

Regulation of *Plasmodium yoelii* Oocyst Development by Strain- and Stage-Specific Small-Subunit rRNA

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ABSTRACT One unique feature of malaria parasites is the differential transcription of structurally distinct rRNA (rRNA) genes at different developmental stages: the A-type genes are transcribed mainly in asexual stages, whereas the S-type genes are expressed mostly in sexual or mosquito stages. Conclusive functional evidence of different rRNAs in regulating stage-specific parasite development, however, is still absent. Here we performed genetic crosses of *Plasmodium yoelii* parasites with one parent having an oocyst development defect (ODD) phenotype and another producing normal oocysts to identify the gene(s) contributing to the ODD. The parent with ODD—characterized as having small oocysts and lacking infective sporozoites—was obtained after introduction of a plasmid with a green fluorescent protein gene into the parasite genome and subsequent passages in mice. Quantitative trait locus analysis of genome-wide microsatellite genotypes of 48 progeny from the crosses linked an ~200-kb segment on chromosome 6 containing one of the S-type genes (D-type small subunit rRNA gene [*D-ssu*]) to the ODD. Fine mapping of the plasmid integration site, gene expression pattern, and gene knockout experiments demonstrated that disruption of the *D-ssu* gene caused the ODD phenotype. Interestingly, introduction of the *D-ssu* gene into the same parasite strain (self), but not into a different subspecies, significantly affected or completely ablated oocyst development, suggesting a stage- and subspecies (strain)-specific regulation of oocyst development by *D-ssu*. This study demonstrates that *P. yoelii* *D-ssu* is essential for normal oocyst and sporozoite development and that variation in the *D-ssu* sequence can have dramatic effects on parasite development.

IMPORTANCE Malaria parasites are the only known organisms that express structurally distinct rRNA genes at different developmental stages. The differential expression of these genes suggests that they play unique roles during the complex life cycle of the parasites. Conclusive functional proof of different rRNAs in regulating parasite development, however, is still absent or controversial. Here we functionally demonstrate for the first time that a stage-specifically expressed D-type small-subunit rRNA gene (*D-ssu*) is essential for oocyst development of the malaria parasite *Plasmodium yoelii* in the mosquito. This study also shows that variations in *D-ssu* sequence and/or the timing of transcription may have profound effects on parasite oocyst development. The results show that in addition to protein translation, rRNAs of malaria parasites also regulate parasite development and differentiation in a strain-specific manner, which can be explored for controlling parasite transmission.

Received 22 January 2015 Accepted 29 January 2015 Published 10 March 2015

Citation Qi Y, Zhu F, Eastman RT, Fu Y, Zilversmit M, Pattaradilokrat S, Hong L, Liu S, McCutchan TF, Pan W, Xu W, Li J, Huang F, Su X. 2015. Regulation of *Plasmodium yoelii* oocyst development by strain- and stage-specific small-subunit rRNA. *mBio* 6(2):e00117-15. doi:10.1128/mBio.00117-15.

Editor Louis M. Weiss, Albert Einstein College of Medicine

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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

A malaria parasite has to complete multistage sporogonic development in the mosquito before being transmitted to a vertebrate host. The parasite enters the mosquito midgut when a mosquito takes blood from an infected vertebrate host; within the midgut, gametocytes quickly differentiate into male and female gametes that fertilize to produce motile ookinetes. The ookinetes then penetrate and cross the mosquito midgut wall, reaching the basal membrane, where they develop into oocysts. For the rodent parasite *Plasmodium yoelii*, sporozoites mature within the oocysts in ~10 to 12 days after fertilization (1) and invade mosquito salivary glands (SGs), where they are injected into a new host when

the mosquito bites again, starting another half of the life cycle in a vertebrate host.

Plasmodium parasites have 4 to 8 nucleus-encoded, structurally distinct, and differentially transcribed rRNA genes, including some specifically expressed in oocyst and sporozoite stages (2–4). The genes encoding rRNA subunits are generally arranged in the order of small-subunit (SSU) rRNA (or 18S rRNA), internal transcribed spacer 1 (ITS1), the 5.8S rRNA, a second ITS (ITS2), and the large-subunit (LSU) rRNA (or 28S rRNA) (3) and are located on different chromosomes (as opposed to tandemly clustered rRNA genes in other eukaryotic organisms). Although all types of

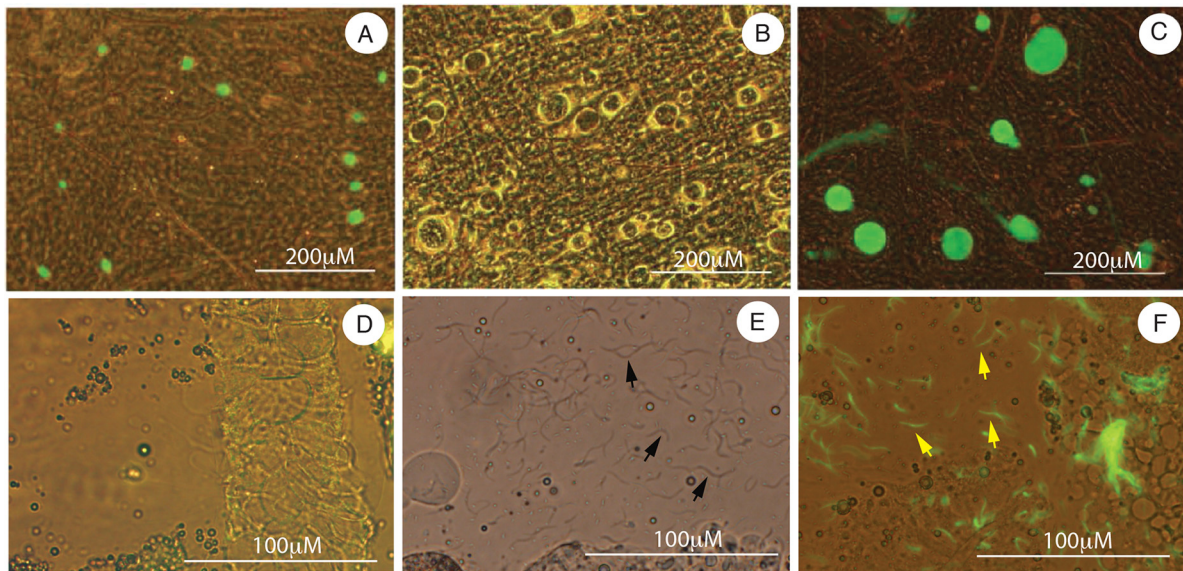


FIG 1 Differences in oocyst and sporozoite developments between *Plasmodium yoelii yoelii* By265, *P. y. yoelii* By265G(o), and *P. y. yoelii* By265G(d). (A) Day 9 oocysts from a mosquito midgut infected with the By265G(d) parasite. (B) Day 9 oocysts from the By265 parasite. (C) Day 9 oocysts from the By265G(o) parasite. (D) No sporozoite was found in the salivary glands of a mosquito infected with By265G(d) on day 11 after feeding. (E) Day 11 salivary gland sporozoites (black arrows) from a mosquito infected with By265. (F) Day 11 sporozoites (yellow arrows) from a mosquito infected with By265G(o).

rRNA genes are transcribed to some level throughout the cycle (5, 6), the malaria rRNA genes can be classified into two types: A-type for maximal expression in asexual stages and S-type for expression in sexual or mosquito stages (3). Moreover, two subtypes of S genes have been recognized in malaria parasites: the C-type (or the original S-type) genes are mostly expressed in late stages of oocysts and sporozoites, whereas the D-type genes (or O-type genes originally found in *Plasmodium vivax*) are largely expressed in maturing ookinetes and young oocysts (3, 7, 8). In the rodent malaria parasites *Plasmodium berghei*, *Plasmodium chabaudi*, and *P. yoelii*, four units containing rRNA genes have been identified: the A and B units of the A-type genes are located on chromosomes 12 and 7, and the C and D units of the S-type genes are present on chromosomes 5 and 6, respectively (9–11).

The stage-specific expression of malaria rRNA genes suggests that different rRNA genes have unique functional roles in the parasites' complex life cycle; however, solid functional evidence of stage-specific roles of the rRNA genes is still missing or controversial. A gene disruption study suggested that the C- and D-type rRNAs might be nonessential for *P. berghei* oocyst and sporozoite development, although significant growth retardation in oocyst development was observed in parasites with disrupted C- or D-type *ssu* (C- or D-*ssu*) genes (6). In a previous study, we obtained a fluorescent *P. yoelii* parasite, By265G(o), after insertion of a plasmid containing the gene encoding green fluorescent protein (GFP) into the genome (12). By265G(o) was an uncloned line and was later found to have its C-*ssu* gene disrupted by the plasmid insertion, but it could still produce normal oocysts and sporozoites (12). After passages of By265G(o) in mice, and for unknown reasons, we obtained By265G(d), which has a defect in oocyst and sporozoite development (small oocysts and lack of infective sporozoites) (see Fig. S1 in the supplemental material). To identify the putative mutation(s), we genetically crossed By265G(d) with a nonfluorescent parasite (*Plasmodium yoelii* nigeriensis NSM/N67,

a subspecies) that produced normal oocysts and sporozoites and linked a segment of DNA on chromosome 6 containing the D-type rRNA unit to the oocyst development defect (ODD). Further fine mapping of the plasmid insertion site in the By265G(d) parent and the progeny, gene expression analysis, and gene knockout (KO) experiments conclusively demonstrated a critical role for the *P. yoelii* D-*ssu* gene in oocyst development for the first time. Unexpectedly, introduction of a plasmid containing the D-*ssu* gene into By265G(d) or wild-type (wt) By265 resulted in total loss of or reduced oocyst numbers. Although more studies are necessary to elucidate the complete mechanism of how the D-*ssu* gene regulates oocyst development, our observations open up an exciting field for studying stage-specific rRNA units in regulating parasite gene expression and development.

