J. Protozool. Res. 13, 10-14 (2003)
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TRANSIENT EXPRESSION OF A GREEN FLUORESCENT PROTEIN GENE IN *BABESIA BOVIS*

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Abstract

In hemoprotozoa, gene transfer technology provides an important tool to aid in the functional study of parasite genes. In this study, a transfer vector containing the enhanced green fluorescent protein (EGFP) gene laid between the *Toxoplasma gondii* GRA1 promoter region and the GRA2 polyA signal region was constructed and transfected into the *in vitro* culture of *Babesia bovis* by the electroporation method. On the first and second days post-transfection, clear positive fluorescences were detected in some parasite bodies, indicating the successful expression of the EGFP gene controlled by the GRA-1 promoter in *B. bovis*. However, the positive parasites were not ubiquitous and finally disappeared until the forth day post-transfection. This is the first time that the expression of a foreign gene has been reported in the *Babesia* parasite.

Introduction

In *Plasmodium* parasites, gene transfer technology has provided an important tool to aid in the functional study of parasite genes [Waterkeyn et al., 1999]. After transfection, the plasmids are transiently maintained as episomes in the parasites, which may eventually integrate into chromosomes after a period of 90-100 days. This period allows for gene expression and promoter dissection experiments [Kadekoppala et al., 2001]. Although the gene transfer technique may be applicable for understanding the molecular biology of hemoprotozoas, it has not been used in the study of *Babesia* parasites.

Babesia parasites are tick-transmitted homoprotozoas of the phylum Apicomplexa. They affect a wide variety of wild and domestic animals and are responsible for enormous economic losses to the livestock industry worldwide [Kuttler, 1988]. During their asexual growth cycle in a natural host, merozoites internalize the host erythrocytes (RBC) via multiple adhesive interactions of several protozoan molecules with the host cell surface [Yokoyama et al., 2002]. Thus, the parasites destroy the infected RBC, which results in severe clinical symptoms, such as high fever, anemia, and haematuria/haemoglobinuria, in the infected hosts. Therefore, understanding of the basic molecular mechanisms of parasite genes involved in the asexual growth cycle may contribute to a faster development of effective therapeutic and preventive methods for babesiosis.

In this study, we constructed a transfer vector containing an enhanced green fluorescent protein (EGFP) gene following the *Toxoplasma gondii* GRA1 promoter region, in which the promoter was used to express the target foreign gene in the transfected *T. gondii* [Xuan et al., in preparation]. By the electroporation method, the vector was transfected into the *in vitro* culture of *B. bovis*, a highly pathogenic hemoprotozoa in cattle [Kuttler, 1988], and the efficacy of EGFP gene expression in the parasite body was evaluated by confocal laser-scanning microscopy.

Materials and Methods

Parasite and culture medium

The Texan strain of *B. bovis* [Suarez et al., 2000] was maintained in purified bovine RBC using a microaerophilus stationary-phase culture system [Levi and Ristic, 1980]. Media M199 (Sigma-Aldrich, Tokyo, Japan) was added with 229 mg/ml N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid hemisodium salt (Sigma-Aldrich) as a pH stabilizer (pH 7.2) [Erp et al., 1980] and then supplemented with 40% bovine normal serum, 60 units /ml penicillin G, 60 μg/ml streptomycin, and 0.15 μg/ml amphotericin B (Sigma-Aldrich) in order to use as a culture medium for *B. bovis*.

Construction of a transfer vector

A transfer vector was constructed on the basis of a plasmid, pKS/GRA5'-GRA3', which contains a promoter sequence upstream of the *T. gondii* GRA1 gene and a polyA signal sequence downstream of the *T. gondii* GRA2 gene in pBluescript KS(+) [Xuan et al., in preparation]. In detail, a 732-bp *Eco*RI DNA fragment containing an open reading frame of EGFP was isolated from pCX-EGFP [Okabe et al., 1997] and inserted into the *Eco*RI site of the pKS/GRA5'-GRA3'. Accurate cloning of the transfer vector was confirmed by nucleotide sequencing using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Japan, Ltd., Tokyo, Japan) with an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The resultant vector, designated as pKS/tEGFP, has the EGPF gene laid between the GRA-1 promoter and the GRA2 polyA signal regions.

Transfection by electroporation

Fifty microliters of the *B. bovis*-infected RBC (5% parasitemia) was washed with phosphate-buffered saline (PBS) and sequentially with a cytomix buffer (25 mM HEPES (pH 7.6), 10 mM K2HPO4/KH2PO4 (pH 7.6), 120 mM KCl, 0.25 mM $CaCl_2$, 5 mM $MgCl_2$) and then resuspended in 470 μl of the cytomix buffer. The suspension was added with 5 μl of 200 mM ATP, 25μl of 100 mM glutathione, and 20 μg of the transfer vector. Four hundred microliters of the suspension was transferred into a sterile 2-mm gap electroporation cuvette (Bio-Rad, Calfornia, USA) and then subjected to electroporation using a BioRad gene pulser II (Bio-Rad) with the following setting: 310 V and 960 μF. The transfected suspension was centrifuged at 1,000 x *g* for 5 min at 4^0C for removing the supernatant and then mixed with 0.9 ml of the culture medium and 0.1 ml of fresh RBC. The culture was incubated in a humidified multi-gas water-jacketed incubator at 37^0C for 4 days, daily replacing with 0.9 ml of fresh culture medium.

