DEVELOPMENT OF A TRANSFECTION SYSTEM FOR GENETIC MANIPULATION OF *BABESIA BOVIS*

By

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To my parents; my husband, Zheng Xia; and my dear son, Chase Qianchi Xia
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To my precious family, I dedicate this dissertation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGMENTS</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>11</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>12</td>
</tr>
</tbody>
</table>

## CHAPTER

### 1 INTRODUCTION

- Background and Significance                                                                                           | 14   |
  - Babesiosis and *Babesia bovis*                                                                                       | 14   |
  - Life Cycle of *B. bovis*                                                                                             | 15   |
- Strategies of Immune Evasion by *B. bovis* and their Major Components                                                  | 16   |
- Mechanisms of Antigenic Variation in Other Similar Organisms                                                          | 17   |
- Mechanisms of Antigenic Variation in *B. bovis*                                                                      | 18   |
- Unique Structure of *ves* gene Pair and Intergenic Region                                                            | 20   |
- Transfection System                                                                                                   | 21   |
- Mechanism Underlying Electroporation                                                                                    | 23   |
- Promoter Structure                                                                                                     | 24   |
- Hypothesis                                                                                                             | 24   |

### 2 FURTHER DEVELOPMENT OF A TRANSFECTION SYSTEM FOR THE GENETIC MANIPULATION OF *BABESIA BOVIS*

- Abstract                                                                                                              | 26   |
- Introduction                                                                                                          | 27   |
- Materials and Methods                                                                                                 | 28   |
  - Parasite Culture                                                                                                     | 28   |
  - Preparation of Bovine Serum and Erythrocytes and *B. bovis* Immune Serum                                            | 28   |
  - Luciferase Plasmid DNA Construction                                                                                  | 29   |
  - EGFP Plasmid DNA Construction                                                                                         | 30   |
- Detailed Procedures for Transient Transfection                                                                       | 31   |
  - Preparation of plasmid DNA for transient transfection of Luciferase construct                                        | 31   |
  - Electroporation and transient transfection of parasites                                                             | 31   |
  - Post transfection maintenance and sample preparation                                                                | 32   |
  - Dual-Luciferase reporter assay (Promega)                                                                              | 33   |
- Biostatistical Analysis of Promoter Activities                                                                        | 35   |
- Transient Tranfection of EGFP Constructs and Live Cell IFA                                                          | 35   |
3 DISSECTION OF THE BIDIRECTIONAL PROMOTER STRUCTURE
EMPLOYED IN THE BABESIA BOVIS VES MULTIGENE FAMILY

Abstract .................................................................................................................................71
Introduction ............................................................................................................................71
Material and Methods .........................................................................................................73
Parasites ...............................................................................................................................73
Cloning of Constructs with LAT Intergenic Region Regulatory Sequences ......................73
Cloning of Constructs with Additional Exons and/or Introns from the
Apposing Gene .......................................................................................................................75
Cloning of Constructs with Intronic Sequences Inverted ....................................................76
Cloning of Intergenic Regions from Two Other ves Donor Loci ........................................76
Transient Transfection and Luciferase Assay .....................................................................77
Biostatistical Analysis of Promoter Activities ....................................................................78
Results ..................................................................................................................................79
Analysis of ves Igr Sequences ............................................................................................79
Analysis of ves Igr Flanking Sequences on Promoter Function ........................................80
Effects of Intronic Sequences Inversion ..............................................................................81
Comparison of Promoter Activities of Sequence Donor Loci with the LAT .......................82
Discussion and Conclusions ...............................................................................................83

4 CONCLUSION .....................................................................................................................98

APPENDIX

A PRIMERS USED IN STUDY ...................................................................................................100

B STABILITY OF TRANSFORMABLE DNA .........................................................................102

LIST OF REFERENCES ..........................................................................................................103

BIOGRAPHICAL SKETCH ......................................................................................................111
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Statistical analysis of promoter activities of calmodulin 5’ and ves1α 5’ sequences</td>
<td>56</td>
</tr>
<tr>
<td>3-1</td>
<td>Statistical analysis of promoter activities in Igr of LAT</td>
<td>88</td>
</tr>
<tr>
<td>3-2</td>
<td>Statistical analysis of promoter activities in Igr of LAT with additional exon(s) and intron(s)</td>
<td>89</td>
</tr>
<tr>
<td>3-3</td>
<td>Statistical analysis of promoter activities affected by intron inversion</td>
<td>90</td>
</tr>
<tr>
<td>3-4</td>
<td>Statistical analysis of promoter activities of Igrs from LAT as well as non-transcribed ves loci</td>
<td>91</td>
</tr>
<tr>
<td>A-1</td>
<td>Primers used in this study</td>
<td>100</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Cloning of regulatory sequences into pGEM-LUC and control constructs</td>
<td>57</td>
</tr>
<tr>
<td>2-2</td>
<td>Scaled schematic representation of luciferase constructs for transient transfection</td>
<td>58</td>
</tr>
<tr>
<td>2-3</td>
<td>Comparison of promoter activities using transient transfection</td>
<td>59</td>
</tr>
<tr>
<td>2-4</td>
<td>Comparison of lysis methods for detection of luciferase activity in <em>B. bovis</em> extracts</td>
<td>60</td>
</tr>
<tr>
<td>2-5</td>
<td>Time course of luciferase expression in <em>B. bovis</em> parasites transfected with plasmid pLAT_Vα5'</td>
<td>61</td>
</tr>
<tr>
<td>2-6</td>
<td>Expression of EGFP in <em>B. bovis</em> C9.1 parasites transfected with pTubulin 5'-EGFP or pVα5'-EGFP</td>
<td>62</td>
</tr>
<tr>
<td>2-7</td>
<td>Live-cell IFA of <em>B. bovis</em> C9.1 parasites transfected with pVα5'-EGFP</td>
<td>63</td>
</tr>
<tr>
<td>2-8</td>
<td><em>In vitro</em> growth inhibition of <em>B. bovis</em> parasites as a function of various drug concentrations, as assessed by tritiated hypoxanthine incorporation</td>
<td>65</td>
</tr>
<tr>
<td>2-9</td>
<td>Schematic representation of the proposed strategy</td>
<td>66</td>
</tr>
<tr>
<td>2-10</td>
<td>Schematic representation of the original <em>ef-1α</em> locus before integration</td>
<td>67</td>
</tr>
<tr>
<td>2-11</td>
<td>Expression of GFP-BSD fusion protein</td>
<td>68</td>
</tr>
<tr>
<td>2-12</td>
<td>Southern blot analysis using pBluescript and GFP-BSD probes</td>
<td>69</td>
</tr>
<tr>
<td>2-13</td>
<td>Southern blot analysis using <em>rap1</em> and <em>ef-1a</em> probes</td>
<td>70</td>
</tr>
<tr>
<td>3-1</td>
<td>A cluster of promoters are revealed in the IG region of LAT</td>
<td>92</td>
</tr>
<tr>
<td>3-2</td>
<td>Enhancing activities are revealed in the individual exon or intron sequences of the apposing genes</td>
<td>93</td>
</tr>
<tr>
<td>3-3</td>
<td>Effects on luciferase expressions when introns are reversely inserted</td>
<td>94</td>
</tr>
<tr>
<td>3-4</td>
<td>Comparable promoter activities revealed in donor <em>ves</em> IG region with LAT</td>
<td>95</td>
</tr>
<tr>
<td>3-5</td>
<td>Alignment of sequences of <em>ves1α</em></td>
<td>96</td>
</tr>
<tr>
<td>3-6</td>
<td>Illustration of a possible <em>in situ</em> switching of transcription activity event in <em>B. bovis</em></td>
<td>97</td>
</tr>
</tbody>
</table>
Transformed *E. coli* recovery as a function of hours post-transfection of bovine RBCs with transformable DNA
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>90% of maximal inhibitory concentration</td>
</tr>
<tr>
<td>Igr</td>
<td>Intergenic region</td>
</tr>
<tr>
<td>IRBC</td>
<td>Infected red blood cell</td>
</tr>
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<td>LAT</td>
<td>Locus of active transcription</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PCV</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>PPE</td>
<td>Percent parasitized erythrocytes</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RLM-RACE</td>
<td>RNA ligase mediated Rapid Amplification of cDNA ends</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>ves</td>
<td>Variant erythrocyte surface gene</td>
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<tr>
<td>VESA1</td>
<td>Variant erythrocyte surface antigen 1</td>
</tr>
<tr>
<td>VYM&lt;sub&gt;s&lt;/sub&gt;</td>
<td>Vega Y Martinez solution</td>
</tr>
</tbody>
</table>
Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

DEVELOPMENT OF A TRANSFECTION SYSTEM FOR GENETIC MANIPULATION OF BABESIA BOVIS

By

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*Babesia bovis* is an intraerythrocytic parasite which establishes and maintains a persistent infection in its bovine host by at least two mechanisms: cytoadhesion and antigenic variation. In the past few years, a transfection system has been initiated in *B. bovis*, providing a useful genetic tool for studying the biology of the parasite. Here I present further development and extension of transfection methodology for *B. bovis*, as a technical basis for targeted genetic manipulation of this parasite. I have determined that *B. bovis* parasites are adequately susceptible to puromycin, blasticidin-S and pyrimethamine to provide for positive selection, using introduction of an exogenous gene imparting resistance, making stable transformation feasible. In developing this approach, I have established a transient transfection system employing firefly luciferase expression to compare promoter activities, and have demonstrated that the IGr’s of the LAT and two transcriptionally silent ves donor gene pairs all display very strong promoter activities, at levels comparable to promoter from the housekeeping gene calmodulin. Taking advantage of this technology to gain insights into the gene regulation in the unique intergenic region of LAT, I have also identified a cluster of promoters within the 434 bp sequences as well as possible enhancing activities embedded in the flanking exonic or
intrinsic sequences. Interestingly, one inverted intron has resulted in significant reduction in promoter activities, suggesting possible DNA binding motifs in that region. Besides, transient expression of enhanced green fluorescent protein whose expression is driven by a ves promoter has revealed there is no strict ves promoter counting in the parasite when only promoter sequences are present. Thus, ves gene expression from the LAT was unaffected while the episomal vector expressing EGFP was replicating and transcriptionally active extrachromosomally. Stable transfection of the parasite has also been achieved, revealing a non-targeted integration event with stable expression of gfp-bsd protein in the transfectants, which suggests it may be difficult to reproducibly achieve the specific genetic alterations desired. These observations provide key background knowledge and reagents for deliberate genetic manipulation of B. bovis.
CHAPTER 1
INTRODUCTION

Background and Significance

Babesiosis and Babesia bovis

Babesiosis is an emerging world-wide vector-borne disease. It is so named because it is caused by protozoan parasites of the genus Babesia, which was in turn named after the bacteriologist Victor Babes who first described the parasite (Babes, 1888). Nowadays, babesiosis has a great impact on the health of a wide range of domestic and wild animals in tropical and sub tropical regions (McCosker, 1981). The major impact is on worldwide livestock production, to which huge loss has already been caused, so the research into the disease carries great economic importance. Human babesiosis is uncommon, but is a potentially fatal parasitic disease. The distribution of human babesiosis cases tends to be focused in the U.S. Northeast and parts of Western Europe (Kjemtrup and Conrad, 2000). In recent years, however, increasing cases have been reported in other areas because of expanded medical awareness (Hunfeld et al., 2008).

