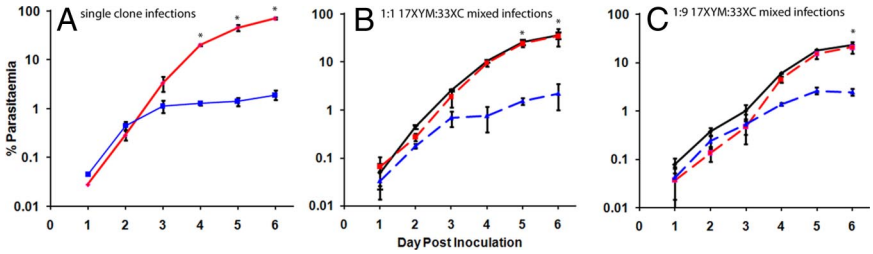

Fig. 1. Single and mixed clone infections of the rodent malaria parasite *P. y. yoelii* 17XYM and 33XC in mice. (*A*) Parasitemias are shown during the course of single clone infections with either 17XYM (red solid line) or 33XC (blue solid line) measured on thin blood smears stained with Giemsa's stain. (*B* and *C*) Clone specific parasitemias (17XYM, red dashed line; 33XC, blue dashed line) and absolute parasitemias (black solid line) are shown in mixed clone infections with parasites of 17XYM and 33XC in proportions of 1:1 and 1:9 (17XYM:33XC), respectively. The clone specific parasitemias in the mixed infections were calculated from the absolute parasitemias measured on thin blood smears stained with Giemsa's stain and from proportions of 17XYM and 33XC in the mixtures as measured by using Pyrosequencing. Error bars give the 95% confidence intervals of the means. Asterisks (*) represent statistically significant differences ($P \ll 0.01$) in parasitemia between 17XYM and 33XC.

blood stage multiplication rate in these parasites. Moreover, sequence analysis of *pyebl* in fast- and slow-multiplying lines of *P. y. yoelii* revealed a SNP at position 2340 in *pyebl* that associates with the multiplication rate phenotypes. This is consistent with the findings of Otsuki and others (14) in suggesting the involvement of *pyebl* in determining multiplication rate phenotype in these malaria parasites. Our studies also indicated effects of at least 1 additional locus on multiplication rate, as did the findings of Otsuki and colleagues on virulence in *P. y. yoelii*.

Results

Blood Stage Parasites of *P. y. yoelii* Line 17XYM Multiply Faster than Those of Line 33XC in Single and Mixed Line Infections in Mice. Growth of the blood stage parasites of cloned lines 17XYM and 33XC of *P. y. yoelii* was studied in mice. Two groups of mice were inoculated with either 17XYM or 33XC and a further 2 groups with mixtures consisting of 17XYM and 33XC in proportions of 1:1 and 1:9, respectively. The absolute parasitemias, as determined by light microscopy of thin blood smears, of the single and mixed clone infections in the mice and the proportions of 17XYM and 33XC in the mixed clone infections, determined by SNP-based Pyrosequencing analysis (15) are shown in Fig. 1. In the single clone infections, parasites of 17XYM achieved much higher parasitemias than those of 33XC by day 4 post-infection ($P < 0.01$; Fig. 1*A*). Likewise, in the mixed clone infections parasites of 17XYM soon outnumbered those of 33XC in infections of either starting proportions of the 2 clones ($P < 0.01$; Fig. 1 *B* and *C*).

Linkage Group Selection Analysis of 2 Independent Crosses Between Lines 17XYM and 33XC of *P. y. yoelii*. The blood stage parasites of the uncloned progeny of 2 independent genetic crosses between 17XYM and 33XC were prepared (see *SI Text*). To apply selection for blood stage multiplication rate differences among the progeny of each genetic cross, they were grown in mice as 3 successive blood infections (Fig. S1). Parasites were harvested from mice at each passage and prepared for genomic DNA extraction and amplified fragment-length polymorphism (AFLP) analysis.

