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Short communication

Integration of a transfected gene into the genome of *Babesia bovis* occurs by legitimate homologous recombination mechanisms

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ABSTRACT

This study examines the patterns of gene integration of *gfp-bsd* upon stable transfection into the T3Bo strain of *Babesia bovis* using a plasmid designed to integrate homologous sequences of the parasite's two identical *ef-1α A* and *B* genes. While the transfected BboTf-149-6 cell line displayed two distinct patterns of gene integration, clonal lines derived from this strain by cell sorting contained only single *gfp-bsd* insertions. Whole genome sequencing of two selected clonal lines, E9 and C6, indicated two distinct patterns of *gfp-bsd* insertion occurring by legitimate homologous recombination mechanisms: one into the expected *ef-1α* orf B, and another into the *ef-1α* B promoter. The data suggest that expression of the *ef-1α* orf B is not required for development of *B. bovis* in cultured erythrocyte stages. Use of legitimate homologous recombination mechanisms in transfected *B. bovis* supports the future use of transfection methods for developing efficient gene function assignment experiments using gene knockout techniques.

Babesia bovis is a tick borne apicomplexan parasite responsible for acute fulminant disease affecting the development of cattle industries worldwide [1,2]. Current live vaccines, based on attenuated *B. bovis*, are mostly able to prevent acute disease but have severe limitations [2–4]. In addition, acutely infected animals can also be effectively treated with babesiacidal drugs, but this is very costly, may also result in the selection of drug resistant parasites, and can lead to the accumulation of undesirable drug residues in the milk and meat [5]. Developing improved methods of control will require a better understanding of the parasite's biology and the identification of targets for the design of novel immunological and drug interventions. Sequencing of the *B. bovis* genome facilitated several possible research avenues, but it also revealed a large percentage of genes with unassigned functions [6]. However, it can be predicted that the recently developed transfection methods for *B. bovis*, [7–10] used in conjunction with novel gene editing, genomic, proteomic and transcriptomic tools will provide valuable approaches for interrogating the *B. bovis* genome. Gene disruption is feasible and exogenous genes that are stably transfected into *B. bovis* integrate relatively efficiently into the genome [7–12]. Furthermore gene knock outs (KO) and recovery of function using sequential transfections methods are now available [9,10]. Experimental evidence collected so far support that integration of transfected genes into the parasite's genome occurs only through homologous recombination (HR) mechanisms [7,10–12], but the molecular mechanisms and patterns of integration remain partially defined. *B. bovis* stable transfection techniques based on blasticidin as a selectable marker were initially developed using a plasmid designed to target integration of a *gfp-bsd* fusion gene into one of the two identical *ef-1α* open reading frames of *B. bovis* [7,8,11–13]. Southern blot and PCR analysis performed on DNA from transfected parasite lines suggests that legitimate integration of the *gfp-bsd* gene into one of the targeted *ef-1α* open reading frames occurs [7,8,11,12]. However, Southern blot analysis performed on some transfected parasite lines demonstrated additional and still uncharacterized insertions of the transfected *gfp-bsd* gene into the genome of transfected parasites [7,8,11,12]. These observations could be explained by the presence of parasite subpopulations in the transfected parasite line containing single or multiple exogenous legitimate or illegitimate gene insertions either in the targeted locus or in other alternative sites of the genome. Therefore it remains unknown if in addition to specific gene target disruption, transfection of foreign genes in the parental *B. bovis* also

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may result in either random, non-specific illegitimate exogenous gene insertions. In addition the possible occurrence of legitimate HR events involving any of the distinct *B. bovis* sequences (promoter, terminators, and flanking insertion sequences) present in the transfection plasmids needs to be investigated. A better understanding of the molecular mechanisms involved in gene insertion and replacement by *B. bovis* transfection techniques will provide insight into the basic molecular biology of the parasite and will help the development of specific gene KO transfection constructs for function assignment. Overall these advances will contribute to the development of novel methods for the control of bovine babesiosis.