RESULTS

A defect in oocyst development. To investigate the molecular basis of the ODD phenotype observed in the By265G(d) parasite, we first cloned the parasite and obtained nine clones, all of which produced small fluorescent oocysts. We infected mosquitoes with By265G(d) (one of the nine ODD clones), By265G(o), and By265 parasites and found that the diameters of day 9 By265G(d) oocysts were less than half the sizes of those from By265 or By265G(o) (Fig. 1A to C). Large numbers of sporozoites were present in the SGs of the mosquitoes infected with By265 or By265G(o) day 9 to 11 postinfection but not in the SGs of the mosquitoes infected with By265G(d) (Fig. 1D to F). Although occasional fluorescent sporozoites could be seen in By265G(d) oocysts in the midgut, no fluorescent sporozoites were found in the SGs of By265G(d)-infected mosquitoes. Repeated injection (3 times) of extract from midgut and SGs of By265G(d)-infected mosquitoes into mice did not lead to infection, confirming no infective sporozoites were produced. Clearly, By265G(d) had smaller oocysts than By265

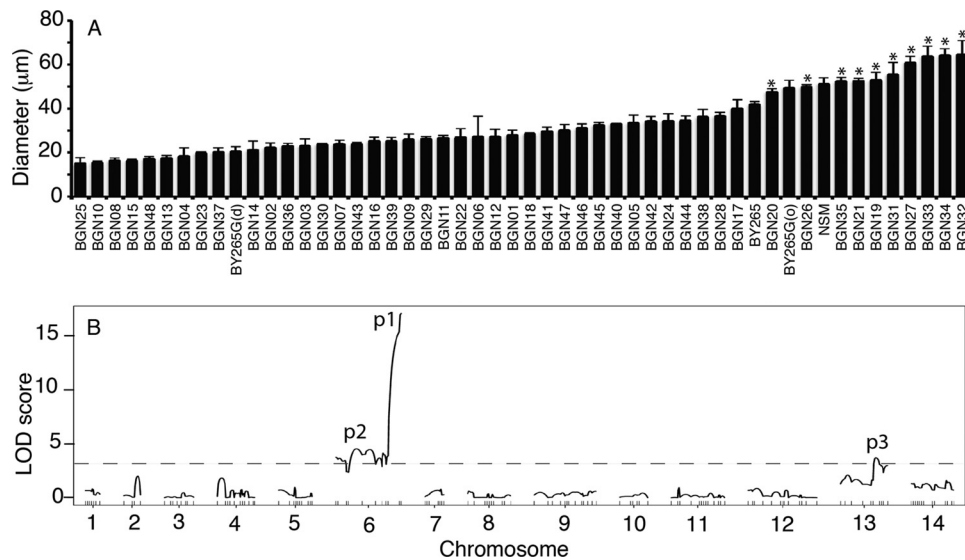


FIG 2 Averaged diameters of day 9 oocysts and logarithm of odds (LOD) scores showing loci linked to the day 9 oocyst diameter. (A) Day 9 oocyst diameters (average and standard deviation from at least 20 oocysts) in micrometers from By265, By265G(d), NSM, and 48 progeny of the By265G(d) × NSM (N67) crosses. An asterisk (*) denotes progeny obtained without pyrimethamine selection. (B) Plot of LOD scores of the markers showing loci significantly (LOD of >3.0) linked to day 9 oocyst size on chromosome 6 and chromosome 13, respectively. p1, p2, and p3 mark peaks with significant LOD scores. QTL analysis was conducted using R/qtl library in R2.12.2 software (<http://www.rqtl.org/>) as described previously (13).

and By265G(o) and could not produce infective sporozoites even 15 days after mosquito feeding.

Genetic crosses and cloning of recombinant progeny. We performed nine independent cross experiments between the defective By265G(d) and NSM (or N67, which was used once but was replaced with NSM because NSM is resistant to mefloquine, representing another phenotype for future mapping), which could produce large numbers of oocysts and infective sporozoites (see Table S1 in the supplemental material). We cloned progeny from mice with or without drug (pyrimethamine) pressure. Theoretically, only recombinant progeny receiving unknown elements critical for oocyst and sporozoite development from NSM and carrying the plasmid with genes encoding GFP and *Toxoplasma gondii* dihydrofolate reductase (*TgDHFR*) from By265G(d) would be present in mouse blood under drug pressure. Indeed, all of the progeny surviving pyrimethamine treatment were fluorescent in the blood smears of mice injected with sporozoites from the crosses (see Fig. S2 in the supplemental material). Without drug pressure, parental NSM progeny would be present in the mouse blood.

After injection of diluted parasites (~1 parasite per 100 μl phosphate-buffered saline [PBS]) into a total of 840 mice, we obtained 192 recombinant progeny (clones), including 48 independent recombinant progeny (IRP) with unique recombination events, after genotyping parasites from 255 infected mice with 14 microsatellite (MS) markers (one on each chromosome) (see Tables S1 and S2 in the supplemental material). Among the 48 IRP, 38 were cloned after applying drug pressure to remove parental parasite clones, and 10 were cloned without drug selection. No progeny with 100% parental genotypes were found in the cloning experiments after drug selection, whereas parental NSM-type progeny were found in the four cloning experiments without drug pressure (see Table S1). These results confirmed that parental By265G(d) cannot pass through the mosquito and that NSM is sensitive to pyrimethamine.

Genome-wide genotyping and phenotyping of recombinant progeny. To identify the genetic loci linked to the ODD, we genotyped the 48 IRP and the parents with additional 217 MS markers (total of 231) located on the parasites' 14 chromosomes (13) (see Table S2 in the supplemental material). To characterize the phenotypic differences between the parents of the crosses and the 48 progeny, we also measured oocyst diameters of the parasites on days 9, 11, 13, 15, and 17 after mosquito feeding, the presence or absence of sporozoites in the midgut and SGs during oocyst development, and the sporozoite infectivity of the mouse (see Table S3 in the supplemental material). Measurements of day 9 oocyst size showed significantly ($P < 0.01$, *t* test) larger oocyst sizes for the 10 progeny (56.8 ± 6.4 μm, mean ± standard deviation [SD]) obtained from crosses without drug selection than for those obtained after drug treatments (27.3 ± 8.1 μm) (Fig. 2A; see Table S3 in the supplemental material). Sporozoites were observed in the SGs of the mosquitoes infected with By265, NSM, and the 10 progeny without drug selection (and no fluorescence) from days 9 to 11 to day 17. Fifteen of the 38 drug-selected progeny had sporozoites within midgut oocysts, with 2 (BGN17 and BGN44) having small numbers of sporozoites in the SGs. Injection of mice with sporozoites from the drug-selected progeny produced no parasites in mice, whereas all progeny from no drug selection had parasites in the blood.

Genetic loci and candidate genes linked to the ODD phenotype. To identify the loci that may contribute to the ODD phenotype, we performed quantitative trait locus (QTL) analysis on day 9 oocyst diameter measurements (when most oocysts mature and contain sporozoites) and 231 MS genotypes from the 48 progeny using the R/qtl package (14). Three peaks with logarithm of odds (LOD) scores of >3.0 were identified (Fig. 2B). A peak (p1) with an LOD score of 17.0 on one end of chromosome 6 in a DNA segment containing MS markers Py2699 and Py2653—as well as two secondary peaks of an LOD of ≈4.5 at the middle of chromosome 6 (p2) and a peak of LOD of 3.7 on chromosome 13 (p3)—

were detected. Additionally, all 10 progeny cloned from crosses without drug selection were nonfluorescent, infective to mice, and carried NSM genotypes for Py1080 and Py2653 (see Table S3 in the supplemental material), supporting a link between NSM genotypes of the markers and normal oocyst development. For the peak p1 on chromosome 6, a crossover between MS markers Py2699 and Py1080/Py2653 in progeny BGN31 defines a locus of approximately 202.5 kb (from Py2699 to the end of the chromosome with gene PYYM_0626300 encoding a YIR protein) containing 50 predicted coding regions/genes (see Table S4 in the supplemental material). The candidate genes in this locus included genes encoding conserved *Plasmodium* proteins and several YIR proteins, and the D-type rRNA genes, one of the targeting sites of the plasmid. In theory, all of the genes in the locus are candidates that can cause the ODD phenotype, and based on the known genetic diversity of NSM and By265 (13), large numbers of nucleotide substitutions and indels are expected to be present between the candidate genes from the two parasites. The only potential difference in the locus between By265 and By265G(d) (except some potentially unknown changes during parasite transformation), however, was insertion of the plasmid into the *D-ssu* rRNA gene that became the prime candidate for the ODD phenotype.