The fast multiplication rate selected progeny from each of the 2 crosses were screened with 108 AFLP markers of the slowly multiplying parent 33XC. The relative intensity indices (RIIs) of the 33XC AFLP markers in each of the multiplication rate-selected crosses were obtained (see *Materials and Methods* for the definition of RII) and plotted against each other for each of the 3 successive blood infections (passages) (Fig. 2). In the selected progeny, 5 33XC AFLP markers had RIIs that were consistently below 0.2 (Table S1 and *SI Text*). Three, including 33XC AG01TA 17XYM, 33XC AG01AG 17XYM, and 33XC CA05TT 17XYM, were strongly reduced in intensity after the first passage; the 2 others, 33XC CA05TA 17XYM and 33XC GT02TT 17XYM, were strongly reduced only after the second and third passages, respectively.

The 3 most strongly reduced markers, 33XC AG01TA 17XYM, 33XC AG01AG 17XYM, and 33XC CA05TT 17XYM, were located as a single linkage group on *P. y. yoelii* chromosome 13 (see *Materials and Methods*), spanning a region of ≈1.1 megabase pairs as estimated by reference to the syntenic se-

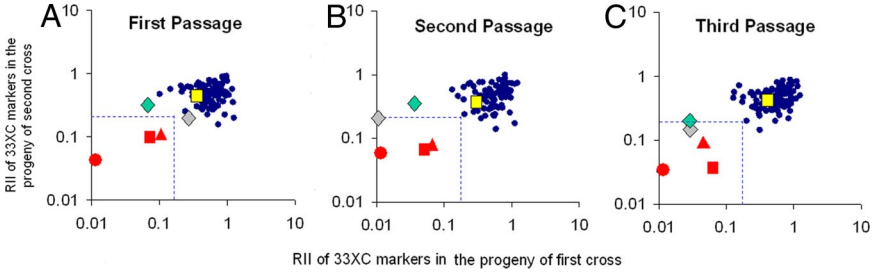

Fig. 2. The RIIs of 33XC (slow multiplying parental strain) AFLP markers in the progeny of the 2 genetic crosses between 17XYM and 33XC of *P. y. yoelii* are shown after multiplication rate selection in mice. (*A–C*) RIIs of the 33XC AFLP markers are plotted against each other for the progeny of each cross in the first, second, and third passages, respectively. Thus, the x- and y-axes represent the RIIs of the 33XC AFLP markers from the first and second cross progeny, respectively. Of the 108 *P. y. yoelii* 33XC AFLP markers analyzed, 3 markers [33XC CA05TT 17XYM (red circle), 33XC AG01AG 17XYM (red square), and 33XC AG01TA 17XYM (red triangle)] had RIIs of <0.20 (area in dashed light blue lines) after the first blood passage in both crosses. Two additional markers, 33XC CA05TA 17XYM (gray diamond) and 33XC GT02TT 17XYM (green diamond), were strongly reduced in both crosses only after the second and third blood passages, respectively. A yellow square represents a marker, 33X AA01CT 17XYM, that was not reduced by growth selection in the progeny of either cross but was found to be genetically linked with the 3 markers, 33XC CA05TT 17XYM, 33XC AG01AG 17XYM, and 33XC AG01TA 17XYM, under early strong growth selection.