Bovine babesiosis may be caused by Babesia bovis, Babesia divergens, Babesia bigemina or Babesia major. The genus Babesia belongs to the phylum Apicomplexa, class Aconoidasida, order Eucoccidiorida, suborder Piroplasmorina and family Babesiidae (Allsopp et al., 1994; Levine, 1971, 1985). The main vectors of Babesia are Boophilus spp. ticks. Boophilus microplus is the most important and widespread vector affecting domestic species like cattle and buffalo (Bock et al., 2004). The parasites can establish persistent infection in the mammalian host. Infection of the bovine host results in the extensive destruction of erythrocytes, causing severe anemia and sometimes a fatal cerebral form of babesiosis (Aikawa et al., 1992).
**Life Cycle of *B. bovis***

Before description of the immune evasion strategies of the parasite that are used to maintain persistent infection, the life cycle of *B. bovis* is briefly introduced here. *B. bovis* has two major phases in its life cycle (Bock et al., 2004). One is in the invertebrate host (ticks) and the other is in the vertebrate host (the cattle). In the first phase, when a competent *Boophilus spp.* tick takes a blood meal from an infected host, babesial parasites in red blood cells are ingested. In the midgut of the tick, some of the parasites undergo sexual development, forming micro- and macrogametocytes. Two populations of ray bodies are developed from the gametocytes and undergo further multiplication. Large aggregations of multinucleated ray bodies form, but once division is complete, single-nucleated ray bodies that are now haploid and assumed to be gametes (Mackenstedt et al., 1995) emerge from the aggregates and then fuse together in pairs (Gough et al., 1998) to form a spherical cell (zygote). The zygote invades and forms a tissue schizont in intestinal cells, releasing kinetes which leave the intestine, migrate through the hemolymph, and penetrate various other organs, such as fat body cells, nephrocytes, ovaries or salivary glands. Primary asexual reproduction may occur in the ovaries of adult female ticks, resulting in infected ova, and ultimately infected larvae. This is referred to as “transovarial transmission”. Within the larvae, the parasite undergoes sporogony, producing up to 100,000 sporozoites, some of which successfully migrate to the salivary glands. At this point the parasite is ready to be transmitted to a new vertebrate host.

In the second phase, when an infected tick feeds, babesial sporozoites are deposited at the feeding site and find their way into the blood stream of the new host near the site of the bite. There, the sporozoites invade the erythrocytes, where they undergo development and division through the ring, trophozoite, and meront stages. Mature merozoites are then
released from infected erythrocytes and invade other red blood cells (Bock et al., 2004). This asexual reproduction, also called merogony, is the portion of the life cycle in which this study takes place.

**Strategies of Immune Evasion by *B. bovis* and their Major Components**

At least two strategies are employed by *B. bovis* to survive immune defenses (Allred and Al-Khedery, 2004) of the host and promote long-term persistent infection (Allred et al., 1994; Calder et al., 1996; Callow, 1963.; Mahoney et al., 1973). One strategy is the capability of *B. bovis*-infected erythrocytes to cytoadhere to bovine endothelial cells and sequester in capillaries and post-capillary venules (Callow, 1963.; Wright, 1972; Wright and Goodger, 1979). The *B. bovis*-IRBC surface Ag called “variant erythrocyte surface Ag 1” (VESA1) has been linked to cytoadhesion in *B. bovis* and may serve the role of parasite ligand (O’Connor and Allred, 2000).

The other significant strategy is the capability of the parasite to undergo antigenic variation, a process of continually changing biochemical, immunological, and structural properties of parasite components on the IRBC surface, thus altering their immunological appearance. This strategy enables the parasites to evade host immune responses and allows variant parasites to persist. As the parasites mature, various isolate-specific epitopes are exported and expressed on the surface of the infected erythrocytes. The host responds to parasite antigens by producing antibodies when the population reaches a sufficient level to trigger immune defenses. Minor parasite populations expressing variant antigen forms are not bound by antibody and may continue development. The variant parasite population may thrive until it, too, reaches levels sufficient to elicit an immune response. New antibodies may target the novel antigen forms expressed on the membrane surface (Allred et al., 1994). With regard to the components of antigenic variation,
previous work has clearly identified VESA1, a size-polymorphic, parasite-derived protein doublet expressed on the IRBC surface, as a key component of clonal antigenic variation (Allred et al., 1994; O'Connor et al., 1997). Therefore, both strategies employed by the parasites to establish persistent infection and evade host immune defense are linked in *B. bovis* by VESA1.

**Mechanisms of Antigenic Variation in Other Similar Organisms**

With regard to the study of mechanisms underlying antigenic variation, evidence in other protozoal parasites like *Plasmodium* *spp.* and *Trypanosoma* *spp.* may provide a framework to think about what is happening in *B. bovis*. Take *Trypanosoma brucei*, for example. It achieves antigenic variation by constantly changing its surface coat, consisting of variant surface glycoprotein (*VSG*). This is accomplished primarily by replacing the actively transcribed *VSG* genes in the active telomeric expression site by a complete *VSG* gene or with segments from different *VSG* genes, or by switching among the approximately 20 potentially available expression sites (Borst and Ulbert, 2001). There are four demonstrated switch mechanisms used by *T. brucei*: duplicative transposition of a non-telomeric gene, telomere conversion, telomere exchange and *in situ* switch (Borst et al., 1998). In the first case, the *VSG* gene can be replaced by a duplicated non-telomeric gene. In the second case, the whole telomere containing the *VSG* gene may be replaced by the duplication of another telomere. In the third one, the telomere containing the *VSG* gene can be exchanged with another telomere. With all of these gene conversion mechanisms working in *T. brucei*, it can still achieve *in situ* switching as well, which involves the epigenetic switching of transcription of one *VSG* expression site to another expression site *in situ* without any associated DNA
rearrangements (Borst et al., 1998). The variety of mechanisms employed in T. brucei suggested the possibility that B. bovis may also employ more than one mechanism.

In the case of the human malarial parasite, Plasmodium falciparum, the multicycopy var gene family is responsible for encoding the antigenically variant erythrocyte-membrane-protein-1 (PfEMP-1) (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). Each haploid parasite has about 60 var genes within its genome (Gardner et al., 2002). Antigenic variation is achieved by in situ switching of the var gene to be expressed, and each time only a single complete gene is expressed while all the others are kept silent (Scherf et al., 1998). In situ switching involves neither modification of the gene or its loss upon switching. So the variant genes remain intact, and within a clonal population the repertoire of variant PfEMP1 molecules can be as many as the number of var genes (Allred and Al-Khedery, 2004). In situ switching in P. falciparum appears to be somewhat dependent of var gene chromosomal location (Chen et al., 1998), and individual var genes have widely differing switch rates that would affect their frequency of expression (Frank et al., 2007; Horrocks et al., 2004).

**Mechanisms of Antigenic Variation in B. bovis**

It has been found that both cytoadhesion and antigenic variation in B. bovis are linked through VESA1 protein. This size-polymorphic, heterodimeric protein is encoded by a large polymorphic ves multigene family (Allred et al., 2000). It is estimated from the analysis of genomic sequences that there are approximately 150 ves genes in the genome of B. bovis (Brayton et al., 2007). From evidence collected so far, only a single ves locus appears to be transcriptionally active at one time (Zupanska et al., 2009). This locus is referred to as the “locus of active ves transcription” (LAT). The genomic organization of
the current LAT is described here, which may facilitate the understanding of the mechanism underlying variation of VESA1 protein. The LAT has been characterized as a quasi-palindromic structure containing two closely related but structurally different head-to-head ves genes. It is located in the B. bovis chromosome 1 in the C9.1 clonal line (Allred and Al-Khedery, 2006). One gene within the LAT is a ves1α gene, which is known to encode the VESA1a subunit. VESA1a contains a ‘cysteine- and lysine-rich domain’ (CKRD), a ‘variant domain conserved sequences’ (VDCS) domain, ‘C-terminal cysteine-rich’ (CTC) domain, and a pair of predicted coiled-coil domains (Allred and Al-Khedery, 2006; Allred et al., 2000). The apposing sequences encode a ves1β gene, containing 12 introns. It has recently been identified to encode VESA1b polypeptides of the VESA1 protein doublet (Xiao, Y and Allred, DR; unpublished data). The VESA1b polypeptide has a CKRD domain, a CTC domain, and a ‘low complexity variant domain’ (LCV) which is typically absent from ves1α genes. The two genes are in a closely juxtaposed, divergently-arranged orientation with a short intergenic region of only 433 bp (Allred and Al-Khedery, 2006).