Fluorescent confocal microscopy

After two washes with PBS, smears of the transfected culture pellet were prepared daily on slides and fixed in 50% acetone-50% methanol solution for 5 min at -20^0C. The slides were mounted in 50% glycerol-PBS and then observed under an ultraviolet (UV) light with confocal laser-scanning microscopy (TCS NT, Leica, Germany).

Results and Discussion

In this study, we constructed and transfected a transfer vector, pKS/tEGFP, in which an EGFP marker gene follows the *T. gondii* GRA1 promoter sequence, into the *in vitro* culture of *B. bovis* by the electroporation method. On the first and second days post-transfection, a clear positive fluorescence was detected in the parasite body. As seen in Figure 1, some ring (A) and also subsequent pear-shaped (B) forms of parasites showed positive signals in the cytoplasm. The ring-stage parasite expressing the EGFP gene was often unstable in shape (Fig. 1A), in contrast with the typical form, which suggests that the first-stage parasite seems to do some damage since the vector was preferably introduced into extracellular merozoites by electroporation. Additionally, the percentage of positive parasites was too low, representing about 0.01% of the total parasites, and the positive reactions finally disappeared until the fourth day post-transfection. On the other hand, no fluorescence was detectable in the parasites non-transfected or transfected with pCX-EGFP (data not shown), in which a mammalian promoter drives the EGFP gene expression [Niwa et al., 1991]. The present finding provides information about the transient but successful expression of the EGFP gene controlled by the GRA1 promoter in *B. bovis*.

EGFP expression was not ubiquitous, and the efficacy of transfection was not considered to be sufficient to apply to the biological study of *Babesia* parasites. Successful stable transfection of *P. falciparum* relies on transfer vectors carrying sequences able to mediate gene expression and confer a selectable phenotype, such as drug resistance [Waterkeyn et al., 1999]. Previously, we reported the inhibitory effect of pyrimethamine on the growth of *Babesia* parasites [Nagai et al., 2003]. Because a resistant marker gene, TgDHFR-ts, against the pyrimethamine has often been used in *Plasmodium* parasites [Kadekoppala et al., 2001], the development of a stable transformation system by using the pyrimethamine selection might be helpful in increasing the efficacy for obtaining the recombinant *Babesia* parasites expressing the foreign gene. Further studies will be required.

In conclusion, this report describes, for the first time, the transfection and resultant expression of the EGFP gene in *B. bovis* with the aid of the *T. gondii* GAR1 promoter. This finding will be available for further development of gene transfer technology in order to study the babesial gene function and contribute to a faster development of vaccines and drugs in the future.

Acknowledgments

We are grateful to Dr. Jun-ichi Miyazaki for kindly providing a plasmid, pCX-EGFP. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and by a grant from The 21st Century COE Program (A-1), Ministry of Education, Culture, Sport, Science, and Technology, Japan.

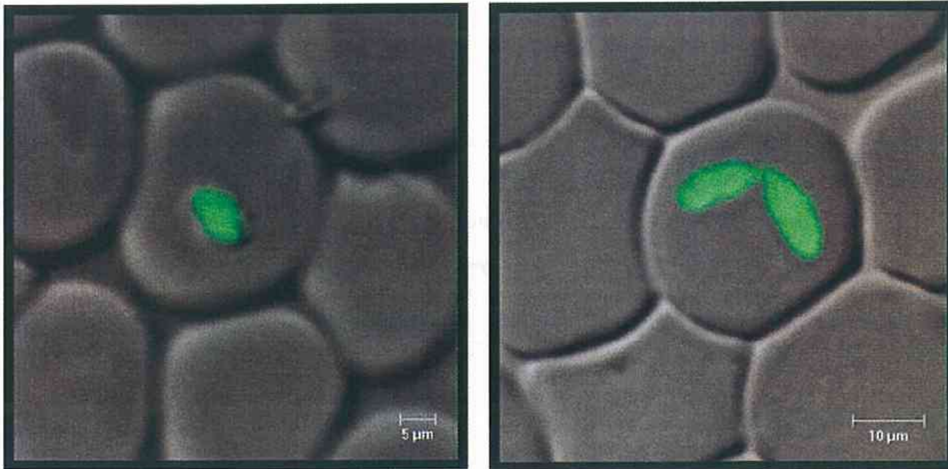

Fig. 1. Detection of EGFP fluorescence using a confocal laser-scanning microscope.
After transfection with pKS/tEGFP, acetone/methanol-fixed smears of the *B. bovis*-infected RBC were prepared and observed by confocal laser-scanning microscopy. Positive fluorescences (green) were visualized under a UV light.
Bar=10 µm.

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