The higher peak at the middle of chromosome 6 was defined by markers Py497 (chromosome position 399300) and Py1006-1 (position 414461) with six candidate genes (see Table S4 in the supplemental material). Among these were genes coding for two conserved *Plasmodium* proteins, an ABC transporter, a mitochondrial ribosomal protein L1-2 precursor, a SNARE protein, and a zinc finger protein. The third peak with a significant LOD score (3.7) was on chromosome 13, defined by MS markers Py2401 (position 1512721) and Py2486 (position 1896471), which spanned ~384 kb and contained 97 candidate genes (see Table S4). Among the candidates were genes encoding three 60S ribosomal proteins (L6, L23, and L23a), the RNA polymerase 3 largest subunit, an RNA binding protein, two Qa SNARE proteins, and several zinc finger proteins or helicases.

We also investigated the possibility of an additive effect or interaction between loci linked to the ODD phenotype by performing two-dimensional genome scans. We evaluated various LOD scores for interaction and additive effect and compared the values with cutoff LOD scores ($P < 0.05$) obtained after 1,000 permutations. The *D-ssu* locus on chromosome 6 (p1) had significant LOD_f (evidence for at least one QTL with allowance for interaction; LOD score of >16 with an LOD_f cutoff of 8.7) values for all the chromosomes. However, the values for LOD_i (evidence for interaction; LOD_i cutoff of 4.8) and LOD_{f1} (evidence for a second QTL allowing for epistasis; LOD_{f1} cutoff of 6.5) were all below the cutoff values.

Mapping plasmid integration site(s). To further determine whether the plasmid was inserted into the *C-* or *D-ssu* gene in the By265G(d) parasite and whether the insertion of the plasmid into the *D-ssu* (or *C-ssu*) gene indeed plays a role in the ODD phenotype, we designed sets of *ssu* copy-specific PCR primers (see Table S5 in the supplemental material) to map plasmid insertion sites in the parasite strains and progeny with fluorescence. The plasmid used for parasite transformation was initially designed for integration into the *C-* or *D-ssu* loci through homologous recombination (6, 15). A search of the *P. yoelii* YM genome (<http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-yoelii.html>) and syntenic maps of rodent malaria parasites also

showed the locations of *P. yoelii* rRNA genes on chromosomes 5 (C-type), 6 (D-type), 7 (B-type), and 12 (A-type) (10). In fact, MS marker Py2653 on chromosome 6 perfectly linked to ODD was located approximately 1 kb from the *D-ssu* sequence (Fig. 3A). DNA samples from parasites By265, By265G(o), By265G(d), NSM, four progeny from the genetic crosses, and the green fluorescent parasite 17XNLG described previously (16) were amplified using copy-specific primers (Fig. 3A; see Table S5 in the supplemental material). The results showed that wild-type *C-ssu* was present in all of the parasites (products from primers LC5 and LC3), but the full *D-ssu* gene (products from LD5 and LD3) was only present in By265, By265G(o), 17XNLG, and NSM parasites, all of which could produce normal oocysts (Fig. 3B). By265G(o) and 17XNLG parasites also had insertions of the plasmid into the *C-ssu* gene (products from LC5/L635 and LC3/L665), whereas By265G(d) and the four progeny had insertion of the plasmid into the *D-ssu* gene only (products from LD5/L635 and LD3/L665) (Fig. 3B). The presence of *C-ssu* products from By265G(o) and 17XNLG suggested that these parasite lines are mixtures of parasites with intact *C-ssu* and *D-ssu* genes as well as interrupted *C-ssu* genes. These results and DNA sequencing of PCR products amplified using primers LD5/L635 and LC3/L665 confirmed the orientation of plasmid insertion as depicted in Fig. 4A (data not shown). Additionally, progeny BGN16 and BGN44 carried the whole NSM-type chromosomes 5, 7, and 12, progeny BGN22 had NSM-type chromosomes 5 and 12, and progeny BGN36 had NSM-type chromosomes 7 and 12 (see Table S2 in the supplemental material). The presence of the NSM alleles of the A-, B-, and/or C-type *ssu* genes in these progeny suggested that these genes were not linked to the ODD, because all of these progeny had the ODD phenotype. These results support the hypothesis that insertion of the plasmid into the *D-ssu* in By265G(d) mediates the ODD phenotype.

To ensure that the A- and B-type *ssu* genes were not disrupted by plasmid insertion, we also used A- and B-*ssu*-specific primers (see Table S5) to amplify DNA samples from the parasites. A PCR product was produced from all the parasites when primers specific for the A-*ssu* (products from LA5 and LA3) or B-*ssu* (LB5 and LB3) gene were used (Fig. 3C). None of the primers produced any PCR products when they were paired with the two primers in plasmid pL0017 (L635 or L665), confirming there was no plasmid insertion, although we could not rule out the possibility that the negative PCR results were caused by nonoptimal amplification conditions.

Lack of *D-ssu* rRNA transcript in oocysts of the ODD parasites. To verify that the parasites with disrupted *D-ssu* genes did not produce full *D-ssu* rRNA transcripts, we amplified cDNA and RNA (without reverse transcriptase) samples from By265, By265G(o), By265G(d), NSM, BGN16, BGN44, BGN22, and BGN36 parasites using primers LD5 and LD3, which could amplify the full-length coding sequence of the *D-ssu* gene. As expected, only By265 genomic DNA control and cDNAs from By265, By265G(o), and NSM produced a PCR product of the expected size from day 5 oocysts (Fig. 3D), showing the lack of intact *D-ssu* rRNA transcript in the parasites with the ODD phenotype—By265G(d), BGN16, BGN44, BGN22, and BGN36.

Impaired oocyst development after disruption of *D-ssu* but not *C-ssu*. To confirm that *D-ssu* is essential for mosquito-stage development, we made a linear construct with a human dihydrofolate reductase (*dhfr*) cassette containing a 5' *pbeef1aa*

