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Short communication

Identification and characterization of interchangeable cross-species functional promoters between *Babesia gibsoni* and *Babesia bovis*

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ABSTRACT

The development of transgenic techniques has been reported in many protozoan parasites over the past few years. We recently established a successful transient transfection system for *Babesia gibsoni* based on *Bg 5′-ef-1α* promoter. This study investigated 6 homologous and 6 heterologous promoters for *B. gibsoni* and *B. bovis* and identified novel interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis*. Ten out of twelve promoters had heterologous promoter function. In particular, *Bg 5′-ef-1α* and *Bg 5′-actin* heterologous promoters resulted in a significantly higher luciferase activity than *Bb 5′-ef-1α* homologous promoter in *B. bovis*. The present study showed that *Bg 5′-actin* promoted the highest luciferase activity in both *B. gibsoni* and *B. bovis*. The study further indicates that heterologous promoter function widely exists between *B. gibsoni* and *B. bovis*. This finding is an important step for future stable transfection construct design and for the production of vaccines based on transfected *B. gibsoni* and *B. bovis* parasites.

1. Introduction

Babesiosis is a tick-transmitted, zoonotic disease caused by hematotropic parasites of the genus *Babesia* (Bonnet et al., 2009). *Babesia* parasites are some of the most ubiquitous and widespread blood parasites in the world (Homer et al., 2000). *Babesia gibsoni* and *B. bovis* are the main parasites responsible for canine and bovine babesiosis in terms of global parasite distribution, with considerable worldwide economic, medical, and veterinary impact (Schnittger et al., 2012).

Transcriptional machinery of Apicomplexan parasites is unable to recognize viral promoters, such as the CMV and SV40 promoters (Meissner et al., 2001; Azevedo and del Portillo, 2007). However, important elements for transcriptional control are interchangeable among some *Plasmodium* species (Crabb and Cowman, 1996; Mota et al., 2001). Recently, two distinct promoters with interchangeable homologous and heterologous promoter function were also identified in *B. bigemina* and *B. bovis* by *Elongation factor-1 alpha* (*ef-1α*) promoter (Silva et al., 2016). *Ef-1α* is a constitutively expressed and abundant protein in eukaryotic protein translation (Suarez and McElwain, 2010). The promoter region of the *ef-1α* gene of *Plasmodium* and *Babesia* parasites has a high level of transcription and a bidirectional activity (Vinkenoog et al., 1998; Suarez et al., 2006).

Genetic manipulation is one of the ways through which novel therapeutic agents such as vaccines can be discovered against parasites. Goo and Xuan (2014) reported that the challenges in developing effective therapy against *B. gibsoni* may be partly attributed to lack of techniques for genetic manipulation of the parasite. Recently, we established a transient transfection of *B. gibsoni* by 5′-intergenic (IG) region-B of the *ef-1α* (*Bg 5′-ef-1α*) promoter (Liu et al., 2017a). This being the only identified promoter for *B. gibsoni* transient transfection, there is

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a need for species specific or cross-species functional analysis of available promoters to advance our understanding of the molecular biology of *B. gibsoni*. Such knowledge will in turn contribute to the establishment of a stable transfection system in the future. This study identified and characterized interchangeable cross-species functional promoters between *B. gibsoni* and *B. bovis*.

2. Materials and methods

2.1. Parasites culture

The *B. gibsoni* Oita strain (Sunaga et al., 2002) and *B. bovis* Texas strain (Brayton et al., 2007) were used in this study. The *in vitro B. gibsoni* and *B. bovis* were cultured in 24-well culture plates (Thermo Fisher Scientific, USA) and grown at 37 °C in humidified CO_2 (5%) and O_2 (5%) incubator (BIO-LABO, Japan). *B. gibsoni* was cultured in 10% canine erythrocytes suspended in RPMI-1640 supplemented with 20% dog serum while *B. bovis* was cultured in 10% bovine erythrocytes suspended in GIT and medium was replaced every day.

2.2. Promoter prediction

Putative promoter regions used in this study were determined using the Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/http://www.cbs.dtu.dk/services/Promoter/).