This study investigates in detail the patterns of exogenous gene insertion in *B. bovis* parasites upon transfection of the parasites using plasmid *pgfp-bsd-ef* [7], designed for targeted integration of the *gfp-bsd* gene into the parasite *ef-1α* locus [13]. Our experimental approach includes Southern blot analyses and whole genome sequencing of two selected *B. bovis* Texas strain T3Bo—derived transfected clonal parasite populations that were generated using a novel cell sorting method.

Parasites of the virulent *B. bovis* Texas strain T3Bo [14] were transfected using plasmid *pgfp-bsd-ef* as described previously [7] (Genbank accession number KT582108). A blasticidin-resistant and green fluorescent transfected line termed Tf-149-6 developed twelve days after the onset of drug selection. These parasites were maintained in *in vitro* cultures [15] for more than four months after transfection and examined repeatedly using fluorescence microscopy, reverse transcriptase PCR, and Western blot to confirm continuous expression of the *gfp-bsd* gene as previously described [7] (data not shown). The insertion patterns of *gfp-bsd* in the cell line Tf-149-6 were initially analyzed using hybridization of undigested and *Bgl*II digested genomic DNA with *gfp-bsd* and *ef-1α* DIG-labeled probes using Southern blots (Fig. 1A). The restriction enzyme *Bgl*II, which cuts twice outside of the *ef-1α* locus generating a fragment of 12,431 bp containing the full *ef-1α* locus in wild type *B. bovis* parasites [7] (Fig. 2A), was selected for the DNA digestions in the Southern blot analysis. However, there is a single *Bgl*II cutting site within the transfection plasmid (Fig. 2A), which was generated upon cloning of the 5′ *ef-1α* orf insertion site in the *Xho*I site of the transfection vector *pgfp-bsd-ef* (Fig. 2, Supplementary Fig. S2). Dig-labeled *gfp-bsd* and *ef-1α* probes were prepared as previously described [7]. Southern blot analysis suggests the presence of at least two distinct types of *gfp-bsd* gene insertions present in 14.4 and 5.5 kbp *Bgl*II restriction fragments in the Tf-149-6 cell line (Fig. 1A and B). In addition, co-hybridization of these identical fragments with an *ef-1α* specific probe (Fig. 1A and B), suggests that both *gfp-bsd* gene insertions are also associated with the *ef-1α* locus (See Fig. 2 for visualizing the localization of the *Bgl*II sites in the transfected plasmid and in the integrated DNA). These two distinct patterns of hybridization could result from the presence of a single transfected parasite population containing two *gfp-bsd* insertions, a mixed population of transfected parasites containing two distinct types of insertion, or a combination of both scenarios. Our strategy to define the mechanism of exogenous gene insertions into the *B. bovis* T3Bo strain was based on examining the pattern of *gfp-bsd* insertion in clonal parasite lines derived from the transfected line Tf-149-6 using a novel cell sorting method, described in detail in Supplementary data. Briefly, *B. bovis* Tf 149-6 cultures with a PPE of ~30% were diluted in a serial 1/10 fashion to obtain a cell density suitable for single cell sorting with a FACSVantage cell sorter (Becton–Dickinson) with Diva Software. Individual infected cells deposited into 96 well culture plates containing fresh erythrocytes and medium were cultured in a low oxygen atmosphere [16]. Screening of individual culture wells for parasite DNA using nested PCR [12] yielded 32 clones.

