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Transfection of the malaria parasite and expression of firefly luciferase

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ABSTRACT **The goal of this work is to develop a method for the functional analysis of malaria genes using the method of DNA transfection. We have developed a transient transfection vector by constructing a chimeric gene in which the firefly luciferase gene was inserted in frame into the coding region of the pgs28 gene of *Plasmodium gallinaceum*. This plasmid DNA was introduced into *P. gallinaceum* gametes and fertilized zygotes by electroporation, and luciferase expression was assayed after 24 hr. This report of successful introduction and expression of a foreign gene in a malaria parasite demonstrates the feasibility of this approach to developing methods for the functional analysis of parasite genes.**

Malaria remains a major threat to world health. Efforts to control the disease have focused on chemotherapy, mosquito control, and, most recently, vaccine development. These efforts have been hampered by the emergence and spread of drug-resistant parasites, the breakdown of malaria control programs due to both insecticide-resistant mosquitoes and upheavals in spraying programs, and the complicated problems of vaccine development and testing. The world faces an increasing threat of malaria in the 1990s, with few new tools to combat the parasite or the disease (1).

One of the underlying problems in developing newer methods of control is that the basic biology of the parasite has not been fully investigated, primarily because of the lack of a method for functional analysis of genes and their products. The goal of the work presented here is to develop a method for the functional analysis of genes that uses the method of DNA transfection. This type of method is a critical next step in the functional analysis of parasite genes and is required for a detailed analysis of the control of expression of parasite genes. Such methods have been critical in dissecting the mechanisms of bacterial pathogenesis and, more recently, in the development of vaccines for several important bacterial pathogens.

The malaria parasite presents a unique challenge for transfection because it is intracellular for most of its life cycle. Introduced DNA must cross multiple membrane barriers before reaching the parasite nucleus. Because these multiple barriers would be likely to reduce the efficiency of introducing DNA into the parasite, we chose a parasite stage which is extracellular, the female gamete and fertilized zygote. Methods had previously been developed for the purification of gametes and fertilized zygotes of the avian malaria *Plasmodium gallinaceum* (2–5). Further, several genes of *P. gallinaceum* have been identified which are expressed at high level in the gamete/zygote stages of the parasite and one of these, pgs28, had been cloned with enough flanking DNA to assume that the necessary 5′ and 3′ controlling elements for the expression of this gene were available for developing a transfection vector (6).

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MATERIALS AND METHODS

Plasmid Construction. The pgs28 plasmid was previously described and the sequence is available in GenBank (accession no. M96886) (6). For these experiments, the pgs28 plasmid was digested with *Pst* I and *Sma* I to remove a small segment of the polylinker containing a *Bam*HI site, incubated with the Klenow fragment of DNA polymerase I under conditions to produce a blunt end at the *Pst* I site, and religated to create the pgs28.1 plasmid, which has a unique *Bam*HI site in the coding region of the pgs28 protein. The luciferase coding region (7) was obtained by PCR amplification of the pGem-*luc* (Promega) plasmid with primers [R1, 5′-GCGGATCCAGAAGACGCCAAAAACATAAAG-3′ (5′ end of gene), and R2, 5′-GCGGATCCAATTTGGACTTTCCGCCCTT-3′] containing synthetic *Bam*HI sites. The *Bam*HI-digested PCR product was incubated with *Bam*HI-digested pgs28.1 under conditions for ligation. The resulting plasmid, pgs28.1LUC (Fig. 1), contains the firefly luciferase coding region inserted in frame with the pgs28 coding sequence. This structure was confirmed by restriction analysis, PCR analysis with primers 69a (5′-GGAAGCTTAACAGCTATGACCATGATTAC-3′) and R2, and DNA sequence analysis. Plasmid pgs28.1LUCR was obtained by digestion of pgs28.1LUC with *Bam*HI and religation. The structure was confirmed by restriction digestion and PCR analysis as above. The pgs28.1GALR plasmid was obtained by a similar approach. The β-galactosidase coding region was purified from the pJ3-β-gal plasmid (gift of C. Cepko, Harvard University) after *Bam*HI digestion. The *Bam*HI-digested product was incubated with *Bam*HI-digested pgs28.1 under conditions for ligation and the resulting plasmid, pgs28.1GALR, has the β-galactosidase gene inserted in the inverse orientation relative to the pgs28 coding region.