2.3. Plasmid constructs

The plasmid schematic diagrams used in this study are shown in Figs. 1 a and 2 a. First, the firefly luciferase and renilla luciferase genes were cloned into the pBluescript (pBS) back-bone plasmid, respectively, using the In-fusion HD Cloning Kit (Takara, Japan). Subsequently, the 3′-untranslated region (UTR) of the *B. bovis rap* gene (*3′-rap*) was cloned into the downstream of the firefly luciferase and renilla luciferase genes, respectively. Then, a total of 12 *Babesia* promoters, including *Bg 5′-ef-1α*-IG-B (*Bg 5′-ef-1α*), *Bb 5′-ef-1α*-IG-B (*Bb 5′-ef-1α*), *Bg 5′-actin*, *Bb 5′-actin*, *Bg 5′-thioredoxin peroxidase-1* (*tpx-1*), *Bb 5′-tpx-1*, *Bg 5′-heat shock protein 70* (*hsp70*), *Bb 5′-hsp70*, *Bg 5′-enolase*, *Bb 5′-enolase*, *Bg 5′-tubulin*, *Bb 5′-tubulin* were amplified from genomic DNA by PCR and cloned into the upstream of the firefly luciferase gene. Finally, to construct an internal control plasmid to normalize the promoter activity, *Bg 5′-ef-1α* and *Bb 5′-ef-1α* were cloned into the upstream of the renilla luciferase gene. All of the primer pairs (Table 1) were designed based on *B. gibsoni* genome (unpublished data), *B. bovis* genome and previous studies (Asada et al., 2012; Hakimi et al., 2016). The constructed plasmids were purified using Qiagen® Plasmid Midi Kit (Qiagen, USA) according to the manufacturer's instructions, and the DNA sequence was confirmed by sequencing before transfection.

2.4. Transfection of parasites

Babesia-infected red blood cells (iRBCs) were pretreated as described (Suarez et al., 2004; Liu et al., 2017a). Transfections were conducted by introducing 20 µg of each firefly luciferase promoter plasmid together with 20 µg of renilla luciferase internal plasmid into *Babesia*-iRBCs. The *B. gibsoni*-iRBCs-plasmid and *B. bovis*-iRBCs-plasmid mixtures were electroporated using Amaxa 4D Nucleofector™ device (Lonza, Germany) and Amaxa Nucleofector™ 2b device (Lonza), respectively, and immediately transferred into 1 ml of culture medium containing 10% fresh RBCs.

2.5. Luciferase assay

The luciferase activity was measured by Dual-Glo® luciferase assay (Promega, USA) at 24 h post transfection (hpt). The pellets were resuspended in 100 µl of freshly-prepared Promega's 1X cell culture lysis reagent (Promega). The pellets were incubated for 15 min at room temperature (RT) for complete lysis and briefly centrifuged to remove the cell debris. The firefly and renilla luciferase activity was measured for a 10 s integration interval using GloMax®-Multi Detection System (Promega), successively. Readings from mock transfected parasites were subtracted from the firefly and renilla luciferase readings and the resulting values of firefly luciferase activity were normalized using the renilla luciferase activity for each sample. To evaluate promoter activities, three independent transfections were done for each promoter and each luciferase assay was performed in triplicate.

2.6. Bio-statistical analysis of promoter activities

The normalized luciferase activities were plotted using GraphPad Prism 6. Activity of each promoter candidates was performed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test with a promoter-less (control) plasmid. Student's *t*-test was used for the comparison between promoter candidates. Differences were statistically significant when $P < 0.05$.

3. Results

A total of twelve promoters, consisting of 6 homologous and 6 heterologous promoters for *B. gibsoni* and *B. bovis*, were investigated.

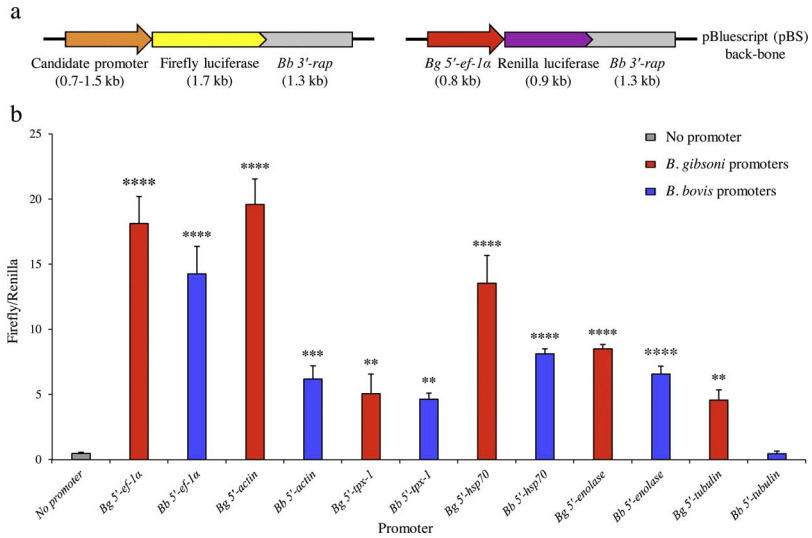

Fig. 1. a, Schematic diagram of plasmid construct to evaluate the promoter activity in *B. gibsoni*, and a Renilla luciferase-expressing plasmid for normalization. **b**, Comparison of luciferase activity in lysates of *B. gibsoni* transfected with different constructs at 24 h post transfection (hpt). A promoter-less plasmid was used as a negative control. The values were presented as means ± S.D. of three independent experiments. Statistical significance between promoter-less plasmid (No promoter) and other promoter candidates was performed by one-way ANOVA analysis of variance, followed by Dunnett's multiple comparison test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).