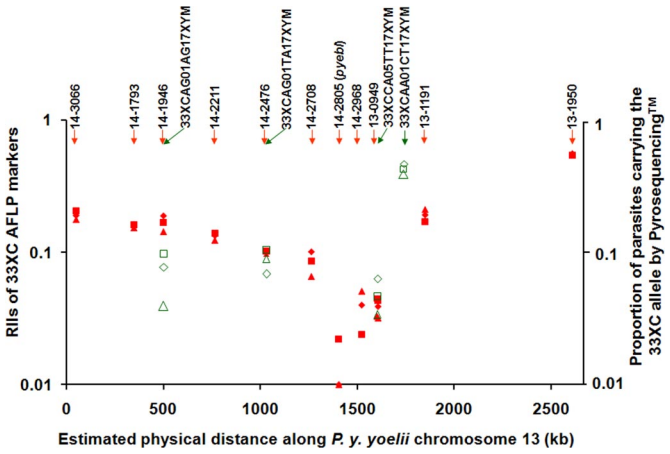

Fig. 3. Proportions of parasites carrying the alleles of *P. y. yoelii* 33XC in the growth selected progeny of the genetic cross (cross 2) between 17XYM and 33XC on chromosome 13 are shown. The RIIs of the four 33XC AFLP markers are represented by open green symbols. The proportions of parasites with the 33XC allele at 11 defined Pyrosequencing loci are represented by filled red symbols. Data obtained from the cross progeny after the first, second, and third rounds of multiplication rate selection in mice are represented by □, ◇, and △ symbols, respectively. Arrows indicate predicted locations of AFLP and Pyrosequencing markers along *P. y. yoelii* chromosome 13. Extrapolation to *P. y. yoelii* chromosome 13 is from synteny between the human malaria parasite *Plasmodium falciparum* and the rodent malaria parasite *P. y. yoelii*. *pyebl* stands for the gene encoding the erythrocyte binding ligand.

quence of *Plasmodium falciparum* (Fig. 3). Another marker, 33XC AA01CT 17XYM, which was not reduced by selection (Fig. 2), was located to a position on *P. y. yoelii* chromosome 13, which was 152 kb downstream of the nearest of the 3 strongly reduced 33XC AFLP markers (Fig. 3), thereby placing an outer limit on the selected region. The 2 other multiplication rate selected 33XC AFLP markers were found in *P. y. yoelii* genomic contigs containing species-specific genes (Table S1). Marker 33XC CA05TA 17XYM, which was strongly reduced only after the second passage, was allocated to a *P. y. yoelii* contig, MALPY02313, which contains a member of a gene family encoding the telomerase reverse transcriptase (TERT) protein (PY06698) (16). The other marker, 33XC GT02TT 17XYM, which was strongly reduced only after the third passage, was located to a contig, MALPY00154, containing a gene coding for the putative *P. yoelii* interspersed repeats (YIR) proteins (PY00559) (16). Because their orthologs have not been reported in the *P. falciparum* genome (17), it was not possible to assign them to locations in the *P. yoelii* genome.

To further determine the physical locations in the *P. y. yoelii* genome of the 5 33XC AFLP markers that were identified above as being under strong multiplication rate selection, we hybridized ^{32}P-radiolabeled DNA probes representing each marker identified above as being under strong multiplication rate selection onto Southern blots of the chromosomes of *P. y. yoelii* 17XYM and 33XC. The 3 strongly reduced markers, 33XC AG01AG 17XYM, 33XC AG01TA 17XYM, and 33XC CA05TT 17XYM, hybridized to a single band corresponding to the co-migrating chromosomes 13 and 14 in 17XYM and 33XC (Fig. S2, lanes 2–4). 33XC AA01CT 17XYM, the marker that was identified in the same linkage group as the 3 linked strongly reduced markers but which was not itself reduced by growth selection, also hybridized to the co-migrating chromosomes 13 and 14 in 17XYM and 33XC (Fig. S2, lane 5). Of the 2 other growth selected 33XC AFLP markers, one, 33XC CA05TA 17XYM, which was located to the gene for the TERT protein, hybridized to a band corresponding to the co-migrating *P. y. yoelii* chromosomes 5 and 6 (Fig. S2, lane 7). An attempt to perform hybridization for the other marker, 33XC GT02TT 17XYM, was unsuccessful as we were not able to design a specific DNA probe for this marker. This was because the marker was found in the gene encoding the YIR protein, the largest gene family in *P. y. yoelii* (16). The chromosomal location of this marker could not, therefore, be determined.

Fine Mapping of the Region Under Growth Selection on Chromosome 13 By Using Pyrosequencing. Eleven quantitative SNP-based Pyrosequencing assays were designed to cover the region on *P. y. yoelli* chromosome 13 that was identified as being under multiplication rate selection by the AFLP markers. These 11 Pyrosequencing markers describe a smooth selection valley in *P. y. yoelii* 17XYM × 33XC cross 2, the trough of which is most closely identified by the Pyrosequencing marker, 14–2805, which measures directly the *pyebl* locus (Fig. 3 and Table S2). This marker was also the most strongly reduced in cross 1 (Table S2).