In B. bovis, one established mechanism of antigenic variation, for which demonstrative evidence is available, is segmental gene conversion. It is similar to a major mechanism in T. brucei (Borst and Ulbert, 2001), Trypanosoma equiperdum (Roth et al., 1989), Anaplasma marginale (Kamper and Barbet, 1992) and many other organisms. Through the duplication of short gene segments from ves donor genes to an actively transcribed ves1α gene, the current LAT progressively becomes a complicated mosaic comprised of a variety of short sequences from many other ves gene copies (Al-Khedery and Allred, 2006). These gene conversion events likely are occurring during replication,
although this has not been established. Thus, the repertoire of potential ves gene products that may be expressed is efficiently expanded by mosaic gene formation without further expansion of the gene family. As in Trypanosoma spp., employment of a gene conversion mechanism does not rule out the possibility of other mechanisms contributing to antigenic variation. Based on similarities between B. bovis and P. falciparum, it would not be unreasonable to postulate that similar mechanisms would operate in both species. Given the high degree of overall similarity between the LAT and some sequence donor gene pairs in B. bovis, it is likely that an in situ switching mechanism may also be used, although unlike P. falciparum, where in situ switching of intact gene copies occurs as the primary mechanism (Chen et al., 1998; Scherf et al., 1998; Smith et al., 1995), the frequency is likely to be low.

**Unique Structure of ves gene Pair and Intergenic Region**

Evidence collected so far suggests that many ves genes share structures and organizations similar to that of the tightly-juxtaposed quasipalindromic ves1α/ves1β gene pair of the current LAT (Al-Khedery and Allred, 2006). Analysis of the available genome sequences of B. bovis revealed 119 ves genes (72α, 43β), together with up to 40 additional ves genes of unknown types predicted to be missing in a chromosome 1 contig gap of about 150 kb. Among known ves genes, 24 loci are similar in their organizations to the current LAT, with paired ves1α/ves1β genes. 9 loci have paired ves1α/ves1α (Brayton et al., 2007). As to which ves genes may become the new LAT, the choices may be limited. The analysis of bulk RNA from in vitro grown C9.1 line parasites detected only one single transcript species, which suggests that transcription among ves genes is mutually exclusive, with silencing of all but one gene copy (Zupanska et al., 2009).
Alignment of the intergenic regions located between 6 of those paired \textit{ves} genes, including the LAT site, has revealed that their 400-450 bp intergenic regions are highly conserved in structure, organization and much of their sequence (Al-Khedery and Allred, 2006). This region is a quasi-palindrome, with each half containing two inverted repeats organized in an asymmetric pattern in each segment.

A variety of evidence is available for the uniqueness of this Igr. When \textit{ves}1\textalpha and \textit{ves}1\textbeta transcripts derived from the LAT were analyzed several years ago, the putative transcriptional start site was identified by a cap independent 5’-rapid amplification of cDNA ends (5’-RACE) method (Allred et al., 2000). Native \textit{ves}1\textalpha and \textit{ves}1\textbeta transcripts have also been observed by 5’- RACE to initiate within the LAT Igr from a variety of different start sites (Al-Khedery B, Allred DR; unpublished data). The transcripts are heavily overlapping, suggesting that \textit{ves}1\textbeta promoter activity is near the \textit{ves}1\textalpha gene coding sequences, and \textit{vice versa}. Based on the facts that \textit{ves}1\textalpha and \textit{ves}1\textbeta genes have overlapping 5’ UTRs, and transcription start sites have been mapped to both halves of the IGr for both genes, it’s not unreasonable to expect some regulatory elements embedded within this region. In order to further study this region and gain insight into the regulation of \textit{ves} gene expression, a transfection technology will be employed to serve the purpose to identify some possible regulatory elements within the Igr.

\textbf{Transfection System}

Transfection indicates the introduction of exogenous DNA into cells, often with a change of phenotype. This is most commonly achieved by the use of calcium phosphate, electroporation or nucleofection to get DNAs across the cell’s plasma membrane (Graham and van der Eb, 1973; Maasho et al., 2004; Neumann et al., 1982). It has been proven to be a robust genetic tool for understanding gene function in several parasites,
including malarial parasites which share the intraerythrocytic lifestyle. Transient transfection has provided the opportunity to rapidly examine control of gene expression in malaria parasites and understand how gene expression is developmentally controlled (Crabb and Cowman, 1996; Goonewardene et al., 1993; Wu et al., 1996). Stable transfection provides the opportunity to express transgenes in parasites, as well as to understand the functions of proteins by disrupting, modifying, or replacing the genes encoding them (Crabb and Cowman, 1996; van Dijk et al., 1996).

Previous work in the *Plasmodium spp.* demonstrated the feasibility of testing promoter activity of exogenous genes in intraerythrocytic parasites transfected by electroporation (Crabb et al., 1997; Crabb and Cowman, 1996; de Koning-Ward et al., 2000). The experience in malarial parasites may shed light on setting up a transfection system in *B. bovis*, but the latter will have its own distinctions and needs for further development.

The development of a transfection system in *B. bovis* was still in the initial stage when this project started. It was first reported in 2004 when a group at Washington State University used transient transfection to test the hypothesis that the *B. bovis* rap-1 IG region could promote extra-chromosomal gene expression *in vivo* (Suarez et al., 2004). Recently, the same group has demonstrated another much stronger constitutive promoter: the intergenic region of *elongation factor 1* alpha (*ef-1α*), which is useful in transient transfection to enhance expression level of exogenous genes (Suarez et al., 2006). Besides conventional electroporation methods, nucleofection has also been demonstrated to work efficiently in *B. bovis* (Suarez and McElwain, 2008). The development of the
transfection technology for both transient and stable expression of exogenous genes was recently reviewed (Suarez and McElwain, 2009b).

This background of information provides a good start for the development of a stable transfection system in *B. bovis*, for which additional efficient constitutive promoters are required. The initial development of stable transfection system as well as preliminary characterization work was published to reveal that integration in the *ef-1α* locus had occurred (Suarez and McElwain, 2009a). However, other recombination events were also noticed but further characterization was not performed, and our understanding of the process is highly inadequate. Therefore, further development of an integration-dependent transfection system for genetic manipulation of *B. bovis* is entailed.

Among the several methods of transfection I mentioned, electroporation is a widely used technology to get DNAs across the cell’s plasma membrane in the study of hemoparasites. Next, I’ll give a brief introduction about electroporation.

### Mechanism Underlying Electroporation

Electroporation is a membrane phenomenon which involves fundamental behavior of cell and artificial bilayer membranes. Electropermeabilization of cells mainly involves the interaction of the electric field with the lipid portions of the cell membrane (Weaver, 1993). The transient aqueous pore theory describes the features of electropermeabilization. The elevated transmembrane voltage creates pores and provides a local driving force for molecular transport of charged molecules (Kinosita and Tsong, 1977). This is the major technology used in my study to load red blood cells with exogenous DNAs.
Promoter Structure

Eukaryotic promoters are diverse and difficult to characterize. They typically lie upstream of the gene and can have regulatory elements several kilobases away from the transcriptional start site (Nelson and Cox, 2008). Core promoters for RNA polymerase II were originally thought to be invariant, however, they have been found to be considerably structurally and functionally diverse, and the diversity makes an important contribution to the combinatorial regulation of gene expression (Butler and Kadonaga, 2002).

Bidirectional promoter is promoter sequences between divergently transcribed neighbouring gene pairs that initiate transcription in both directions. Computational analysis of human genome sequences and full-length cDNA libraries has identified gene pairs that are arranged head-to-head on opposite strands with less than 1000 base pairs separating their transcription start sites (Adachi and Lieber, 2002). A bidirectional promoter regulates the transcription of a gene pair whose levels may need to be coordinately expressed (Hansen et al., 2003; Maxson et al., 1983; Schmidt et al., 1993).

The technology of transient transfection has made it possible to investigate the control of gene expression. Take Plasmodium spp. for instance. Several promoter regions of P. falciparum have been dissected, including those from the HSP86, HRP3, DHFR-TS, and calmodulin (CAM) (Crabb and Cowman, 1996; de Koning-Ward et al., 1999; Wu et al., 1996).

Hypothesis

Expanding upon evidence collected in the past, we proposed the hypothesis that in B. bovis, antigenic variation may occur by in situ switching of transcriptional activity from the current LAT to another previously silent gene pair. This project was aimed at
providing a test of that hypothesis. To achieve this goal, a transfection system was proposed in *B. bovis* to be used to manipulate the expression of *ves* genes. Functional and mechanistic analysis of the parasite genes was proposed to be accomplished by using positive selection for maintenance of circular plasmids carrying drug selectable markers. Specifically, I attempted to induce a switching of the LAT by positive selection for the activation of a different *ves* locus. Given the mutually exclusive transcription of *ves* locus, I hypothesized that such a switch would be accompanied by the silencing of the current LAT. For success in this strategy, it would be necessary to be able to achieve integration-dependent stable transformation of *B. bovis*. As no technology existed to achieve this goal at the time this project was started, I first initiated the development of this technology for *B. bovis*.