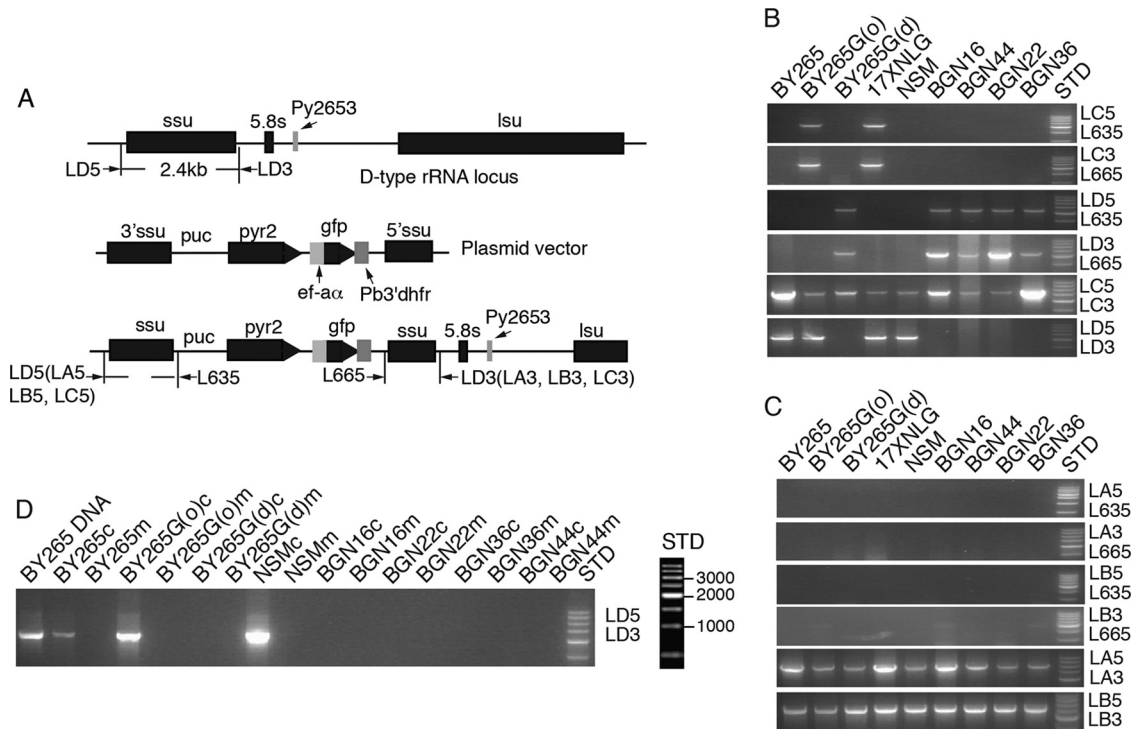


FIG 3 Mapping plasmid insertion sites within the *ssu* loci. (A) Diagram showing gene arrangement in the *Plasmodium yoelii* D-type rRNA locus (top), plasmid vector used to create insertion in the *ssu* locus (middle), and the *D-ssu* locus after plasmid insertion. Relative positions and directions (arrows) of the primers for the amplification of the D-type rRNA (LD5 and LD3) are as indicated. Similarly, LA5 and LA3 for type A, LB5 and LB3 for type B, and LC5 and LC3 for type C are designed to amplify the A, B, and C genes, respectively. LD5 is paired with L635 to detect 5' integration into the D gene, and LD3 is paired with L665 to detect 3' integration. Again, L635 can also be paired with LA5, LB5, or LC5 for the detection of potential 5' integration, and L655 can be paired with LA3, LB3, or LC3 to detect 3' integration into the A, B, or C gene, respectively. (B) PCR amplifications of C- and D-*ssu* genes from various parasite lines and progeny carrying a plasmid insertion in the C- or D-*ssu* genes. LD5/LC5 and L635 detect 5' insertions into D-*ssu* and C-*ssu*, respectively. Similarly, LD3/LC3 and L665 detect 3' insertions. Parasite names are on the top, and the primer pairs used are marked on the right side of the gel images. (C) PCR amplification A- or B-*ssu* genes as in panel B. (D) Amplification of cDNA (marked with "c" at the end of parasite name) and mRNA (no reverse transcriptase controls, marked with "m") from day 5 oocysts of various parasite lines and four progeny using primers LD5 and LD3. STD, DNA size standard.

untranslated region (UTR) and 3' *pbdhfr* UTR as selection marker flanked by two *D-ssu* segments to disrupt the *D-ssu* gene. Six parasite clones (DssuKO1, DssuKO2-1, DssuKO2-2, DssuKO3-1, DssuKO3-2, and DssuKO3-3) were obtained from three independent transfections after WR99210 selection and limiting dilution cloning (Table 1). Insertion of the *hdhfr* cassette into the *D-ssu* locus in the parasite clones was confirmed after PCR amplifications of parasite DNAs using one primer in the *hdhfr* cassette (p8 or p9) and another on the parasite chromosome outside the insertion construct (p7 or p10) (Fig. 4A and B) or the primers flanking the *D-ssu* (Fig. 4C [larger PCR products after insertion]). Amplification of cDNA and mRNA from day 5 oocysts (midgut extracts) from By265G(d) and the three *D-ssu* KO clones showed no *D-ssu* transcript of the expected size (Fig. 4D), confirming the absence of *D-ssu* expression. No insertion into the *C-ssu* gene was detected in the three KO parasites, with amplification of the full *C-ssu* gene of the same size from all the parasites (Fig. 4E), and no PCR products were obtained using primers detecting 5' and 3' integration into *C-ssu*, respectively (Fig. 4F).

Repeated mosquito-feeding experiments with DssuKO1 produced no oocysts; however, smaller and fewer day 9 oocysts (compared with wild-type By265) were found from clones of the DssuKO2 and DssuKO3 parasites, a phenotype similar to that of

By265G(d) (Table 1). No SG sporozoites were observed from DssuKO2 and DssuKO3 parasite clones, and infection of mice with day 15 (and day 20 or 23) SG extracts produced no mouse infection. These results further confirm that *D-ssu* plays an essential role in *P. yoelii* oocyst development.

We also performed two independent *C-ssu* KOs using two different approaches to confirm that *C-ssu* does not cause the ODD phenotype. One approach inserted a linearized plasmid, pL0017 (SacII digestion), carrying a GFP gene into the *C-ssu* locus, as was done for By265G(o) (see Fig. S3A in the supplemental material); another approach used a linear PCR product for double crossovers to replace the *C-ssu* gene, as was done for *D-ssu* KO, except using *C-ssu* flanking sequences (see Fig. S3B). Proper integrations of the constructs into the *C-ssu* locus were detected in two clones (By265/pL0017C1 and -C2) after insertion of the pL0017 plasmid and three clones (By265CKOC1, -C2, and -C3) with replacement of the *C-ssu* gene by the linear PCR products (see Fig. S3C and D). Green oocysts and SG sporozoites were observed in the By265/pL0017C1 and -C2 clones (see Fig. S3E and F). Oocysts of normal sizes and SG sporozoites were also observed in the three By265CKO clones (see Fig. S3G and H). Infection of mice with the SG sporozoites also resulted in blood-stage infection (Table 1), demonstrating that the *C-ssu* deletion does not cause the ODD.

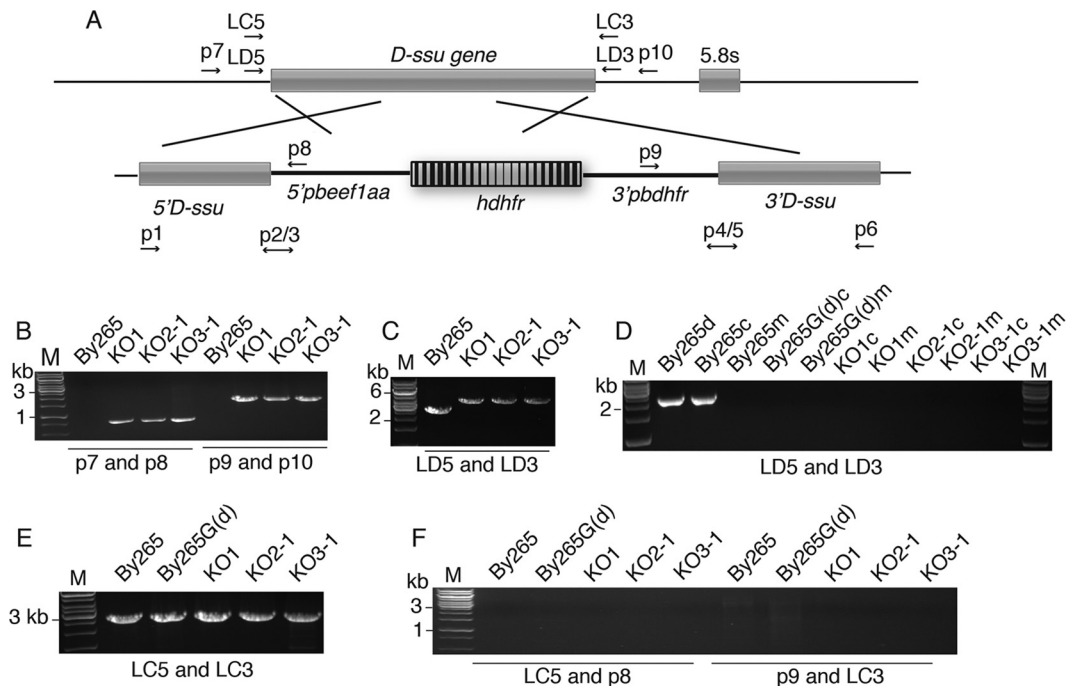


FIG 4 Disruption of the *D-ssu* gene in *Plasmodium yoelii yoelii* By265 and lack of *D-ssu* transcript in KO parasites. (A) Diagram of a linear DNA construct containing the human dihydrofolate reductase gene (*hdhfr*) driven by the *Plasmodium berghei* elongation factor 1 α A promoter (*5' pbeef1aa*) and 3' untranslated region of the *P. berghei dhfr* gene (*3' pbdhfr*). Arrows indicate relative positions of primers (not to the scale of actual sequences) used in PCR amplifications. p1 to p6 are primers used to make the linear construct; p2/3 and p4/5 are pairs of reversed and complemented primers used to connect segments of PCR products. (B) PCR products using primers p7 and p8 (lanes 2 to 5) or p9 and p10 (lanes 6 to 10). Bands of PCR products indicate 5' or 3' integration into the chromosome. Only data from DssuKO1, DssuKO2-1, and DssuKO3-1 are presented here. (C) PCR products from primers p7 and p10. Parasites with an inserted cassette had a larger PCR product than those with wild-type *D-ssu* sequence. (D) RT-PCR detection of RNA transcript from day 5 oocyst using primers LD5 and LD3. (E) Amplification of *C-ssu* genes showing the presence of *C-ssu* in all parasites. (F) No specific PCR band was produced using primers to detect 5' and 3' insertion into the *C-ssu* gene.