Physical Location of *pyebl* in the *P. y. yoelii* Genome. Inclusion of the *pyebl* locus in our study was made after a communication from Otsuki and colleagues (14) informing us of the results of their recent experiments, which, by using gene replacement transfection, showed that *pyebl* is a major genetic determinant of virulence (lethality of an infection) in *P. y. yoelii* in 17XYM, which is the same fast multiplication rate phenotype clone used in our present analysis. We, therefore, attempted to locate *pyebl* in the *P. yoelii* genome to be able to test whether this gene might also be segregating with the multiplication rate phenotypes in our LGS analysis. By genomic database searching (*SI Text*), the *pyebl* gene was predicted and confirmed, by using long PCR (Fig. S3) and direct genomic mapping (Fig. S2, lane 6), to be located at position 1406 kb on *P. y. yoelii* chromosome 13, which is, within the limits of resolution of our method, at the center of the region identified as being under strong multiplication rate selection by LGS analysis.

Sequence Analysis of *pyebl*. We sequenced the entire *pyebl* gene in 3 slow-multiplying lines of *P. y. yoelli*, 33XC, A/C, and 17X(NIMR), and in our fast-multiplying line, 17XYM, which, as we have shown, is congenic with 17X(NIMR) (8). We observed 2 SNPs in the complete gene sequence between all 4 clones, both of which were nonsynonymous. All of the slow multiplying lines were identical at position 2340 and distinct at this position from the fast multiplying and virulent 17XYM. The other polymorphism (at nucleotide position 401), did not associate with the multiplication rate phenotype in these lines of *P. y. yoelii* (Fig. S4). These findings are consistent with position 2340 of *pyebl* being a genetic determinant of the multiplication rate phenotype in this parasite. Position 2340 of *pyebl* is the mutation shown by Otsuki and colleagues (14), in their transfection experiments, to determine virulence in these parasites.

Inheritance of the 17XYM and A/C Alleles at Loci on *P. yoelii* Chromosome 13 in Recombinant Progeny Clones of a Genetic Cross Between 17XYM and A/C. We also analyzed the inheritance of parasite clone specific markers in 6 cloned recombinant progeny of a previously conducted genetic cross between *P. y. yoelii* lines 17XYM (the same line as used in the present study) and A/C (a slow-multiplying line related to, but genetically distinct from, 33XC) (10). Each progeny clone, which had been phenotyped as being either virulent (fast multiplication rate) or avirulent (slow multiplication rate) (Table S4), was examined for the inheritance of SNPs or insertion or deletion mutations at 9 loci on chromosome 13 spanning the region identified here as under selection (see *Materials and Methods*).

Among the cloned cross progeny, there was complete cosegregation of the fast and slow multiplication phenotypes with markers of the fast and slow multiplying parents, respectively, at

Table 1. Inheritance of 9 parasite clone-specific markers in 6 recombinant clones of the progeny of a genetic cross between 17XYM and A/C on chromosome 13

Parasite clones	Multiplication rate	Predicted physical location on *P. y. yoelii* chromosome 13, kb								
		52	350	502	1032	1406 (*pyebl*)	1602	1705	1850	2609
17XYM (fast-growing parental clone)	Fast	F	F	**F**	**F**	**F**	**F**	**F**	F	F
604	Fast	F	F	**F**	**F**	**F**	**F**	**F**	S	S
605	Fast	F	F	**F**	**F**	**F**	**F**	**F**	F	F
645	Fast	F	F	**F**	**F**	**F**	**F**	**F**	F	F
606	Slow	F	F	**S**	**S**	**S**	**S**	**S**	S	S
611	Slow	S	S	**S**	**S**	**S**	**S**	**S**	F	F
648	Slow	S	S	**S**	**S**	**S**	**S**	**S**	F	F
A/C (slow-growing parental clone)	Slow	S	S	**S**	**S**	**S**	**S**	**S**	S	S

Markers of the fast and slow multiplying parental clones are represented by the letters F and S, respectively. The loci marked in bold type show perfect segregation with fast and slow multiplication genotype.

the 5 loci [positions 502, 1032, 1406 (corresponding to the *pyebl* gene), 1602, and 1705 kb on chromosome 13] (Table 1) spanning the region found here to be under the strongest selection. This result provides further evidence that a locus that determines variation in multiplication rate of the blood stage parasites, lies within the region of *P. y. yoelii* chromosome 13 that contains *pyebl*.