In addition, I proposed to take advantage of transient transfection to study the transcriptional regulation of *B. bovis ves* genes by pursuing another specific hypothesis: regulatory elements are embedded within the uniquely organized compact Igr of LAT.
CHAPTER 2
FURTHER DEVELOPMENT OF A TRANSFECTION SYSTEM FOR THE GENETIC MANIPULATION OF BABESIA BOVIS

Abstract

*Babesia bovis* is an intraerythrocytic parasite which establishes and maintains a persistent infection in its bovine host by at least two mechanisms: cytoadhesion and antigenic variation. In order to facilitate the study of genetic mechanisms, I further developed a transfection system in *B. bovis* as a technical basis for targeted genetic manipulation of this parasite. I have determined that *B. bovis* parasites are adequately susceptible to puromycin, blasticidin-S and pyrimethamine, providing the possibility of positive selection and maintenance of transfected parasites and making stable transformation feasible. In developing this approach I have established a transient transfection system employing firefly luciferase expression to compare promoter activities, and have demonstrated that the Igr’s of the LAT and two transcriptionally silent *ves* donor gene pairs all display very strong promoter activities, at levels comparable to promoter from the housekeeping gene calmodulin. Transient expression of enhanced green fluorescent protein whose expression is driven by a *ves* promoter has revealed there is no strict *ves* promoter counting in the parasite when only promoter sequences are present. Thus, *ves* gene expression from the LAT was unaffected while the episomal vector expressing EGFP was replicating and transcriptionally active extrachromosomally. Stable transfection of the parasite has also been achieved, revealing a non-targeted integration event with stable expression of gfp-bsd protein in the transfectants, which suggests it may be difficult to reproducibly achieve the specific genetic alterations desired. These observations provide key background knowledge and reagents for deliberate genetic manipulation of *B. bovis*. 
Introduction

Babesiosis is an emerging zoonotic disease which has a great impact on the health of a wide range of domestic and wild animals, mainly in tropical and subtropical regions (Gray et al., 2002). The bovine hemoparasite, Babesia bovis, causes huge losses of livestock on a worldwide basis. Therefore, research into the disease carries the potential for having considerable economic impact.

In the past few years, a transfection system has been initiated in B. bovis in order to obtain insight into the function of parasite genes. Transient transfection of B. bovis was first reported when the rhoptry associated protein-1 (rap-1) intergenic region was used to promote transient gene expression (Suarez et al., 2004). However, rap-1 promoter was relatively weak, when compared to the intergenic region of ef-1α (Elongation Factor - 1α) genes. The ef-1α gene was found to contain strong promoters able to promote expression of foreign genes efficiently in transiently transfected parasites (Suarez et al., 2006). Taking advantage of this ef-1α-IG region, stable transfection was then attempted. Stable integration of the chimeric gfp-bsd gene, encoding a Green Fluorescent Protein and blasticidin deaminase fusion protein, into the B. bovis genome was recently achieved. In this study, ef-1α-IG region was able to drive the expression of gfp-bsd, which was integrated mostly through double crossover recombination (Suarez and McElwain, 2009a). However, there was sign of additional non-homologous site of integration revealed using one of the probes, together with evidence of double crossover recombination. This indicated the occurrence of other re-arrangements involving the ef-1α locus. During my attempt of stable transfection using the same construct, I found it hard to reproducibly achieve these specific genetic alterations in B. bovis. Moreover, in
order to explore more biologically significant questions, there’s a need for additional promoters and more efficient regulatory sequences, so as to determine if the ves multigene family could be targeted. Here, I present additional promoters, including a ves promoter, all of which are able to drive the transcription of firefly luciferase efficiently. These compliment those already available and may broaden the possible applications as a result.

The development of both transient and stable transfection technologies, together with the release of the full genome sequence of the T2Bo isolate of *B. bovis* (Brayton et al., 2007), will greatly improve the opportunities for genetic manipulation of the parasite and significantly contribute to our understanding of its biology.

**Materials and Methods**

**Parasite Culture**

*B. bovis* parasites of the C9.1 clonal line were cultivated *in vitro* under microaerophilous conditions, as described before (Allred et al., 1994). Briefly, parasites were cultured in a settled layer of bovine erythrocytes at 10% packed cell volume (PCV) in M199 supplemented with 40% adult bovine serum and 26 mM sodium bicarbonate (M199) under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 37 °C. Culture volumes were 1000 μl in 24-well plates. Cultures were maintained at a parasitaemia between 1% and 5% by daily dilution with uninfected erythrocytes in culture medium.

**Preparation of Bovine Serum and Erythrocytes and *B. bovis* Immune Serum**

Blood collected from a normal bovine donor was defibrinated by shaking with glass beads, followed by centrifugation for 30 min at 4 °C at 4000x g to pellet erythrocytes. Serum was centrifuged again to remove remaining particulates, frozen at –
20 °C, and stored until use. Erythrocytes were washed four times in VYM buffer, kept at 4 °C and used for maximally 45 days.

**Luciferase Plasmid DNA Construction**

A number of different plasmids were used in the transient transfection experiments. A promoterless pGEM-LUC reporter vector was purchased from Promega (Madison, WI). Presumptive terminator sequences to the β-tubulin gene were amplified by PCR from genomic DNA of *B. bovis* C9.1 clonal line, using oligonucleotides (XW23: 5′-C[GAGCTC]ACATAGTATAACCTTATTGCATAAGTTCAC-3′) and (XW24: 5′-C[GAGCTC]AGAAGCGTGAATATGCCTTG -3′). The resulting PCR product was digested with *Sac*I and cloned into the *Sac*I site downstream of luciferase coding sequences in pGEM-LUC. A construct with correctly oriented β-tubulin 3′termination sequences was denoted as pLUC-T3 (Figure 2-1D; Figure 2-2B). It was used as a promoterless negative control, as well as the template for all pLUC constructs.

For the construction of plasmids using the 5′ regulatory sequences of housekeeping genes, the 1397 bp of 5′ sequences of calmodulin was amplified using (XW17: 5′-CC[AAGCTT]TACCGAGAAGAGCCTGCAAC-3′) and (XW18: 5′-CC[AAGCTT]GTATTTAATAATATTAAATTGCTAATACTG-3′). The resulting amplicons were digested with *Hind*III, and cloned to the corresponding site in pLUC-T3 to yield plasmids pC5′-LUC. For the construction of plasmid using *ves* gene 5′ sequences, the 678 bp upstream of start codon of *ves1α* gene was amplified by PCR from phagemid 6-1, as described previously (Allred and Al-Khedery, 2006), using oligonucleotides (XW25: 5′-GC[AAGCTT]GGAATCATACAGTAGGTCCTTC-3′) and (XW26: 5′-GC[AAGCTT]TGTCAGTGCTTCTAGGAGTACTCAG-3′). The resulting PCR product was digested with *Hind*III, and cloned into the *Hind*III site of pLUC-T3 to
yield plasmid pLAT_Vα5’. In addition to Igr sequences, the construct contained exon 1 and intron 1 of ves1β. The orientation of insertion was determined by digestion mapping. Plasmids with correctly inserted 5’ sequences were used as major constructs. Plasmids with reversely inserted 5’ sequences served as reverse promoter controls. The resulting constructs were designated as pC5’F-LUC, pC5’R-LUC, pLAT_Vα5’, pLAT_Vα5’R. (C: Calmodulin, V: ves1; F: forward orientation, R: reverse orientation). Figure 2-1-A and B as well as Figure 2-2-A show these plasmids. Figure 2-1-C shows the positive control construct pEF1α-IG, which is a gift from Dr Carlos Suarez. It has previously been shown to contain a strong promoter (Suarez et al., 2006).

For the construction of an internal control plasmid, a fragment of 2726 bp containing 1699 bp of LUC gene plus 996 bp of β-tubulin 3’ sequences were removed from pC5’F-LUC construct by digestion with BamHI and SacI. Consequently, this was replaced by a fragment of 1595 bp containing 933 bp of R. reniformis luciferase gene plus 662 bp of polyadenylation sequences from P. falciparum calmodulin. This fragment was obtained by digestion of pPfrluc plasmid DNA with BamHI and SacI. pPfrluc was a gift from Dr Diane Wirth (Militello et al., 2004; Militello and Wirth, 2003) and provided to us by Dr. Tonya Bonilla. The resulting plasmid was named pC5’-Renilla-C3’ as shown in Figure 2-1E and Figure 2-2C.

EGFP Plasmid DNA Construction

The 1635 bp of 5’ upstream sequences of β-tubulin were amplified by PCR from genomic DNA extracted from B. bovis clonal line C9.1, using oligonucleotides (XW21: 5’- CC[AAGCTT]GAAACTCGCATCGCTCTAAAC-3’) and (XW22: 5’- CC[AAGCTT]CTATTGTTACACTACAGAATGTAACATGAAC -3’). This was cloned
into the HindIII site of pEGFP-1 (BD Biosciences Clontech, San Jose, CA). A similar construct was created with the 678 bp of 5’ upstream sequences from the ves1α gene fragment described earlier. Figure 2-6-A shows these two plasmids.

All plasmids described above were confirmed by restriction mapping. The inserts were confirmed for proper construction by DNA sequencing. All constructs maintain the start codon of the firefly luciferase gene.

**Detailed Procedures for Transient Transfection**

**Preparation of plasmid DNA for transient transfection of Luciferase construct**

Plasmid DNAs were prepared using EndoFree Plasmid Purification Mega Kit (Qiagen) or Maxi Kit (Qiagen) following manufacturer’s instructions. Plasmid DNA was dissolved in 0.5ml endotoxin-free Buffer TE. To determine yields, DNA concentrations were measured by UV spectrometry, and quantitative agarose gels were also run to confirm concentrations by comparison with standard markers. Before each transfection, isolated plasmid DNA was diluted in cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl₂, final pH 7.6) and stored at 4 °C.

**Electroporation and transient transfection of parasites**

The day before transfection, smears were made and stained with a Giemsa-like quick stain to determine the percent parasitized erythrocytes (ppe). Cultures were adjusted by feeding appropriate amount of RBC and media to give 6-15% ppe the next day.

For transfecting IRBCs, parasite cultures were harvested into sterile 15ml conical tubes and centrifuged at 4000xg for 10 min at 4 °C. The supernatant was discarded and the cell pellet was washed two times with excess cytomix to remove any antibiotics,
which may reduce transfection efficiency. For each transfection, 100 µl packed infected
RBCs (ppe: approx. 10%) were electroporated with 11.5 pmol of transfection construct
dNA and 3.8 or 7.7 pmol of pC5’-Renilla-C3’ mixed together in approx. 150 µl
DNA/VYM suspension at 1.25 kV 25 µF capacitance, and 200 Ω resistance in a 2mm
gap cuvette (Fisher Biotech). Transfected cells were then transferred into 6 well plates
containing 3ml complete medium containing uninfected erythrocytes at 2.5% PCV
(Packed Cell Volume).