Electroporation. White Leghorn chickens (3–5 weeks old) were inoculated with 0.1 ml of *P. gallinaceum*-infected blood and the parasitemia was followed daily. Blood was withdrawn (parasitemia 60–80%), and exflagellation and purification of fertilized zygotes and gametes were as previously described (2–5, 8, 9). The zygote/gamete fraction (1–2 × 10^7 cells) was resuspended in Cytomix (10) and incubated in the presence or absence of plasmid DNA with or without electroporation. Unless otherwise indicated, all electroporations were performed under conditions of 2500 V/cm, 25 μF, with a time constant of 0.4. The cells were then washed and resuspended in ookinete maturation medium and incubated for 24 hr (11).

Luciferase Activity. The parasites were examined microscopically after 24 hr, pelleted, and suspended in luciferase

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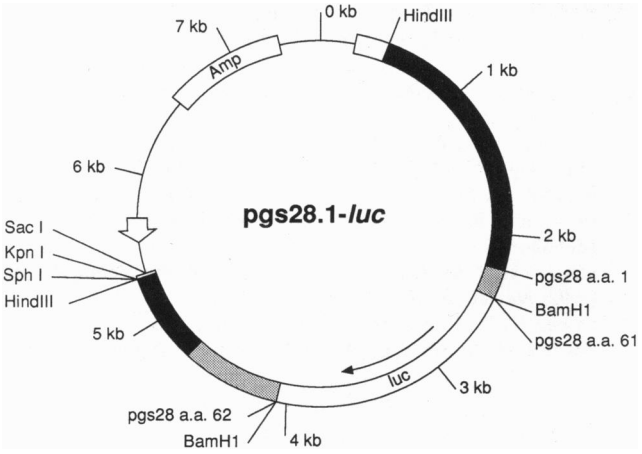

FIG. 1. Map of pgs28.1LUC. Amp, ampicillin-resistance gene; luc, luciferase gene; a.a., amino acid.

lysis buffer [25 mM Tris, pH 7.8 with H_3PO_4/2 mM 1,2-cyclohexanediaminetetraacetic acid/2 mM dithiothreitol/10% (vol/vol) glycerol/1% (vol/vol) Triton X-100]. In each experiment, an aliquot of the ookinete maturation medium was analyzed for sterility by plating in rich bacterial medium. No contamination was observed in any experiment. In each case, a standard curve was obtained at the same time for firefly luciferase (Sigma catalogue no. L5256, 18.9×10^6 light units/mg of protein) using the same substrate. The luciferase assay was performed with a luciferase assay kit (Promega, no. E1500) according to the manufacturer's instructions [substrates were 270 μM coenzyme A (lithium salt), 470 μM luciferin, and 530 μM ATP in 20 mM Tricine/1.07 mM $(MgCO_3)_4Mg(OH)_2$/2.67 mM $MgSO_4$/0.1 mM EDTA/33.3 mM dithiothreitol, final pH 7.8]. The results presented are in light units measured with an LKB BioOrbit 1251 luminometer. The amount of luciferase activity in each assay was calculated by using the standard curve which was assayed simultaneously. Each assay represents 10 μl of a 50-μl reaction mixture. For several experiments, an additional aliquot of each reaction mixture was assayed on a Lumat Berthold LB9501 luminometer.