Discussion

We have used LGS analysis to investigate the genetic determinants of differences in the multiplication rate of blood stage parasites between lines of the rodent malaria parasite *P. y. yoelii*. LGS analysis, which combines classical genetic crossing and phenotypic selection of the uncloned cross progeny with genomics-era bioinformatics, is a rapid and efficient method for such purposes (12, 13)

Two independent genetic crosses were generated between a slowly multiplying parasite (33XC) and a fast multiplying parasite (17XYM), and the resulting uncloned recombinant progeny were grown through several rounds (between 12 and 16) of asexual multiplication in mice. The selected progeny were screened with large numbers of quantitative, genome-wide, markers of the parental clones to identify regions of the genome under selection. The results revealed that a genetic locus with a major effect on the multiplication rate phenotype in these parasites is located within a region of less than 500 kb on *P. yoelii* chromosome 13.

Within this region lies the gene encoding the EBL protein, which has been identified by Otsuki and colleagues (14) as a major genetic determinant of virulence differences between lines of *P. y. yoelii* during blood infections in mice. In our LGS analysis, a genetic marker of the *pyebl* locus in the slowly multiplying parent, 33XC, was that most consistently and strongly reduced during growth selection of the cross progeny. This suggests that *pyebl*, or a gene closely linked to it, is the target of the multiplication rate selection. We also characterized 6 recombinant progeny clones from a previous genetic cross between the fast-multiplying (17XYM) and slow-multiplying (A/C) lines of *P. y. yoelii* (10). The 17XYM and A/C alleles of *pyebl* segregated with fast and slow multiplication phenotypes, respectively, in these recombinant progeny clones. These results demonstrate that a single genetic locus on *P. y. yoelii* chromosome 13, containing *pyebl*, is a major genetic determinant of the fast and slow multiplication rate phenotypes of these parasites in blood infection in mice. Furthermore, sequencing the *pyebl* gene revealed a SNP at position 2340 that distinguished all of the slow multiplying lines, which were identical at this position, from the fast multiplying line. This SNP leads to an amino acid substitution in region 6 of the *Py*EBL protein and is the same SNP as was identified as controlling the virulence phenotype differences between 17X and 17XYM by Otsuki and colleagues (14). Together, these results strongly imply that the SNP at position 2340 of *pyebl* is the major genetic determinant of both the multiplication rate and virulence difference phenotypes in the lines of *P. y. yoelii* that have been investigated in our two studies.

The original description of the 17XYM line reported that fast multiplying parasites emerged in a single step during blood passage of 17X, a slowly multiplying line of *P. y. yoelii* (3). This suggests that a single mutation event may have been responsible for the major effect in determining the fast multiplying phenotype. These circumstances and this hypothesis are consistent with the above analysis indicating the involvement of a single major genetic locus. However, in their replacement transfection experiments, Otsuki and colleagues found that, although the *pyebl* gene constituted a major determinant of the multiplication rate phenotype, the influence of other genes was also likely (14). Moreover, although our LGS analyses, which involved genome-wide coverage, found no direct evidence of strong multiplication rate selection outside the region containing the *pyebl* gene on *P. yoelii* chromosome 13, two other AFLP markers (33XC CA05TT 17XYM and 33XC GT02TT 17XYM) were, nevertheless, found to be under strong selection after the second and third rounds of infection of the uncloned cross progeny in mice. Unfortunately these markers could not be located in the genome through database mining, partly because they were found to consist of sequences from members of multigene families encoding TERT and YIR proteins, respectively. Nevertheless, direct genomic mapping enabled the marker that encoded the *tert* gene to be located to either of *P. yoelii* chromosome 5 or 6, implying the possible presence of other genetic determinants of multiplication rate in this region as was also suggested by Otsuki and colleagues (14). Likewise, we cannot exclude the possible involvement of other genes within the *pyebl*-associated selection valley on chromosome 13. Sequencing of this region in congenic fast- and slowly multiplying parasite lines in parallel (8) could resolve this question.