An attempt was also made to pre-load erythrocytes with transfection constructs to
determine whether parasites may be passively transfected. For the RBC pre-loading
method, 200µl packed RBCs were transfected with 11.5 pmol DNA and 7.7 pmol pC5’-
Renilla-C3’ in approx. 200 µl DNA/VYM suspension at 0.31 kV 1.07 mf (maximum)
capacitance 200 Ω resistance in a 2 mm gap cuvette and transferred into one well in a 6
well plate containing 3 ml complete medium. Then, 2 ml infected RBCs with 10% pcv
and over 5% ppe were added into each well to allow parasites to take up DNA
spontaneously.

**Post transfection maintenance and sample preparation**

On the day following transfection, the medium was changed and 50µl of packed
RBCs were added into each well. At the desired time point post-transfection, RBCs were
collected into 2ml microcentrifuge tubes and sedimented by centrifugation at 4000xg for
10 min, followed by two washes under the same conditions, with 1x VYM. Parasites
were then released by NH₄Cl-Tris lysis (Martin et al., 1971). A 0.8% (w/v)-ammonium
chloride solution was prepared using deionized water. To each 90 ml of this solution 10
ml of 0.17 M Tris buffer (pH 7.65) were added, and the final pH of the buffered
ammonium chloride solution was adjusted to 7.4 at room temperature. The solution was
warmed to 37 °C prior to use. The microcentrifuge tubes were inverted gently about 70
times, then incubated on ice for 10 min. The lysates were verified visually to be
translucent before they were centrifuged at 12000xg for 10 min. Parasites were pelleted
and washed with 1xVYM two times under the same conditions, then lysed with 100µl
freshly prepared Passive Lysis Buffer (Promega, Madison, WI) for 25 min.

**Dual-Luciferase reporter assay (Promega)**

The required volume of Passive Lysis Buffer working stock was prepared
immediately before use by adding 1 volume of 5x Passive Lysis Buffer to 4 volumes of
distilled water. After each use, 400µl was saved for buffer control. According to the
manufacturer’s protocol, the Firefly and *Renilla* luciferases contained in the cell lysates
prepared with PLB are stable for at least 6 hours at RT, and up to 16 hours on ice, up to
one month at -20 °C or long term storage at -70 °C.

Luciferase Assay Reagent II (LAR II) was prepared by resuspending the provided
lyophilized Luciferase Assay Substrate in 10 ml of the supplied Luciferase Assay Buffer
II (Promega). According to the manufacturer’s instructions, aliquots of LAR II reagent
are stable for one month at -20 °C or for one year when stored at -80 °C according to
manufacturer’s manual. Accordingly, LARII was stored for a maximum of one month at -
20 °C prior to use.

The required volume of Stop&Glo Reagent (Promega) was prepared right before
each assay by adding 1 volume of 50x Stop&Glo Substrate to 49 volumes of Stop&Glo
Buffer. According to manufacturer’s instructions, aliquots may be stored at -20 °C for up
to 15 days.

Both Firefly and *Renilla* luciferase activities were quantified with a MicroBeta Jet
scintillation spectrometer (PerkinElmer, San Jose, CA). A twenty-four well white plate
counting protocol was set up to perform a 10-second measurement for each reporter assay without any pre-measurement delay or mixing. Lysates from the same triplicate group were carefully laid out in the manner they were to be assayed, so as to be read simultaneously. No samples were positioned immediately next to each other to avoid crosstalk between wells.

To enhance accuracy and minimize background, readings were performed in several steps. First, the white 24-well plate support frames 1450-102 (PerkinElmer) were cleaned by ethanol and luminescence was measured for the empty support frames. The frame with the lowest background was chosen. Secondly, the 24-well sample plates 1450-402 (Wallac Oy, Finland) were inserted into this support frame one by one, and luminescence was measured for each sample plate. Thirdly, thawed or freshly prepared lysates were centrifuged for 2 min at 12000 xg at room temperature. The 100 µl of cleared extracts were dispensed into the wells. Background liquid luminescence was recorded three consecutive times. The average reading of each well from the last step was then subtracted from luciferase activity measurements taken from the same well.

Before starting luciferase assay procedures, both reagents were warmed to room temperature. A multichannel pipetter and reagent reservoirs were used to dispense 100µl LAR II to all wells to ensure simultaneous reaction of luciferin substrate and firefly luciferase. Firefly luciferase activity was measured, immediately followed by the addition of 100µl Stop&Glo reagent to all wells by multichannel pipetting. Renilla luciferase readings were then taken. Readings from mock transfected parasite lysates were subtracted from all renilla luciferase readings. After the measurement and processing of all renilla luciferase activity, the level of firefly luciferase activity was normalized to the
level of *Renilla reniformis* luciferase activity. Triplicate experiments were repeated at least three different times.

**Biostatistical Analysis of Promoter Activities**

The normalized luciferase activities were plotted using Microsoft Excel as the mean ± standard deviation, calculated from the triplicate samples. Differences in promoter activities were determined by two-sided two sample t-test, using SPSS program (SPSS, Chicago, IL). The calculated P values are listed in Table 2-1. If the P value is below the threshold value chosen for statistical significance (P = 0.05 for this study), the two promoter activities are considered significantly different.

**Transient Tranfection of EGFP Constructs and Live Cell IFA**

Similarly to the luciferase constructs, 11.5 pmol of EGFP constructs were transfected into *B. bovis* C9.1 IRBCs to allow uptake of the DNAs and expression *in vivo* as described in the previous sections. Expression of green fluorescent protein was detected by fluorescence microscopy. Live-cell immunofluorescence assays (live-IFA) were performed essentially as described (Allred et al., 1993), using polyclonal antibody R6a-v1β765 as primary antibody. Bound antibody was localized with Chicken anti-rabbit IgG-Alexa594, and the signal was visualized by fluorescence microscopy.

**Antibiotic Sensitivity Assay**

The sensitivity of *B. bovis* to various antibiotics was performed by measuring the inhibition of hypoxanthine incorporation, as described in (Desjardins, 1979). Briefly, C9.1 parasites were cultured to a parasitemia of 0.5%. Drug powder was initially dissolved in distilled water to make a 10mg/ml stock. VYM (1x) was used to make two-fold serial dilutions ranging from 1mg /ml to 1µg/ml for puromycin, threefold dilutions ranging from 12mg/ml to 0.2 µg /ml for pyrimethamine and fourfold dilutions ranging
from 64mg/ml to 1 µg/ml for blasticidin. A constant volume of 225µl of the parasitized erythrocyte suspension and 25µl of drug solution were distributed into the wells of a 96-well plate, using a multichannel pipette. Therefore, the final drug concentration in the well was 1/10 that of the working stock. The first row in each column contained no drug, serving as a parasites-only negative control. The last row in each column served as non-parasitized erythrocyte control, without drug or parasites. After a 24h incubation period, 25µl of [³H]hypoxanthine in culture medium (0.5µCi) was added to each well. After an additional 18h of incubation, parasites were harvested, using an automatic cell harvester following manufacturer’s instructions. Scintillation counting was performed on a MicroBeta Jet scintillation spectrometer (Perkin-Elmer). Each compound is present in triplicate columns. Data were averaged in OriginPro 8. IC₅₀ and IC₉₀ values were determined by fitting a sigmoidal inhibition curve to the data points. OriginPro 8 (Originlab; Northampton, MA) was used to perform analysis and plotting of the data.

**Stable Transfection and Drug Selection**

Electroporation procedures for stable transfection were performed in the same way as described in the previous sections, except that the plasmids were linearized by adding restriction enzyme in the electroporation cuvette first. Four hours post transfection, the cells were placed under selection with 6.25, 12.5 or 25µg/ml of blasticidin, and monitored for up to 6 weeks for surviving parasites. Fresh drug in fresh complete medium was replaced on a daily basis. Fresh RBCs were supplied every 3 days at 2.5% PCV in complete medium. Ten days post transfection, drug pressure was removed for 11 days, but was resumed at the original concentration as soon as parasites became detectable by light microscopy. Parasites expressing GFP-blasticidin-s-deaminase fusion protein were detected by fluorescence microscopy. Drug pressure was increased up to
50µg/ml (108.8µM), which was lethal to wild type parasites. Maintenance of the transfected parasites is normally performed at 25µg/ml (54.4µM) of blasticidin.

**Western Blot Analysis**

To confirm the expression of GFP-BSD fusion protein, stably transfected parasites were subjected to SDS-PAGE and analyzed by Western blot, as described previously (Allred et al., 2000). Antigen was detected with anti-GFP antibody (Roche, Indianapolis, IN) at a dilution of 1:2000. The immunoblots were developed with goat-anti-mouse-HRP antibody and detected with SuperSignal (Thermo-Pierce; Rockford, IL).