Strain-specific interference with oocyst development by complemented episomal *D-ssu*. To elucidate the function of *D-ssu* in oocyst development, we cloned the full *D-ssu* gene plus 978 bp of its 5' flanking sequence and 527 bp of the 3' flanking sequence from By265 and N67 parasites into plasmid pL0007,

which contains the *hdhfr* gene conferring resistance to WR99210, generating plasmids pL0007_ *dssuB* (plasmid containing *D-ssu* from By265) and pL0007_ *dssuN* (plasmid containing *D-ssu* from NSM), respectively (see Fig. S4 in the supplemental material). The circular pL0007_ *dssuB* and control pL0007 plasmids were intro-

TABLE 1 Oocyst counts and mosquito infectivity of parasites with or without *D-ssu* gene

| Parasite | No. of mosquitoes with oocysts/ dissected ^a | Avg no. of oocysts (SD) | Diam of day 9 oocysts (SD), μ m | Day salivary sporozoites observed ^b | Mouse infection ^c |
|----------------------|--|-------------------------|-------------------------------------|--|------------------------------|
| By265 | 98/121 | 24.7 (19.9) | 41.7 (2.4) | 11 | Yes |
| By265/pL0017C1 | 22/30 | 23.2 (20.2) | 37.4 (2.4) | 11 | Yes |
| By265/pL0017C2 | 51/67 | 9.6 (8.0) | 38.4 (2.4) | 11 | Yes |
| By265CKOC1 | 46/54 | 18.8 (13.4) | 38.0 (2.4) | 11 | Yes |
| BY265G(d) | 33/35 | 27.7 (20.8) | 20.3 (2.3) | No | No |
| DssuKO1 ^d | 0/373 | 0 | | | |
| DssuKO2-1 | 29/38 | 3.4 (3.8) | 20.3 (1.7) | No | No |
| DssuKO2-2 | 45/55 | 8.9 (7.9) | 18.6 (2.7) | No | No |
| DssuKO3-1 | 33/44 | 4.8 (4.4) | 19.2 (1.5) | No | No |
| DssuKO3-2 | 31/42 | 4.3 (3.7) | 18.2 (1.6) | No | No |
| DssuKO3-3 | 27/45 | 3.4 (3.7) | 18.4 (2.6) | No | No |

^a Number of mosquitoes with oocysts/total number of mosquitoes dissected.

^b Day when salivary sporozoites were observed. For the KO parasites, no sporozoites were found until day 17 postinfection of mice.

^c "Yes" indicates the presence of blood-stage parasites in mice after injections of salivary gland extract day 15 postinfection of mosquitoes.

^d All mice were injected with 10E-6 iRBCs and were fed to mosquitoes 96 h after injection, except for the DssuKO1 group. For this group, mosquitoes from 15 individual feedings at different feeding times (from 72 to 110 h), dosages (10E-6 or 10E-7), and different mouse strains (BALB/c or CD-1) were dissected. In "DssuKO" designations, the first number after "DssuKO" indicates one of three independent KOs, and the second number indicates the individual clone.

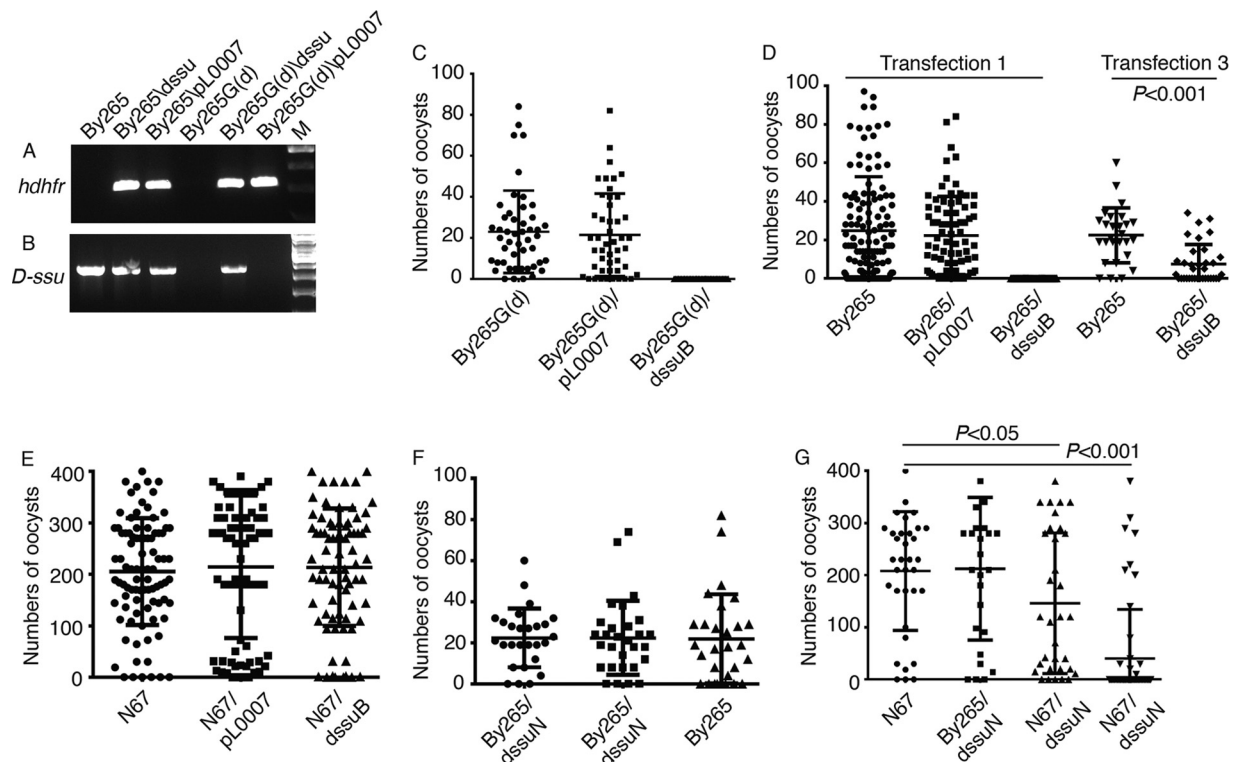


FIG 5 Strain-specific interference with oocyst development by episomal *D-ssu* sequence. Plasmids containing the *D-ssu* gene from *Plasmodium yoelii yoelii* By265 or *P. yoelii Nigeriensis* N67 parasites were introduced into By265G(d), By265, or N67 parasites. (A) PCR detection of the introduced human dihydrofolate reductase (*hdhfr*) gene in parasites transfected with plasmid pL0007 or pL0007_ *dssuB*. (B) PCR detection of *D-ssu* genes in wild-type By265 or the parasite transfected with pL0007_ *dssuB*. (C) Oocyst counts from 1 of 10 feeding experiments. Each dot or square represents the oocyst count from a mosquito infected with By265G(d) or By265G(d) transfected with pL0007 or pL0007_ *dssuB*. (D and E) The same experiments as in panel C but using By265 or N67 parasites, respectively. Shown in panel D are feedings from two independent transfection experiments. Each group in panel E represents the combined oocyst counts from 3 to 4 feedings. (F) Introduction of *D-ssu* from N67 into By265 did not affect oocyst counts in feedings of two independent transfections. (G) Introduction of *D-ssu* from N67 into itself (N67) significantly reduced oocyst counts in feedings of two independent transfections. Significance tested using the *t* test in Excel. The middle level bars are means of oocyst counts, and the upper and lower bars are standard deviations, respectively.

duced into By265G(d) parasites *in vitro*, and the transfected parasites were injected into mice. After 26 h, mice injected with parasites were treated with WR99210 to keep the plasmids in the parasites (see Materials and Methods). The introduced *hdhfr* gene was detected in all the transfected parasites 5 to 7 days postinfection (Fig. 5A), but the *D-ssu* gene was detected only in the wild-type By265 and the parasites transfected with pL0007_ *dssuB*, as expected (Fig. 5B). To our surprise, repeated feeding (10 independent experiments) of the By265G(d) parasites complemented with pL0007_ *D-ssuB* failed to produce any oocyst in 224 mosquitoes dissected, whereas ~80% of the mosquitoes fed on By265G(d) or By265G(d) complemented with the control plasmid pL0007 were infected with an average of >22 oocysts (Fig. 5C and Table 2). We next introduced the pL0007_ *dssuB* into the wild-type By265 parasite. In six feeding experiments using By265 parasites containing pL0007_ *dssuB* from three independent transfections, five produced no oocysts; one had significantly ($P < 0.01$) fewer oocysts than those fed with untransfected By265 or transfected with pL0007 (Fig. 5D and Table 2). Again, 76.7% and 85.0% of the mosquitoes fed on By265 or By265 complemented with pL0007 had averages of >20 oocysts per mosquito, respectively. We also introduced the pL0007_ *dssuB* into the N67 parasite and found that the By265 *D-ssu* had no effect (i.e., produced normal oocysts) on N67 oocyst development (Fig. 5E).

Similarly, introduction of pL0007_ *dssuN* (*D-ssu* from the NSM parasite) into the By265 parasite had no effect on the oocyst numbers (Fig. 5F), but significantly reduced numbers of oocysts ($P < 0.05$) were obtained when the pL0007_ *dssuN* plasmid was introduced into the N67 parasite (Fig. 5G and Table 2). These results suggest that the presence of episomal *D-ssu* may interfere with ribosome assembly and/or the expression of the genes critical for oocyst development and that the effect is strain sequence specific. The reciprocal experiments confirmed the results that the loss of oocyst development was observed only when the episome matched the parasite with the genetic background from which the *D-ssu* gene was derived. The observations also represent an important discovery with major biological implications.