Proteins that are the expression products of several distinct gene families of malaria parasites have been implicated in erythrocyte invasion processes (18). Genetically based structural differences (polymorphisms) in these proteins might be expected to affect the rate of multiplication of the parasites in the blood of a host. Among them are the *Duffy*-binding-like/erythrocyte-binding-like (DBL/EBL) family of proteins to which *Py*EBL belongs. The DBL/EBL proteins, originally defined by their binding specificity for the Duffy antigen on human erythrocytes (19, 20) are characterized by a conserved 35-kDa cysteine-rich domain, known as the *Duffy*-binding-like domain (21, 22), which was first described for the *ebl* gene of *Plasmodium vivax* (23). Although a number of such proteins, including EBL, EBA175, BAEBL, JSEBL, MAEBL, and others, are known in *P. falcipa-*

rum (24), the only known DBL-domain-carrying protein in *P. yoelii* is *Py*EBL itself, implicated as the major gene-determining multiplication rate in the present study and virulence in that of Otsuki and colleagues (14).

Apparently unrelated to the above group of erythrocyte-binding proteins implicated in host RBC invasion are a family of reticulocyte-binding proteins (RBLs) found in *Plasmodium spp.* There are 6 members of the RBL homolog proteins in *P. falciparum* (Rh1, Rh2a, Rh2b, Rh3, Rh4, and Rh5). In a genetic analysis of *P. falciparum*, a polymorphism in the gene for Rh5, of the RBL homolog protein family, was found to determine multiplication rate of the blood stage parasites in *Aotus* monkeys (25). These proteins share significant similarity to the *P. vivax* reticulocyte-binding protein 2 (26) and also have some similarity with a 500-residue region within the C terminus of the 235-kDa family of rhoptry proteins of *P. yoelii* (*Py*235) (26). It has recently been proposed that the differences in erythrocyte invasion preference in the fast-multiplying (virulent) and slow-multiplying (avirulent) lines of *P. y. yoelii* and, by implication, therefore, in their blood stage multiplication rate differences, could be associated with differences in expression of members of the *Py*235 multigene family which encode the 235-kDa rhoptry proteins (18, 27). Iyer and colleagues (28) have reported increased expression of specific *Py*235 genes during blood infections of 17XYM that coincide with the time of sudden change from slow to rapid multiplication rate that characterizes this parasite line and phenotype. This observation led to the proposal that the RBC invasion properties and multiplication rates of the parasites in the blood are influenced by the level of expression of *Py*235 genes.

Our findings do not rule out the possible involvement of *Py*235 genes in the multiplication rate/virulence phenotype. Two of the markers that were found to be under multiplication rate selection in our LGS analysis could not be located to a chromosomal position possibly because they are present subtelomerically and, therefore, difficult or impossible to locate using presently available databases. It is known that some *Py*235 genes are located subtelomerically (29, 30). Therefore, until we are able to locate and identify within the *P. yoelii* genome the 2 other AFLP markers that were implicated as being linked to genes controlling blood stage multiplication in this parasite, our present findings will not be able to contribute to hypotheses concerning a role for *Py*235 genes in the blood stage multiplication rates of this parasite.

The investigations reported or discussed here show that genetic analyses of malaria parasites are able to identify genes and proteins involved in the multiplication of these parasites in a host. Over 30 years ago, Meir Yoeli and colleagues (3) expressed their hope of "using genetic recombination experiments…. to investigate the possible genetic control of virulence in *P. b. yoelii*." Here, in conjunction with the results of Otsuki and colleagues (14), we present evidence that the major genetic determinant of multiplication rate and virulence phenotypes of *P. y. yoelii* is at a single genetic locus; that containing the *pyebl* gene on *P. y. yoelii* chromosome 13.

Materials and Methods

Parasites, Mice, and Mosquitoes. The *P. y. yoelii* parasites used in this study were originally isolated from thicket rats, *Thamnomys rutilans*, caught in the Central African Republic (31, 32). Slow-growing cloned lines of *P. y. yoelii* used were 33XC, 17X(NIMR), A/C, 606, 611, and 648 (8–10). Fast-multiplying cloned lines of *P. y. yoelii* used were 17XYM, 604, 605, and 645 (10). The origin and characteristics of each clone are summarized in Table S4. The mice used were inbred female CBA/Ca mice, aged 6–8 weeks (on primary infection). Mosquitoes were from a laboratory-bred colony of *Anopheles stephensi*.