RESULTS AND DISCUSSION

Two lines of experimentation were pursued simultaneously: the development of a system for the introduction of DNA into the gametes and zygotes and the construction of a transient expression vector which could be used to assay for expression of introduced DNA. Chickens were infected with *P. gallinaceum*, infected blood was withdrawn and allowed to undergo gametogenesis, and the gametes and fertilized zygotes were harvested and purified. These parasites were then suspended either in "suspended animation" (SA) buffer (10 mM Tris/170 mM NaCl/10 mM glucose, pH 7.4) or in Cytomix and subjected to electroporation under various conditions. The parasites were washed and incubated in ookinete development medium (11) for 24–48 hr. Ookinete development was then assayed by microscopic examination. Ookinete development was dependent both on the concentration of cells during development and on electroporation. The latter resulted in a lower yield of mature ookinetes. By using several different electroporation conditions and then assaying for ookinete development by microscopic examination, we determined electroporation conditions (2500 V/cm, 25 μF in Cytomix) which routinely gave an ≈50% reduction in the yield of mature ookinetes (data not shown).

The transient-transfection vector was constructed as follows. A 3.0-kb *Hin*dIII fragment including both the pgs28 coding region and flanking DNA was cloned into pUC13 and used for further development of the transient-transfection vector (6). DNA sequence analysis had identified a unique *Bam*HI site in the codons for amino acids 61 and 62 of pgs28 protein (6). Primers were prepared which allowed for the insertion of the firefly luciferase gene in frame at this position (plasmid pgs28.1LUC, see Fig. 1). Analysis of the resulting plasmid by PCR, restriction digestion, and sequence analysis has confirmed the in-frame insertion of the luciferase gene. A second plasmid (pgs28.1LUCR), in which the luciferase gene was inserted in the inverse orientation, was used as a negative control. A third plasmid (pgs28.1GALR), containing the bacterial β-galactosidase gene, was also used as a negative control.

Table 1. Expression of firefly luciferase in *P. gallinaceum*

Exp.	Cell no. $\times 10^{-7}$	Electroporation*	Plasmid (100 μg)	Luciferase, light units
1	2	+	pgs28.1LUC	154.7
	2	+	None	15.6
2	2.5	+	pgs28.1LUC	447.3
	2.5	−	pgs28.1LUC	7.9
3	1.3	+	pgs28.1LUC	179.2
	1.3	+	pgs28.1GALR	8.2
	1.3	+	None	8.8
4	1.1	+	pgs28.1LUC	707.3
	1.1	+	pgs28.1LUCR	8.5
5	1	1500 V/cm	pgs28.1LUC	86.3
	1	2500 V/cm	pgs28.1LUC	340.5
	1	3000 V/cm	pgs28.1LUC	21.5
6	0.75	+	pgs28.1LUC	746.5
	0.75	+	pGEM-LUC	8.8

*At 2500 V/cm, 25 μF unless otherwise noted.

All of the plasmids used for electroporation were purified by CsCl gradient centrifugation. Gametes/zygotes (2×10^7 cells) were electroporated in the presence of 100 μg of plasmid pgs28.1LUC. In control experiments, the same number of parasites were incubated in the presence of plasmid DNA without electroporation, electroporated in the absence of exogenous DNA, or electroporated in the presence of control plasmids (pgs28.1LUCR, pgs28.1GALR, pGEM-*luc*). The cells were washed and suspended in maturation medium. The cells were harvested after 24 hr and assayed for luciferase activity (Table 1). Luciferase activity was detected only in lysates of those parasites which had been electroporated in the presence of pgs28.1LUC. The amount of luciferase activity was dependent on electroporation conditions. No activity was detected in parasites incubated with that plasmid without electroporation or in parasites which had been electroporated with any of the control plasmids. In all experiments, an aliquot of each tube was assayed for sterility and no bacterial or fungal contamination was detected.

These results demonstrate transient expression of a reporter gene in the malaria parasite. The method constitutes the first step in the development of a transfection system. Clearly, the next step will be development of stable transfectants by using a selectable marker. Further, because the pgs28 gene is expressed only in sexual stages, modification of the flanking sequences to remove stage-specific controlling elements or identification of another gene expressed in other or all life-cycle stages will be necessary for analysis of gene expression in asexual or sporozoite stages. Nonetheless, the successful introduction and expression of a foreign gene in a malaria parasite demonstrate the feasibility of this approach to developing methods for the functional analysis of parasite genes.

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