Variations in the *D-ssu* genes between the By265 and N67 parasites. The observations of strain-specific interference of oocyst development after complementation of N67 and By265 parasites suggest that differences in *D-ssu* sequence can affect its function. We therefore obtained the *D-ssu* sequences from By265 and N67 after PCR amplification using the *D-ssu*-specific primers and PCR product direct sequencing (see Table S5 in the supplemental material). Comparison of the sequences detected 26-bp differences mostly clustered in two regions in the 2,067-bp *D-ssu* genes (1.26%) between the parasites (see Fig. S5A in the supplemental material). Although the precise secondary structures of the

TABLE 2 Mosquito infection rates and oocyst counts from parasites complemented with the episomal *D-ssu* gene or control plasmid pL0007

| Parasite | Plasmid | No. of transfections ^a | No. of feedings ^b | No. of mosquitoes dissected | No. (%) of mosquitoes infected | Avg no. of oocysts (SD) ^c |
|-----------|----------------------|-----------------------------------|------------------------------|-----------------------------|--------------------------------|--------------------------------------|
| By265G(d) | pL0007 | 1 | 6 | 148 | 124 (83.8) | 23.0 (20.0) |
| | pL0007_ <i>dssuB</i> | 2 | 4 | 73 | 59 (80.8) | 21.5 (20.1) |
| | | | 10 | 224 | 0 (0.0) | 0 |
| By265 | | | 6 | 163 | 125 (76.7) | 24.3 (26.3) |
| | pL0007 | 1 | 3 | 80 | 68 (85.0) | 22.3 (20.2) |
| | pL0007_ <i>dssuB</i> | 3, a | 4 | 217 | 0 (0.0) | 0 |
| | | b | 2 | 62 | 37 (59.7) | 7.69 (10.1)† |
| | | c | 2 | 49 | 0 (0.0) | 0 |
| | pL0007_ <i>dssuN</i> | 2, a | 1 | 29 | 25 (86.2) | 22.4 (18.0) |
| | | b | 2 | 60 | 51 (85.0) | 25.3 (20.6) |
| N67 | | | 5 | 218 | 209 (95.9) | 219.3 (115.9) |
| | pL0007 | 1 | 4 | 107 | 100 (93.5) | 219.5 (138.3) |
| | pL0007_ <i>dssuB</i> | 2, a | 4 | 132 | 123 (93.2) | 205.2 (116.3) |
| | | b | 1 | 27 | 24 (88.9) | 212.4 (136.8) |
| | | | | | | |
| | pL0007_ <i>dssuN</i> | 2, a | 2 | 65 | 53 (81.5) | 132.8 (117.4)* |
| b | | 3 | 115 | 60 (52.2) | 70.0 (93.2)† | |

^a Number of independent transfections conducted. The letters “a” to “c” indicate individual transfection experiments.

^b Number of independent mosquito feedings.

^c †, $P < 0.001$, and *, $P < 0.05$, by *t* test compared with untransfected controls.

D-ssu genes will require further investigations, structural prediction of the *D-ssu* sequences from By265, N67, and *P. berghei* ANKA modeled on the secondary structure of the *P. falciparum* A-type rRNA (<http://www.rna.cccb.utexas.edu>) (17, 18) showed that the two major variant regions were located at the eukaryote expansion segment ES6S (nucleotides 1645 to 1700) (Fig. S5B to D) of the central domain and ES9S (nucleotides 2464 to 2513) of the 3' major domain at the ribosome head (19, 20). There were also 58 differences in 1,505-bp flanking sequences (3.85%). The lower level of nucleotide substitution in the *D-ssu* genes compared with the flanking regions suggested functional constraint on the *D-ssu* gene. The differences in the sequences and secondary structures could affect binding of the *D-ssu* genes to mRNAs and/or ribosomal proteins and, therefore, the correct assembly of functional ribosomes and efficient protein translation.

Higher transcript levels of *D-ssu* in parasites complemented with episomal *D-ssu*. One potential explanation for the loss of oocysts after *D-ssu* complementation is that the introduced *D-ssu* gene altered the normal transcription dynamics, which may interfere with the programmed processes of oocyst development. To investigate this possibility, we extracted RNAs from blood stages (a mixture of asexual stages and gametocytes), ookinetes prepared as described previously (21), and day 2, 4, and 6 oocysts (midguts), and amplified transcripts of the *D-ssu* and *C-ssu* genes, as well as a heat shock protein 70 (*hsp70*) gene (PYYM0712000, as a control) using gene-specific primers (see Table S5 in the supplemental material). Interestingly, the *D-ssu* transcript could be readily detected at the ookinete stage in the parasites transfected with pL0007_*dssuB* but not (or at a very low level) in By265 or the parasite transfected with pL0007 (Fig. 6A). In contrast, *D-ssu* transcripts were detected in day 4 and day 6 oocysts in By265 and the parasite transfected with pL0007 but not from the parasite transfected with pL0007_*dssuB*, likely due to the lack of oocyst development. Transcripts for *C-ssu* and *hsp70* were detected in all of the parasite stages except day 6 mosquito midguts infected with pL0007_*dssuB* (and day 4 midgut for *hsp70*) (Fig. 6B and C).

These results suggested that the “unusually” high level of *D-ssu* transcripts in the ookinete from the episomal *D-ssuB* could disrupt the programmed process for normal oocyst development. The same sets of experiments were also performed on RNA samples from the N67 parasite complemented with *D-ssu* from N67 (*D-ssuN*) or the pL0007 plasmid (Fig. 6D to F). Similarly, *D-ssu* was not detected in the asexual or gametocyte stages but was detected at a higher level at the ookinete stage when N67 was transfected with the plasmid containing *D-ssuN* (Fig. 6D). However, the expression of the *D-ssu* gene was also detected at all other time points because some oocysts developed normally, although the numbers of oocysts were reduced significantly (Fig. 5G). No obvious changes in *C-ssu* or *hsp70* expression were found (Fig. 6E and F).

DISCUSSION

Our study demonstrates conclusively that *P. yoelii* *D-ssu* is essential for normal oocyst development and that disruption of *D-ssu* leads to growth retardation or complete loss of oocysts for the first time. First, QTL analysis of 48 progeny significantly linked a locus on chromosome 6 containing the *P. yoelii* *D-ssu* gene to the ODD phenotype (LOD score of 17). There were 12 progeny that carried NSM genotypes across the whole chromosome 5 and also had the ODD phenotype, confirming that C-type genes do not cause the ODD phenotype. In particular, the progeny BGN16, BGN22, and BGN44 carried NSM-type genes on chromosome 5 (C-type), chromosome 7 (B-type), and chromosome 12 (A-type) and also had the ODD phenotype, ruling out the requirement of having the defect in both C-type and D-type genes for the ODD phenotype. Additionally, all the progeny that could produce infective sporozoites were not fluorescent and contained the NSM genotype at the *D-ssu* locus. Second, fine mapping of plasmid insertion sites and analysis of *D-ssu* expression in young oocysts of different parasite lines and progeny corroborated the linkage of plasmid insertion into *D-ssu* and the ODD phenotype. There was no evidence of plasmid insertion into the A- or B-type genes in parasites