Mixed Clone Infections of *P. y. yoelii* 17XYM and 33XC. Two groups of 4 CBA mice were infected i.p. with a mixture of 1×10^6 parasitized RBCs containing the blood stage parasites of 17XYM and 33XC in proportions of either 1:1 or 1:9. Two further groups of 5 mice were inoculated i.p. with 1×10^6 parasitized RBCs of either clone 17XYM or 33XC. The infections were examined on thin blood smears stained with Giemsa's solution. Blood was collected from the tail vein of the mixed clone infected mice and applied to FTA cards (Whatman) before DNA extraction. Proportions of 17XYM and 33XC in the mixed cloned infections were measured by using a Pyrosequencing assay (15) for the 17XYM and 33XC alleles of the gene for apical membrane antigen-1 (see also *SI Text*). Pyrosequencing primers and the SNPs used in the assay are given in Table S3. Clone specific parasitemias were obtained by multiplying proportions of 17XYM and 33XC, as determined by Pyrosequencing, by the total parasitemias of mixed clone infections as measured by microscopic examination of thin blood smears. Confidence intervals (95%; 2× standard error of mean) were calculated. Analysis of variance was performed by using SPSS (version 11.5; SPSS). Differences were deemed significant with a P value < 0.01.

Linkage Group Selection Analysis of Genetic Crosses Between Lines 17XYM and 33XC of *P. y. yoelii*. Two independent genetic crosses between *P. y. yoelii* 17XYM and 33XC were made (see *SI Text* and Table S5). Four days after inoculations of sporozoites of each of the 2 crosses into mice, blood stage parasites were harvested to produce blood inocula containing the uncloned recombinant progeny of each cross. A total of 1×10^6 parasitized RBCs of the uncloned progeny of each cross were inoculated i.p. into 3 CBA mice. The infections were followed by microscopic examination of thin blood smears taken from the mouse tail vein (see Fig. S1). Blood stage parasites were harvested and passaged twice into CBA mice when mean parasitemias of 20% or above were achieved. Blood stage parasites of the uncloned progeny of cross 1 were harvested on day 4 post-infection in each of the 3 blood passages. The blood stage parasites of the uncloned progeny of the second cross were harvested on day 6 post-infection in the first blood passage and on day 5 post-infection in the second and third passages. Parasites grown in these mice were prepared for DNA extraction as previously described (33).

Genomic DNA of the multiplication rate selected cross progeny and that of the 2 parental clones, 17XYM and 33XC, were analyzed by AFLP as previously described (33). AFLP bands which were present in one parental clone and absent in the other were identified as polymorphic. RIIs of AFLP markers for the slow-multiplying parental clone 33XC in the uncloned progeny of the 2 genetic crosses in each of the 3 passages were measured as previously described (34). The RII is defined as the intensity of a polymorphic band divided by the intensity of a specific nonpolymorphic band (intensity index), divided by the equivalent index of the same bands measured from the pure parental strain. The RII of an individual AFLP marker is known to be directly proportional to the percentage of parasites carrying that marker in a complex genetic mixture (34).

Genomic Mapping of AFLP Markers in the *P. y. yoelii* Genome. DNA fragments were extracted from AFLP bands as previously described (13). AFLP marker sequences were located in the 5-fold (5×) coverage genomic contigs of *P. y. yoelii* clone 17XNL (16) as follows. *P. y. yoelii* contigs containing sequences highly similar to AFLP marker sequences were identified by BLASTN (DNA versus DNA) search, which can be accessed at the Institute for Genomic Research web site (http://www.tigr.org/tdb/e2k1/pya1/). *P. y. yoelii* genes on these contigs were used to locate orthologous loci in the genome of the human malaria parasite *P. falciparum* clone 3D7 (35) by BLASTP (DNA versus Protein) search, which can be accessed at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/sutils/blast_table.cgi?taxid = Protozoa&database). The genomic locations of the orthologs in *P. falciparum* were, in turn, used to predict chromosomal locations of the AFLP markers in *P. y. yoelii* by using a genome-wide synteny map for the human and rodent malaria species (17).