The presence and the patterns of insertion of the transfected *gfp-bsd* genes were initially characterized by Southern blot analysis using a *gfp-bsd* probe on *Bgl*II digested genomic DNA extracted from 10 selected blasticidin-resistant, GFP-expressing clonal lines (Fig. 1B). Each of the selected clones exclusively demonstrated hybridization with either ~5.5 or ~15 kbp *Bgl*II restriction fragment (Fig. 1B). Importantly, none of the analyzed cloned parasite lines contained more than a single *Bgl*II restriction fragment hybridizing with the *gfp-bsd* probe (Fig. 1B). We concluded from these experiments that the clonal lines were truly derived from single parasites and that the transfected parasite line Tf-149-6 contains at least two distinct types of transfected parasite populations containing the *gfp-bsd* gene integrated into the *ef-1α* locus in two different configurations. Two of these clonal lines, termed E9, which pro-

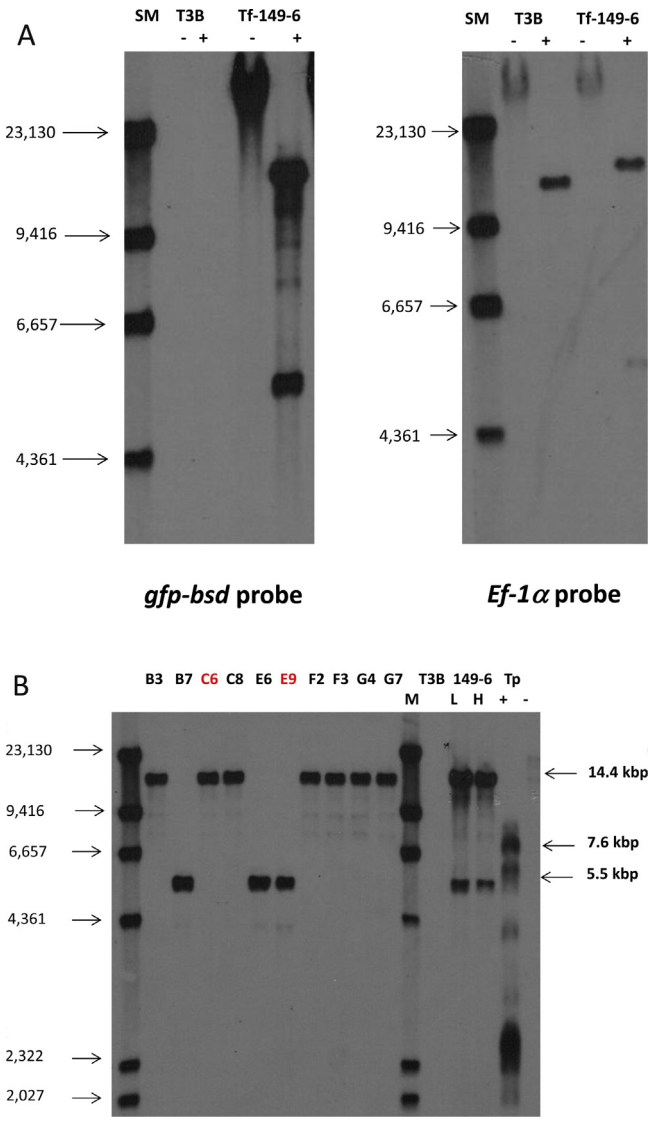

Fig. 1. (A) Southern blot analysis of genomic DNA extracted from the *B. bovis* T3Bo and transfected line Tf-149-6 using *gfp-bsd* (left panel) or *ef-1α* (right panel) dig-labeled specific probes. The DNA analyzed was either not treated (−) or digested with *Bgl*II (+). SM represent dig-labeled size marker DNA. Sizes of the markers are indicated by arrows located at the left side of each panel. (B) Southern blot analysis performed on *B. bovis* gDNA extracted from ten selected clonal lines termed B3-G7, T3Bo and transfected line 149-6, and from transfection plasmid *pgfp-bsd-ef* (Tp) upon digestion with *Bgl*II, with a dig-labelled *gfp-bsd* specific probe. SM represent dig-labeled size marker DNA. The sizes of the markers are indicated by arrows at the left side. The positions of the *Bgl*II digestion fragments of 14.4, 7.6 and 14.4 kbp are indicated on the right. The gDNA from the cell line 149-6 was obtained from cultures maintained with high (H) (64 μg/ml), or low (L) (3.2 μg/ml) levels of the selectable marker blasticidin. The clonal lines C6 and E9, marked with red font, were selected for further analysis.