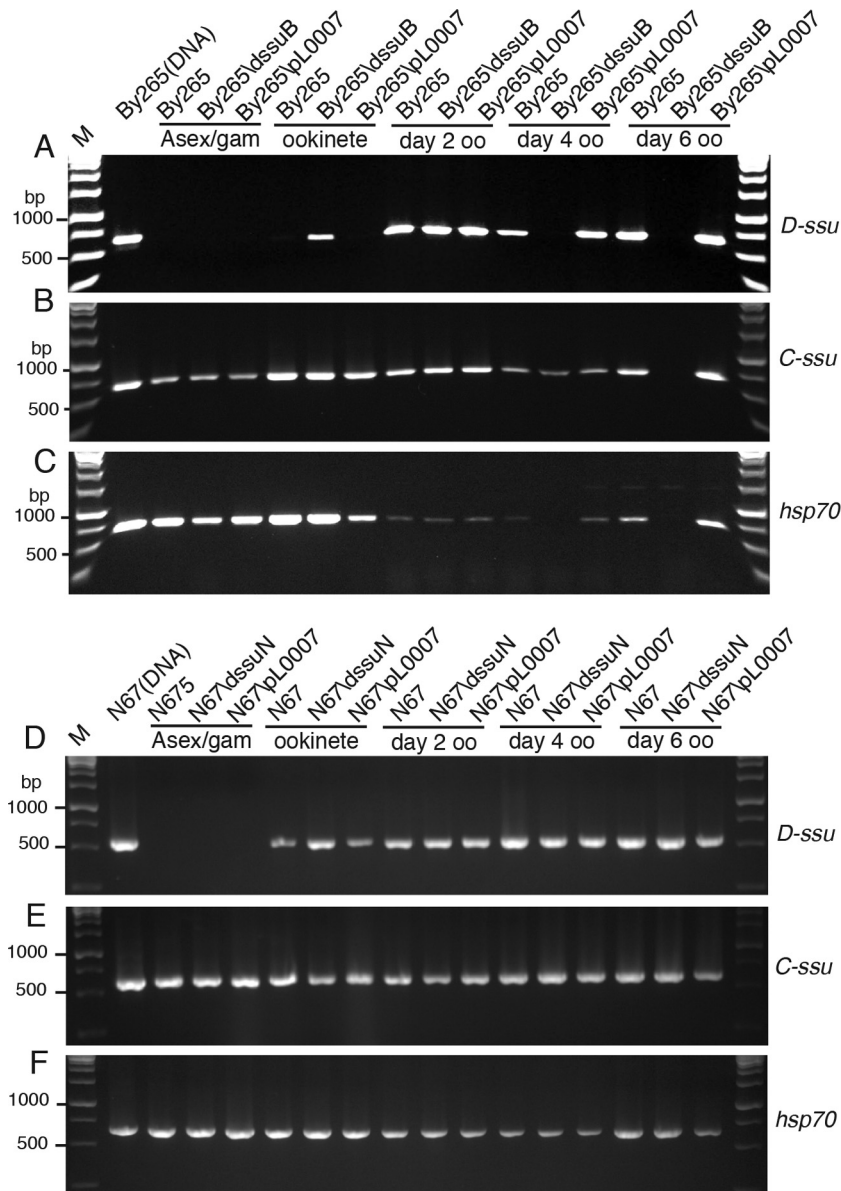


FIG 6 Detection of *C-* and *D-ssu* RNA in preparations containing various parasite developmental stages with or without plasmid complementation. (A) Amplification of *D-ssu* genes from mixtures of asexual stages and gametocytes (asex/gam), cultures with induced ookinetes, and mosquito midguts (oocysts) on days 2 (day 2 oo), 4 (day 4 oo), and 6 (day 6 oo) postfeeding. (B) Amplification of *C-ssu* from the same samples as in panel A. (C) Amplification of a heat shock protein 70 gene (*hsp70*) from the same samples as in panel A. The *C-ssu* and *hsp70* genes act as controls for the presence of parasite materials/RNAs, although the amplifications were not quantitatively performed. By265, *Plasmodium yoelii yoelii* By265 parasite; By265/dssuB, By265 parasites transfected with plasmid pL0007_ *dssuB*; By265/pL0007, By265 parasites transfected with plasmid pL0007. (D to F) The same sets of experiments but using N67 parasite and *D-ssu*N from N67.

with the ODD phenotype. Third, independent gene knockout experiments also showed total loss of oocysts or smaller oocysts, as seen in By265G(d) after disruption of *D-ssu* but not *C-ssu*. These results demonstrate that a functional *D-ssu* gene is required for normal *P. yoelii* oocyst development and maturation, which can be explored for the development of drugs targeting this critical stage of parasite transmission.

To our surprise, episomal complementation of the *D-ssu* gene did not restore normal oocyst development; instead, the introduction of the *D-ssu* gene ablated or disrupted oocyst development of By265G(d) and By265. Although the results were not what we

expected, they again support the conclusion that *D-ssu* plays a role in oocyst development and represent a novel discovery. Unlike a protein-coding gene, an rRNA acts as the central core of a nucleoprotein complex. The formation of a ribosome complex depends on precise processing steps that have to occur in a particular order for the ribosome to be active. Transcriptional analysis showed a higher level of *D-ssu* transcript in the pL0007_ *dssu*-complemented parasite than in wt By265 at the ookinete stage and the absence of *D-ssu* transcript in day 4 and day 6 oocysts. These results suggest that the presence of episomal *D-ssu* and/or a high level of the *D-ssu* transcript may interfere with the

ordered assembly of the ribosome complex specifically required for early oocyst development. Although we still do not know how the endogenous *D-ssu* expression is regulated in the parasite, the “unregulated” expression of the introduced gene could be due to the “unnatural” promoter (we used an ~1-kb *D-ssu* 5' upstream sequence). This issue of an “unusual” high level of *D-ssu* transcript and inhibition of oocyst development requires further investigation and confirmation.

Our data showing stage-specific function of the *D-ssu* gene are also consistent with those from a previous report testing catalytic difference in yeast between the 28S (LSU) rRNA genes expressed in the blood-stage parasites and those expressed in the mosquito (22). By introducing plasmids carrying chimeric genes having A- or S-type 28S rRNA GTPase center from *P. falciparum*, Velichutina et al. (22) were able to obtain yeast cells with a pure population of ribosomes with an A-type GTPase center but not cells containing the S-type chimeric gene. S-type chimeric rRNA was found to severely inhibit yeast growth in the presence of wt yeast rRNA and cause lethality in the absence of the wt yeast rRNA (22). The results showed distinct catalytic properties between the A- and S-type genes, indicating that the same could be true in the parasite.

It is possible that the roles of *D-ssu* in oocyst development are different in other malaria species. Indeed, a previous study concluded that both the *C-ssu* and *D-ssu* genes were not essential for oocyst development in *P. berghei*, although significantly smaller day 10 oocysts were also observed in both *C-* and *D-ssu* KO parasites, including the absence of oocysts in one of the *D-ssu* KO parasites (6). The authors attributed the smaller oocysts in the *P. berghei* *C-* and *D-ssu* KO parasites to dose effect: i.e., both genes contribute to oocyst development, and loss of one gene could retard oocyst growth (6). In our study, disruption of *C-ssu* in By265G(o), 17XNLG, By265CKO1, and By265/pL0017C1 parasites did not prevent production of normal oocysts, which is different from the results obtained from *C-ssu*-disrupted *P. berghei* (6). On the other hand, our observations are consistent with some of the results from the *P. berghei* study: disruption of *D-ssu* leads to smaller oocysts, although the phenotypes (oocyst size and production of infective sporozoites) can vary among the parasites with disrupted *D-ssu* gene. Variation in the phenotypes after *D-ssu* KO can be explained by contributions of other genes in the genome, such as the candidate genes under the peaks of chromosome 13 and at the middle of chromosome 6 (Fig. 2B). Phylogenetic analysis of the *ssu* sequences on chromosome 5 and 6 (*C-* and *D-*types) from *P. berghei* ANKA and *P. yoelii* YM parasites (available in public databases) showed clustering of the *P. berghei* *ssu* gene on chromosome 6 (*D-*-type) more closely with *P. yoelii* *D-ssu* on chromosome 6 than the *P. berghei* *C-*-type gene, and the *P. berghei* *C-*-genes clustered with the *P. yoelii* *C-*-type gene on chromosome 5 (see Fig. S5E in the supplemental material). These results also show that the *P. berghei* *ssu* genes are quite different from those of *P. yoelii*. The differences in the rRNA sequences and the secondary structures may contribute to the binding specificity of some mRNA critical for oocyst development and the observed phenotypic variations.

Our observations of strain-specific interference of oocyst development by episomal *D-ssu* are consistent with the hypothesis that differences in *D-ssu* sequence can affect its function in different genetic backgrounds. These observations also predict differences in ribosomal proteins or mRNAs and their binding to the

D-ssu genes between the two parasites. Differential expression of distinct rRNAs having different upstream regulatory regions in response to environmental changes has been described in *Escherichia coli*, suggesting a potential means to regulate translation in response to environmental factors (23, 24). The observation of strain- and stage-specific *D-ssu* genes for *P. yoelii* oocyst development adds another example for the “ribosome filter” hypothesis that ribosomal subunits themselves are regulatory elements or filters mediating interactions between particular mRNAs and components of the translation machinery, and differences in the interactions or filters can affect translation of particular sets of mRNA (25). Our observations open up an interesting area for studying ribosomal genes in regulating protein translation, gene expression, and parasite development, which may lead to strategies for blocking parasite transmission.

MATERIALS AND METHODS

Parasites, mice, and mosquitoes. All parasite strains used in this study have been described previously (12, 13). By265G(o) was a parasite generated previously by inserting a plasmid (pL0017 by A. P. Waters and colleagues) carrying the gene encoding a green fluorescent protein (GFP) from the jellyfish *Aequorea victoriae* and the *Toxoplasma gondii* dihydrofolate reductase (*TgDHFR*) gene into the genome of the By265 parasite (12). Another green fluorescent parasite, 17XNLG (*P. yoelii* 17XNLG), used in this study was a gift from Ana Rodriguez (New York University School of Medicine) (16). By265, N67, and NSM are abbreviated names for *P. yoelii* By265, *P. yoelii* N67, and *P. yoelii* NSM. NSM was derived from N67 after mefloquine selection, and these two parasites are essentially the same (26). The three parasites are all sensitive to pyrimethamine. A colony of *Anopheles stephensi* mosquitoes (Nijmegen strain) was maintained at 23°C and 70 to 80% relative humidity and fed with 5% sugar solution. Kunming (KM) outbred mice or BALB/c inbred mice (ages 6 to 8 wk), purchased from the Institute of Laboratory Animals of the Third Military Medical University, the Laboratory Animal Center of Xiamen University, China, or Charles River Laboratories, were used in the experiments. A summary of parasite origin and relationship, oocyst phenotypes, and the general strategy of this study is presented in Fig. S1 in the supplemental material. All animal procedures were performed in accordance with the approved protocols by the following Institutional Animal Care and Use Committees (IACUC): the Institutional Animal Care and Use Committee of the Third Military Medical University (permit no. syxk-PLA-2007035), the Laboratory Animal Management and Ethics Committee of Xiamen University (permit no. xmulac20120016), and the National Institute of Allergy and Infectious Diseases (NIAID) Division of Intramural Research ACUC (permit no. LMVR11E) following the guidelines of the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (27) and AAALAC.