**Southern Blot Analysis**

Genomic DNA was extracted by a classic sodium dodecylsulfate and proteinase K, phenol-chloroform procedure essentially as described (Tripp et al., 1989), with the modification that most hemoglobin had been released by treating C9.1 IRBCs with 0.05% saponin in PBS. The parasite pellet was then lysed in 0.5ml TE buffer containing 1% Sodium dodecylsulfate (SDS). Lysates were then treated by 100µg/ml proteinase K for 2 hours and 40µg/ml RNaseA for an additional 1 hour. Digestions were followed by phenol and chloroform extractions, and ethanol precipitation. To prepare blots, 1.5 µg gDNA and 0.5 ng plasmid DNAs were digested with NotI and BglII for 3 hours. Digested DNAs were fractionated on 0.8% agarose gels in TAE buffer at 70V for 4~5 hours. Gels were exposed to intense UV light for 5 minutes to nick DNA to increase transfer efficiency. The locations of size standard markers were spotted on the gel before the gel was rinsed briefly in distilled water. DNAs were then denatured with denaturing solution (1.5M NaCl, 0.5 N NaOH) in two 15-min incubation. The gel was neutralized in neutralization buffer (1 M Tris-HCl, 1.5 M NaCl, pH 7.45) for 30 min, followed by 10x SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0) for 10 min. DNA was then transfered onto Hybond N+
membranes (GE healthcare; Piscataway, NJ) in 10x SSC overnight following standard procedures (Sambrook, 2001). To fix the DNA on the membrane, the blots were subjected to UV crosslinking in a Stratalinker 2400 UV Crosslinker (Stratagene; La Jolla, CA) at 120mJ. Probes were labeled with $\alpha$-$[^{32}P]dCTP$ by random priming extension with Klenow polymerase, following manufacturer’s instructions (DECAprime™II Random Priming DNA Labeling Kt; Ambion). The blots were incubated in prehybridization solution supplemented with denatured 50 µg/ml salmon sperm DNA for at least 3 hours at 55ºC. (1 liter pre-hybridization solution was made by mixing 250 ml 1M NaH$_2$PO$_4$, pH 6.0, 300 ml 20 × SSC, 15g Na$_4$P$_2$O$_7$•10 H2O, 200 ml 50 x Denhardt’s solution, 25ml 20% SDS and 225 ml distilled water, 0.45 µm filter-sterilized). Blots were then hybridized overnight with probe in 5ml hybridization solution (same recipe as pre-hybridization solution) at 65 ºC. The blots were washed stringently three times for 20 min. each with 0.2XSSC/0.5%SDS at 60 ºC. Finally, the membrane was exposed to film (Hyperfilm MP; GE Healthcare) at -80 ºC for 8 to 24 hours before developing.  

**Fluorescence microscopy**

The fluorescence was detected on an Olympus BX50 microscope fitted with a 100x oil-immersion (NA 1.3) phase contrast objective. Images were captured with a Retiga 1300B cooled CCD camera (QImaging; Surrey, BC). Images were processed using IP Lab (Scanalytics, Inc.; Rockville, MD) and ImageJ version 1.8.2 (http://rsb.info.nih.gov/ij/) software.

**Experimental Results**

**Expression of Luciferase and Comparison of Heterologous Promoter Activity**

In order to determine whether regulatory elements could be detected in the 5’ sequences of housekeeping genes or the ves1α gene, I cloned the upstream regulatory
sequences from both gene classes, in both orientations, into the luciferase expression vector, upstream of the luciferase coding sequences (Figure 2-1A, B; Figure 2-2A). The abilities of these sequences to drive the transcription of exogenous genes \textit{in vivo} by transient transfection were then tested. The flanking regions of β-tubulin and calmodulin were chosen as they are likely to be transcribed throughout development in \textit{B. bovis} and with minimal fluctuation. The results are shown in Figure 2-3 and P values calculated from t-test are shown in Table 2-1. Calmodulin 5’ sequences generated significant amounts of firefly luciferase activity compared to promoterless control when introduced into \textit{B. bovis} parasites (P=0.0063), indicating a promoter embedded in this region. Reversely inserted upstream sequences of calmodulin gene did not effect expression of the marker gene; the amount of luciferase activity was not significantly different from the promoterless; negative control (P=0.3974). Surprisingly, \textit{ves1α} 5’ sequences were able to promote a very high level of luciferase activity when the promoter sequences tested were placed in their normal orientation relative to coding sequences (P=0.0156). Firefly luciferase luminescence values, normalized with the \textit{Renilla} luciferase values from the same samples, were almost twice as high as those of the positive control containing the demonstrated strong promoter, \textit{ef-IG}-fragment-B (Suarez et al., 2006) (P=0.0364). This result indicates that this region contains a very strong promoter.

The cloned upstream sequences of the \textit{ves1α} gene are 678 bp in length, containing not only the 434 bp shared intergenic region of the LAT \textit{ves} gene pair, but also exon 1 and intron 1 of the apposing \textit{ves1β} gene. Therefore, it was not surprising that reversely-oriented controls did not display much promoter activity (P=0.0743). When inserted in the reverse orientation, the exon 1 and intron 1 sequences would lie between \textit{ves1β} 5’
sequences and the start codon of the luciferase gene, resulting in interference with normal folding of protein structure. The coding sequences of the ves1β gene may disrupt the correct folding and stability of luciferase protein.

**Transfection Methodology**

While working out the transient transfection system, a series of experiments aimed at optimizing transfection conditions were performed, as well as alternative strategies for loading DNA. In *P. falciparum*, successful transient transfection has been achieved without even exposing the parasites to electric current, i.e., by preloading red blood cells with exogenous DNA and allowing parasites to invade afterwards (Deitsch et al., 2001). It was determined previously that, using the condition of 150 V, 1000 µF, and 70 Ω to transflect, it is possible to load bovine RBCs with exogenous DNA, which is degraded exponentially, with a $t_{1/2}$ of approximately 10.25 hours (Appendix Figure B-1). However, from numerous trials using various electroporation conditions, I have found that preloading bovine erythrocytes with exogenous DNA to facilitate transfection of *B. bovis* parasites does not result in the transfection of *B. bovis* at detectable levels. Another distinct difference in the sample preparation of transiently transfected *B. bovis*, in contrast to *Plasmodium spp*., is that saponin lysis does not work well in *B. bovis*, in contrast to *Plasmodium spp*. Saponin lysis is widely used in *Plasmodium spp* to release extra hemoglobin before lysis of the parasite. However, the use of saponin on *B. bovis* IRBC resulted in the extensive loss of luciferase protein together with the release of hemoglobin (Figure 2-4). The luciferase expression is not significantly different from mock transfected parasites ($P>0.05$ data not shown). This loss did not occur when IRBCs were lysed by the NH$_4$CL-Tris lysis method (Martin et al., 1971). It is apparent that each of the
different species presents distinct advantages as well as disadvantages for investigating parasite biology.

Transient DNA stability in transfected red blood cells may be confirmed by a “zap twice” strategy. Bovine erythrocytes were preloaded with exogenous DNA first under low voltage/high capacitance conditions 150 V, 1.07 mf, 200 Ω, and parasites were allowed to invade the loaded erythrocytes immediately. Four hours later, erythrocytes were collected from the wells, washed three times with 1x VYM to remove residual DNA from the medium. The parasitized erythrocytes were put back into an electroporation cuvette and electroporated again under high voltage low capacitance conditions. This was followed by standard procedures of parasites culture, sample preparation and luciferase assay. Among the numerous tests in preliminary experiments, I detected luciferase expression at an appreciable level only once (data not shown), and at a much lower level than the luminescence signals recovered from parasites loaded by the standard method. Therefore, this approach is yet to be optimized before any conclusion about feasibility could be drawn.

Besides the loading strategy, I also tested the kinetics of luciferase expression after transfection with expression vector with ves promoter, in an attempt to optimize the time point to collect transfected parasites for luciferase assay. The time course of luciferase expression after transfection with pLAT_Vα5’ plasmid was determined, with samplings at 1, 2, 3, 4, 9, 12, 24, 48, 72 and 96 h (Figure 2-5). Of the time points examined, detectable luciferase signal was observed as early as 1h after transfection. Importantly, the peak signal appeared at 24h after transfection, with easily quantifiable luciferase expression from 8 h to 24 h post transfection. However, when it has passed the time point
of 24 h, the expression declined afterwards in an almost linear fashion. The pattern of *Renilla* luciferase expression was a little different from that of Firefly luciferase expression. This is not surprising as these two reporter genes were regulated by different promoters. In this experiment, Firefly luciferase was driven by the *ves* promoter (including exon1 and intron 1 of the apposing *ves1β* gene), whereas *Renilla* luciferase was driven by the calmodulin promoter. The time point of peak signals for Firefly luciferase expression with *ves* promoter was 8-24 hours, consistent with previous observation of luciferase expression driven by *ef-1α* IG-B promoter (Suarez and McElwain, 2008). The peak signal for *Renilla* luciferase expression appeared even earlier - at 8 hour post transfection- with a slow decrease after that. Luciferase activities were still considerable at 48 and 72 h, indicating that a significant fraction of transfected parasites expressing luciferase remain viable and able to infect erythrocytes. The ratio of Firefly luciferase expression over *Renilla* luciferase expression started to rise at 1h, forming a plateau from 4 to 7 hours, then started rising again until it reached a peak at 24 hour followed by a gradual drop. Therefore, the time point we chose to collect and assay all the samples was set at 24 hours post transfection.

**Coexpression of EGFP and VESA1**

In addition to transient expression with the luciferase gene, a second reporter gene encoding enhanced green fluorescent protein, EGFP, was also tested. EGFP is a red-shifted enhanced fluorescence yield mutant form of *Aequorea vitoria* green fluorescent protein. The 5’ sequences of β-tubulin and *ves1α* were subcloned into the promoterless vector pEGFP-1. Green fluorescence could be detected within a small proportion of the parasites at 24h post transfection, as shown in Figure 2-6. Immediately following that, a live cell immunofluorescence assay was performed on the parasites transfected with
plasmid containing ves1α promoter, which was able to provide information on the
developmental timing of EGFP expression and its correlation with VESA1 protein
expression on the IRBC surface. Polyclonal antibody R6 and monoclonal antibody
4D9.1G1 were used to recognize VESA1b antigen and VESA1α antigen, respectively, on
the surface of red blood cells (Y Xiao and D.R. Allred, submitted data). Significantly, as
shown in Figure 2-7, this assay revealed red fluorescent IRBCs, which labeled parasites
expressing from LAT, harboring parasites of both the trophozoite and merozoite stages
which also demonstrated green fluorescence. B. bovis parasites continue to express ves
genes from the LAT while an episomal vector driven by LAT ves promoter sequences is
maintained extrachromosomally in an active state.

**Antibiotic Sensitivity Assay for Developing Stable Transfection in B. bovis**

Besides the further development of the transient transfection system, we also
worked on the stable transfection system, with characterization of the stably transfected
parasites.

In order to develop an integration-dependent transfection system, with the
employment of a transfection vector carrying a selectable marker, it is essential to use
well-characterized susceptible and resistant strains of the parasite in the genetic
manipulation of the genome. The antibiotic sensitivity assay provided quantitative
measurements of the competence of compounds to inhibit parasite growth, based on the
inhibition of incorporation of a radiolabeled nucleic acid precursor by the parasites during
short-term cultures in microtitration plates.