Direct physical mapping was performed by pulsed-field gel electrophoresis (PFGE) and Southern hybridization (see *SI Text*). Chromosomal DNA were fractionated by PFGE, as previously described (29, 30). DNA was transferred to Nylon transfer membrane, Hybond-N+ (Amersham Biosciences) under standard conditions. Southern hybridization of DNA probes specific to the 33XC AFLP markers, and *pyebl* is given in *SI Text*.

Mapping of the *pyebl* Gene in the *P. y. yoelii* Genome. We obtained an approximately 4.6-kb contig sequence containing *pyebl* of *P. y. yoelii* clone 17XNL, deposited in the GenBank database under accession number AABL01001466 (16). We identified an ortholog of *pyebl* among the 8-fold (8×) coverage contigs of the rodent malaria parasite *Plasmodium chabaudi chabaudi*, by BLASTN search (performed in Oct, 2008), which can be accessed at the Sanger Institute web site (http://www.sanger.ac.uk/Projects/P_chabaudi/). The *P. c. chabaudi* contig sequence with the highest sequence similarity to that of *pyebl* was used to locate the 2 nearest annotated genes

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upstream and downstream of the ortholog of *pyebl* and identify loci in *P. falciparum* containing orthologous genes, by BLASTP search. By using a genome-wide synteny map (17), the corresponding loci of the nearest annotated genes upstream and downstream of *pyebl* in the *P. y. yoelii* genome were identified, and those were used to predict a chromosomal location of the *pyebl* in *P. y. yoelii*.

Long PCR for Confirmation of Physical Location of *pyebl*. PCR assays were designed to determine possible physical linkage between *pyebl* and the 2 nearest flanking genes as predicted by genome database searching (see above). The genes PY07221 and PY01695, deposited in the GenBank database under accession numbers XM_722945 and XM_724397 (16), respectively, were predicted to be the 2 nearest genes upstream and downstream of *pyebl*. Details of primer sequences and reaction conditions are given in *SI Text*.

Proportional Quantification of 17XYM and 33XC Alleles on *P. y. yoelii* Chromosome 13 by Pyrosequencing. Eleven loci located to positions 52, 349, 502, 767, 1035, 1264, 1406 (corresponding to *pyebl*), 1524, 1602, 1850, and 2609 kb on *P. y. yoelii* chromosome 13 were selected to develop Pyrosequencing assays (15) to measure the proportions of parasites carrying alleles from either parent in the multiplication rate-selected uncloned progeny of the genetic crosses between 17XYM and 33XC. The respective *P. falciparum* chromosomal locations orthologous to loci on *P. y. yoelii* chromosome 13 used for each of the 11 Pyrosequencing assays can be found in Table S2. The primers and SNPs used for these assays are given in Table S3.

Sequence Analysis of *pyebl*. The *pyebl* gene was amplified from genomic DNA prepared from the blood-stage parasites of *P. y. yoelii* cloned lines, 17XYM, 33XC, A/C, and 17X(NIMR) by using PCR. The amplicons were analyzed and sequenced as described in *SI Text*. The full-length sequences of the *pyebl* gene from 17XYM, 17X(NIMR), 33XC, and A/C of *P. y. yoelii* were deposited in the GenBank database under the accession numbers FJ610238, FJ610239, FJ610240, and FJ610241, respectively.

Genotyping the Parental Alleles on *P. y. yoelii* Chromosome 13 in the Cloned Progeny of the 17XYM × A/C Genetic Cross. Six recombinant clones, 604, 605, 606, 611, 645, and 648, of the progeny of a genetic cross between *P. y. yoelii* lines 17XYM and A/C (10) and the 2 parental clones, 17XYM and A/C, were grown in donor CBA mice to harvest blood stage parasites. Genomic DNA of 17XYM and A/C were prepared and amplified with oligonuclotide primers designed for identification of SNPs and/or insertion or deletion mutations at 9 defined loci, corresponding to positions 52, 350, 502, 1032, 1406 (corresponding to *pyebl*), 1602, 1705, 1850, and 2609 kb, on *P. y. yoelii* chromosome 13. Primers and cycling parameters for each of the 9 PCRs as well as SNPs or insertion or deletion mutations identified between the 2 parental clones are shown in Table S6. These assays were subsequently used to identify the parental origin of SNPs and insertion or deletion mutations in the recombinant clones of the 17XYM × A/C cross progeny.

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