Parasitemia and infection of mice and mosquitoes. To initiate a blood-stage infection, 100 μ l of donor mouse blood containing approximately 1×10^6 infected red blood cells (iRBCs) was injected intraperitoneally (i.p.) into a recipient mouse. Parasitemia and gametocytemia were measured as previously described (28). The mosquitoes were dissected on days 9, 11, 13, 15, and 17 after the blood meal, and mosquito midguts were examined for oocysts under a light microscope. Sporozoites within developing oocysts and in the SG were also recorded.

Genetic crosses and parasite cloning. Genetic crosses were performed according to procedures described previously (13, 29). For mapping the ODD phenotype, By265G(d) parasites that produced no functional oocysts or sporozoites were crossed with NSM (or N67) parasites that produced an average of ~200 oocysts per mosquito with large numbers of sporozoites (28). Before performing genetic crosses, we cloned By265G(d) and obtained nine clones. The parasites were genotyped with 14 microsatellite (MS) markers (see Table S2 in the supplemental material) and were shown to be clonal. Single-clone infections of By265G(d)

and NSM were initiated individually in donor mice. Parasitemia was monitored daily 4 days postinoculation. Nine independent genetic crosses were performed using two different parental ratios: By265G(d) to NSM (N67) ratios of 15:1 or 10:1 (see Table S1 in the supplemental material).

Drug selection for recombinant progeny. To improve the frequency of obtaining recombinant progeny, we also treated parasite mixtures after crossing with pyrimethamine (5 mg/kg in PBS) to kill progeny from self-fertilization of parental genotypes (see Fig. S1 in the supplemental material). In theory, only recombinant progeny carrying the plasmid from By265G(d) and a putative element (gene) critical for oocyst development from the NSM parasite will pass through the mosquito and survive drug treatment. Blood-stage parasites from the mouse infected with sporozoites after a genetic cross were transferred to new mice. Some mice were given pyrimethamine intramuscularly 26 h postinoculation for 3 consecutive days, whereas others were not treated before cloning. The parasites were cloned when parasitemia was <1%. When parasitemia reached 5 to 10%, the parasites were either frozen for further use or lysed for DNA extraction.

MS typing and QTL analysis. DNA samples were extracted from 100 to 200 μ l of iRBCs using a commercial blood sample extraction kit (Qiagen). PCR primers for the MS markers were described previously (30). To facilitate separation and detection of PCR products in a regular agarose gel, we selected MS markers that produced large differences (>8 bp) in PCR products between the cross parents. PCR in a 15- μ l volume was performed under conditions described previously (31). The cycling program included 94°C for 2 min for initial denaturation, followed by 94°C for 20 s, 55 to 50°C for 20 s, and 60°C for 30 s for 40 cycles and a final extension at 60°C for 2 min. PCR products were separated on 2% to 4% agarose gels, and the sizes of the PCR products were estimated and recorded to generate genotype maps for each recombinant progeny.

Quantitative trait locus (QTL) analysis was conducted using the R/qtl library in R2.12.2 software (<http://www.rqtl.org/>) (14) as described previously (13). Oocyst size measurements from the 48 progeny of the N67/NSM and By265G(d) crosses and genotypes from 231 MS markers were analyzed using standard interval mapping (the Expectation Maximization algorithm). A single-QTL genome scan was done first with all the MS markers with day 9, 11, and 17 oocyst size measurements. A two-dimensional scan and finally two-QTL genome scans were performed to detect potential epistatic interactions between the loci significantly linked to the ODD phenotype.

Mapping plasmid insertion sites. Because the pL0017 plasmid was designed for insertion into the *C*- and/or *D-ssu* genes, we designed sequence-specific PCR primers to map the plasmid insertion sites (see Table S5 in the supplemental material). The PCR conditions were as follows: 94°C for 2 min for initial denaturation, followed by 94°C for 20 s, 55 to 50°C for 20 s, and 60°C for 30 s for 40 cycles and a final extension at 60°C for 2 min. PCR products were separated on 1% agarose gels and photographed.

Parasite transformation and gene KO. To further confirm that mutations in the *D-ssu* gene play a role in ODD, we made a linear DNA construct to knock out the *D-ssu* gene in the By265 parasite by connecting three pieces of DNA segments using a PCR strategy described previously (32). The construct consisted of segments of 5' and 3' *D-ssu* sequences flanking a drug-resistant cassette containing the gene encoding human DHFR (hDHFR) driven by *P. berghei* elongation factor 1 α A (*pbeef1aa*) promoter (5') and 3' untranslated region (UTR) of the *P. berghei dhfr/fts* gene (*pbdhfr/fts*) (Fig. 5A). Parasites from mouse blood were cultured in RPMI 1640 supplemented with 20% fetal bovine serum, 3% NaHCO₃, 0.1% gentamicin, and 0.5% neomycin for 12 h *in vitro*. Cultured parasites were transformed in 100 μ l of culture medium containing 10 μ g of DNA using the Amaxa Nucleofector Device II (Lonza). The transfected parasites were then injected into three mice that were treated with WR99210 (i.p. injection of 5 mg/kg of body weight) 26 h later for 3 days. DNA from blood stages was extracted for detection of integration into the targeted DNA locus using PCR. One of the PCR primers was located outside the

homologous targeting region and the other within the *dhfr* cassette. After detection of integration, the parasites were cloned using limiting dilution to obtain parasites with disrupted *D-ssu* genes. Parasites with disrupted *D-ssu* genes were further evaluated for oocyst development phenotypes in mosquitoes. Disruption of the *C-ssu* gene was performed similarly, except with *C-ssu*-specific primers.

Gene complementation. To complement the ODD gene defect and to confirm the function of *D-ssu* in oocyst development, we PCR amplified (primers *dssu_EcoRI* and *dssu_NotI* [see Table S5 in the supplemental material]) the full *D-ssu* gene plus 978 bp of its 5' flanking sequence and 527 bp of the 3' flanking sequence from By265 or N67 parasites and cloned the PCR products into the plasmid pL0007 through the NotI (GCGGCCGC) and EcoRI (GAATTC) sites. The plasmid pL0007 obtained from MR4 contained DNA sequence encoding hDHFR driven by the *pbeef1aa* promoter (5') and 3' untranslated region (3' UTR) of *pbdhfr/fts*, generating plasmid pL0007_ *dssuB* from By265 and pL0007_ *dssuN* from N67, respectively (see Fig. S4 in the supplemental material). The parasites were transfected and selected as described above. The presence of the transfected plasmids and the *D-ssu* genes was detected using primers *hdhfrs/hdhfrs* (*dhfr* primers) and D-F1/D-R1 (*D-ssu* primers) (see Table S5 in the supplemental material). RNA samples from mixtures of asexual and gametocytes and ookinetes were prepared as described previously (21), and mosquito midguts containing oocysts were extracted using TRIzol reagent (Life Technologies). The RNA samples were reverse transcribed using random hexamers and amplified using primers for *D-ssu* (D-F1/D-R1), *C-ssu* (C-F1/C-R1), and *hsp70* (HSP70-F1/HSP70-R1) (see Table S5 in the supplemental material). The PCR cycling conditions were 94°C for 2 min, followed by 94°C for 10 s, 50°C to 55°C for 10 s each, and 65°C for 30 s for 32 cycles and a final extension at 65°C for 2 min.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00117-15/-/DCSupplemental>.

Figure S1, PDF file, 0.1 MB.

Figure S2, PDF file, 1.1 MB.

Figure S3, PDF file, 0.9 MB.

Figure S4, PDF file, 0.04 MB.

Figure S5, PDF file, 2.8 MB.

Table S1, XLSX file, 0.01 MB.

Table S2, XLSX file, 0.2 MB.

Table S3, XLSX file, 0.02 MB.

Table S4, XLSX file, 0.02 MB.

Table S5, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China no. 81271858, 81220108019, and no. 81201324, by Project 111 of the State Bureau of Foreign Experts and Ministry of Education of China (B06016), by the Fundamental Research Funds for the Central Universities (2011121031), and by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

We thank Brenda Rae Marshall, DPSS, NIAID, for editing.

Y.Q., F.Z., R.T.E., Y.F., L.H., and S.L. performed genetic crosses, gene knockout, and other experiments. M.Z. and S.P. designed experiments and analyzed data. T.F.M., W.P., F.H., J.L., and W.X. designed experiments, supervised daily laboratory activities, and wrote the paper. X.-Z.S. conceived the project, designed experiments, analyzed data, and wrote the paper.

The authors declare they have no conflicts of interest.

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