Based on previous work in the lab as well as experience from *Plasmodium spp.*
(Mamoun et al., 1999), I chose to test the following drugs: blasticidin-S, hygromycin,
puromycin, pyrimethamine. As shown in Figure 2-8, puromycin and blasticidin-S were
both found to be effective and to provide adequate selection for the two day assay. *B. bovis* was found to be very sensitive to puromycin at concentrations as low as 2.1 µM after only 5 cycles of reproductive replication. The IC<sub>50</sub> for puromycin was found to be 0.74 µM and the IC<sub>90</sub> 8.9 µM. Transfection constructs carrying the *pac* (Puromycin-N-Acetyltransferase) gene will encode resistance to puromycin (de Koning-Ward et al., 2000). The *bsd* gene encodes a deaminase that converts the potent inhibitor blasticidin-S into a nontoxic deaminohydroxy derivative (Yamaguchi et al., 1975). *B. bovis* C9.1 parasites were found to be susceptible to blasticidin-S with an IC<sub>50</sub> of approximately 8.7 µM and IC<sub>90</sub> of about 58.8 µM. *B. bovis* is sensitive to pyrimethamine at 59.5 µM, with an IC<sub>50</sub> of approximately 1.73 µM but IC<sub>90</sub> over 764 µM. Pyrimethamine was found to be most effective after an extended period of incubation (data not shown). However, pyrimethamine may be more prone to the selection of naturally drug-resistant mutants, and spontaneous mutations in the DHFR-TS gene may easily be selected, as has occurred in malaria parasites (Gatton et al., 2004). This could potentially be overcome by the use of WR99210, which also inhibits parasite DHFR-TS (dihydrofolate reductase-thymidylate synthase), but is less prone to the development of natural resistance. Data points were fitted to hill curves, which is an analytic method frequently used in pharmacology to describe the response of an organism as a function of drug concentration (Heidel and Maloney, 1999). Each of the three drugs was considered to be useable in stable transfection of *B. bovis* to provide strong selection of transfected cells. However, to avoid potential problems, I chose to avoid the use of pyrimethamine in favor of blasticidin-S or puromycin. In contrast, neomycin, hygromycin, and chloramphenicol were all found to be without effect in *B. bovis* (data not shown).
**Drug selection and Stable Expression of GFP-BSD Fusion Protein**

With a functional promoter and suitable selection conditions, the transfection system can be applied to more complicated projects. Bovine erythrocytes infected with *B. bovis* C9.1 parasites were chosen as the target cells for introduction of DNA and development of stable transfection. To select transgenic parasites, constructs harboring a *bsd* gene that could impart blasticidin resistance were tested. For the two constructs tested, as shown in Figure 2-9A and B, the *bsd* gene was flanked by the 434 bp of 5' sequences of *ves1α* and 3' intergenic region lying between β-tubulin and the cell differential family protein coding sequences. One construct, pDS-BSD (kindly constructed by Daniele Swetnam), additionally possessed 1 kb of sequences from the 5' end of a *ves1α*-like gene 1.8 kb downstream of the LAT (Figure 2-9A). It was intended to target the plasmid into the parasite’s genome by single crossover homologous recombination. Figure 2-9B shows a second form of the plasmid carrying truncated targeting sequences, which was included in an attempt to enable essentially random targeting to nearly any *ves* Igr. The schematic representation of how pDS-BSD is anticipated to target the genome is given in Figure 2-9C.

Plasmid pgfp-bsd-ef, which was a gift from Dr Carlos Suarez and had previously been shown to stably transform *B. bovis* (Suarez and McElwain, 2009a), was used as a positive control. A schematic representation of this vector, the expected integration site, as well as how the genomic locus would be anticipated to look after integration are given in Figure 2-10, which is adapted from (Suarez and McElwain, 2009a). Parasitized erythrocytes were transfected with the constructs in linearized form. This was done because linearization increases the frequency of recombination as linear DNA ends appear more recombinogenic (Cruz and Beverley, 1990; Lee and Van der Ploeg, 1990;
Nunes et al., 1999; ten Asbroek et al., 1990). Four hours after transfection, the parasites were exposed to 13.6 µM or 27.3 µM blasticidin-S. Drug pressure was removed 11 days post transfection, but resumed at original concentration as soon as parasites became detectable again under microscope. Initially, drug selection at lower concentrations was attempted, but only spontaneously resistant parasites were selected (data not shown). Following selection at the higher concentrations of drug, drug resistant parasites in the well transfected with the positive control plasmid, pgfp-bsd-ef, first showed up around 20 days post transfection. The parasites grew very well under high drug pressure up to 54.5 µM, which was lethal to routinely cultured normal C9.1 parasites, as well as to mock-transfected parasites. Expression of green fluorescence protein was consistently detected by routine fluorescent microscopy analysis (Figure 2-11A). Expression of a GFP fusion protein was demonstrated by Western blot analysis of the stably transfected parasite line using monoclonal rabbit antibody against GFP. As shown in Figure 2-11B, mouse anti-GFP antibodies bound a protein of ~39 kD, compatible with the expected size of the GFP-bsd fusion protein. Anti-GFP antibody did not react with any protein in wild type C9.1 strain parasites trials.

Nothing grew from wells containing parasites transfected with constructs driven by ves promoters during the first 3 attempts. The LAT sequences present in the transfection constructs may have been silenced immediately after transfection during these failures. However, in a most recent attempt to transfect parasites with pDS-BSD linearized with EcoRI, under continuous drug selection at 27.3 µM of blasticidin, highly drug-resistant parasites suddenly expanded 17 days after transfection in the well. However, I did not
have time to characterize the newly expanded drug resistant transfectants. Only those transfected with pgfp-bsd-ef, which came up months earlier, were further studied.

**Characterization of Genomic Locus of the Integrated gfpbsd Gene**

Southern blot analysis using pBluescript, ef-1αA5’, rap1-3’ and gfp-bsd specific [³²P]-labeled probes was used to determine whether the gfp-bsd gene was integrated into the genome of the *B. bovis* transfectants, whether episomal or integrated plasmid was still present in the transfected parasite cell line, or where the gfp-bsd gene targeted in the genome. Genomic DNA was extracted from stably transfected parasites as well as the original, non-transfected C9.1 parasites. Genomic DNAs, 1.5 µg each, and 5 ng each of transfection plasmids pgfp-bsd-ef or pBluescript, were doubly digested with *Bgl*II and *Not*I (+), or left undigested (-), then analyzed by agarose gel electrophoresis. *Bgl*II cuts two times outside the *ef-1α* locus and *rap1* locus, but not within the *eflGB-gfp-bsd-rap1* 3’ cassette. *Not*I cuts twice in the flanking pBluescript backbone of the pgfp-bsd-ef construct, but not within *rap1* or *ef-1α* locus sequences. These cut sites and the sizes of expected fragments are shown in Figure 2-10.

As shown in Figure 2-12, *gfp-bsd* probe detected a single band of ~12 kb in the digested gDNAs without showing any sign of residual episomal DNA. This observation suggests successful integration of the plasmid construct into the genome. The 3 kb pBluescript backbone plasmid detected only faintly a band of 3 kb. This may result from the integration of a concatemer into the genome, or could suggest a small amount of residual episomal DNA replicating extrachromosomally. As shown in Figure 2-13, the *rap1*-3’ probe also detected a single band of ~12 kb in addition to the wild type 8.6 kb band. However, the *rap1* locus does not appear to have been disrupted. A confusing result was observed on the blot probed with *ef-1α* orfA 5’ sequences. The probe detected
a band of the same size as expected in wild type C9.1, which was also around 12 kb in
length, suggesting that the ef-1α locus was also not disrupted. However, the probe
additionally detected two smaller bands of comparable signal intensity. This suggested
that either some episomal plasmid persisted extrachromosomally or the construct
integrated into the genome as a concatemer.

Therefore, initial characterization of the stably transfected parasite lines revealed
that successful integration of the construct into the genome had been achieved. However,
it appears that the construct may have targeted to an ectopic location within the genome.

Discussion

The ability to transfect B. bovis will provide an invaluable means to elucidate the
mechanism of antigenic variation and gain insights into some important babesial
processes that are specific to cattle infections. Previous work provided groundwork for
establishing the transfection system (Suarez and McElwain, 2009a; Suarez et al., 2006;
Suarez et al., 2004), but required further optimization—something recognized by the
authors as well (Suarez et al., 2007). Through the results provided herein, the conditions
for transient transfection system are now better established, and several additional
suitable promoters to drive transgene expression have been identified. When the
constructs were transfected into B. bovis IRBCs to allow uptake of the DNAs and
expression in vivo, the 5’ sequences of calmodulin and tubulin all displayed significant
competence in directing the expression of exogenous genes (Figure 2-3; Figure 2-6). The
direct comparison of our pLUC-T3 series constructs with pEF1αIG (-LUC-rap3’)
construct provided here is not completely appropriate, as different termination sequences
were used which may result in differences in the regulation of gene expression in vivo.
However, it is clear that significant promoter activity has been detected from these transfection constructs.

The type of promoter used to drive transgene expression needs to be carefully chosen for transfection because the promoter may influence the timing of expression as well as the subcellular localization of the encoded protein. For instance, in *Plasmodium spp.*, the stage-specific *P. berghei* AMA-1 promoter limited expression of the apical membrane antigen-1 of *P. falciparum* (Pf83/AMA-1) AMA-1 to the rhoptries in mature schizonts, but the constitutive *P. berghet* PbDHFR-TS promoter led to aberrant expression of the protein throughout schizogony as well as in gametocytes (Kocken et al., 1999). Another example in the expression study with PBS21 as the transgene has shown that truncation of the promoter region can lead to loss of stage-specific expression of the PBS21 gene such that constitutive gene expression was observed in the asexual blood stages of the parasite (Margos et al., 1998). Here, I have provided a range of promoters to choose from, for different purposes such as driving the transcription of several marker genes in one construct or targeting different positions in the genome of *B. bovis*.

Previous efforts at comparing relative promoter activities suffered from a lack of any means of data normalization (Suarez et al., 2006). Significantly, promoter activities are now more convincingly quantifiable through the inclusion of an internal control, using *R. reniformis* luciferase. This is a significant improvement of the transient transfection system in *B. bovis*, as without normalization of transfection conditions, it is not possible to directly compare samples. With normalization, as provided herein, the assumptions introduced when making comparisons can be minimized. In *Plasmodium spp.*, it was reported several years ago that simultaneous expression of both Firefly and
*Renilla* luciferase genes provided an opportunity to standardize experimental samples in co-transfection experiments, using *Renilla* luciferase as a transfection efficiency control (Militello et al., 2004; Militello and Wirth, 2003). Now, using a *Renilla* control construct driven by *B. bovis* calmodulin 5’ regulatory sequences together with Firefly luciferase constructs, we have found equivalent levels of expression of both luciferases, even though the two constructs possess different 3’ termination sequences. The optimized amount of experimental and control reporter plasmid is at a 3:2 molar ratio, however, the *Renilla* luciferase standard control is highly sensitive and reliable, even at 3:1 molar ratio. To save plasmids, this ratio is used in Figure 2-3. Besides its use for transient transfection experiments, this *Renilla* luciferase gene could also be integrated into the genome in order to study transcriptional regulation of a number of genes in *B. bovis*, such as testing the bidirectional promoter function of the intergenic region of *ves* gene pair by flanking the regulatory sequences with both Firefly and *Renilla* luciferase genes.

There is one point I want to make about how to ensure consistency in efficiency of plasmid DNAs from experiment to experiment. Plasmid DNAs should be either freshly prepared or aliquoted after each DNA preparation. Multiple freeze-thaw cycles should be avoided to lower the chance of sheering of circular dsDNA, which may result in variation in the amount of circular DNA loaded, and hence, making the actual molar ratio of Firefly to *Renilla* luciferase plasmid DNA constant.

Another important finding was that the 5’ regulatory sequences of *ves1α*, the majority part of which is derived from the intergenic region (Igr) of the LAT, drives significant expression of both of the exogenous marker genes used here, luciferase and EGFP, when replicated in the parasite episomally. Interestingly, this was achieved
without apparent effect on VESA1 expression, suggesting that there may be no strict ‘promoter counting’ system in *B. bovis* controlling transcription of the ves multigene family as described for *VSG* genes in *Trypanosoma brucei* (Navarro and Gull, 2001). However, it is not possible to draw a firm conclusion in this regard, as only ves 5’ regulatory sequences were present, and monoparalogous transcription may depend upon the presence of 3’ or internal sequences. We had anticipated that ves Igr-containing constructs would rapidly become silenced by the machinery silencing the rest of the ves gene repertoire. But this did not happen, which strongly suggests that the episomal ves promoter sequences failed to be recruited to the silencing mechanism. Transcriptional activity of the LAT may involve association of the LAT Igr with sequence segments from other ves genes (A. Bouchut and D.R. Allred, unpublished data). This may provide a framework to dissect maintenance of transcriptional activity at the LAT and silencing of the remainder of the ves multigene family, as well as insights into what may be a unique mechanism of transcriptional control.

As babesial parasites spend essentially all moments of their life cycle intracellularly, the ability to successfully target DNA across cell membranes to the parasite nucleus while maintaining the integrity of the host cell is impressive. Besides conventional electroporation as was used here, nucleofection has also been used to transfect *B. bovis* successfully. Nucleofection was more efficient than electroporation for transfecting smaller amounts of DNA (less than 20 µg; (Suarez and McElwain, 2008)). This was not attempted in this study due to a lack of available equipment, but could be a useful method to reduce costs in transfection/transformation studies. On the other hand, alternative strategies for loading DNA into parasites which could be performed with more
commonly available equipment were tested here. The strategy of preloading RBCs did not appear to achieve transfection of B. bovis, although it is useful for P. falciparum. However, the “ZAP twice” method may be an alternative. Preliminary results showed that if the first electroporation is performed under low voltage/high capacitance conditions and the second electroporation under high voltage/low capacitance conditions, an appreciable level of luciferase expression can be achieved.

According to the time course of expression of Firefly and Renilla luciferases, different patterns of luciferase expression may result from a series of influences, one of which is the promoter involved. In the experiments described here, firefly luciferase was driven by a ves promoter, Renilla luciferase was driven by calmodulin promoter. The promoter driving expression of a gene may influence the timing as well as the overall level of expression. Consequently, there can be significant differences in the developmental control of two reporter genes. This effect was evident in this study, as seen in Figure 2-5. Interestingly, these kinetics were similar to those of the EF1αIG promoter (Suarez et al., 2007; Suarez and McElwain, 2008), but in contrast with those of the rap-1 promoter (Suarez et al., 2004), in which luciferase expression by transfected parasites was still very limited at 24h, reaching a peak at 48 h after electroporation. This dissimilarity can also be explained by different mechanisms involved in regulation of expression of rap-1 and ves1α gene.

The success of EGFP constructs in transient transfection has shown great significance. As this reporter can be visualized in live individual cells, it has many potential applications in dissecting different aspects of cell biological processes of B. bovis. Individual parasites that express EGFP can be counted and sorted from non-
fluorescent cells by fluorescence-activated cell sorting (FACS), so as to select a particular population of the parasites. EGFP, when fused to a marker gene, will find good application in stable transfection to observe transgenic parasites. A good example is *T. gondii*, where GFP has been utilized to localize proteins to different organelles (de Koning-Ward et al., 2000).

I have determined that *B. bovis* parasites are adequately susceptible to puromycin, blasticidin-S and pyrimethamine to provide for positive selection and maintenance of transfected parasites. Puromycin inhibits protein synthesis by interacting with the A site of the large ribosomal subunit of eukaryotic ribosomes (Vazquez, 1979), whereas Blasticidin-s blocks peptide bond formation by the ribosome (Barbacid et al., 1975). Pyrimethamine interferes with folic acid synthesis by inhibiting the enzyme dihydrofolate reductase. Among these drugs, puromycin has particularly high potency, but blasticidin-S was the first one demonstrated for successful selection and integration in *B. bovis* (Suarez and McElwain, 2009a). We chose to clone the *bsd* gene in the transfection vector. During the first three trials, I used lower drug concentrations of 6.8 µM or 13.6 µM for selection, and ended up selecting spontaneously resistant parasites only. In the most recent trial, I raised the initial drug selection concentration to 27.3 µM. Parasites expanded suddenly on Day 17 post transfection and survived very well in 54.5 µM, which is lethal to wild type parasites. However, it is yet to be determined if the parasites are naturally resistant or not. Therefore, it could be a better choice to use a more potent drug like puromycin, which may provide a more stringent condition and render the possibility of selecting spontaneously resistant parasites less likely.
We have initiated a stable tranfection strategy in an attempt to induce transcriptional switching from the LAT by integration at another ves locus, using a transfection vector carrying a selectable marker driven by LAT Igr sequences. The parasites were administered drug 4h post transfection, in an attempt to select integrated parasites before the integrated sequences were silenced. Growth of parasites with selection through many generations for blasticidin resistance might have yielded stably maintained though probably episomal plasmids with assembled chromatin structure. However, it is likely that such episomes would be lost when drug pressure was removed. Advantage has been taken of this behavior in an “on-off” selection strategy to facilitate the recovery of parasites in which episomes have integrated (Crabb and Cowman, 1996; Fidock et al., 1998; Wu et al., 1996). Using this strategy, we have achieved stable integration of the same pgfp-bsd-ef construct reported by Suarez and McElwain (Suarez and McElwain, 2009a). The following observations are consistent with stable integration of the gfp-bsd gene into the B. bovis genome: A) sustained growth in culture media containing concentrations of blasticidin that are otherwise inhibitory for wild type parasites; B) identification of expression of a gfp-bsd fusion protein in transfected merozoites by western blots; C) detection of fluorescence in transfected parasites more than 5 months post electroporation; and D) evidence of integration into the genome in Southern blots, although possibly not at the expected locus.

Gene targeting in P. falciparum was first achieved with circular constructs, while integration into P. berghei genome occurred if the incoming DNA is linearized (Nunes et al., 1999). In B. bovis, it was claimed that integration can be achieved by both circular and linearized constructs (Suarez and McElwain, 2009a). We have confirmed the ability
to achieve stable integration using a linearized construct. Circular constructs may also work, although it didn’t work for me during the first trials.

These developments have provided the groundwork for future studies, such as stable transfections targeting loci of interest, including \textit{ves} loci, and may facilitate future strategies involving targeted insertional mutagenesis of \textit{B. bovis}. Of particular interest to this laboratory, the stable transfection system offers the potential to further our understanding of the switching nature of LATs, and the molecular mechanisms used to vary the structure and antigenicity of VESA1 polypeptides subunits. Ultimately, this technology will facilitate the study of parasite biology, including the functions of cellular components, vaccine development, and the development of chemotherapeutic strategies to control \textit{B. bovis} and other related parasites.
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Figure 2-1. Cloning of regulatory sequences into pGEM-LUC and control constructs. A) Major constructs with Calmodulin 5’ or LAT ves1α 5’ sequences; B) Reverse promoter control constructs with reversely inserted Calmodulin 5’ or LAT ves1α 5’ sequences; C) Positive control with demonstrated eflα IG-fragment-B promoter; D) Promoterless control construct with Tubulin 3’ sequences only; E) Internal Renilla control with Calmodulin 5’ and Plasmodium faciparum Calmodulin 3’ sequences.
Figure 2-2. Scaled schematic representation of luciferase constructs for transient transfection. A) Major constructs pC5'-LUC pLAT_Vα5’ with calmodulin 5’ or ves1α 5’ sequences. B) Promoterless negative control construct pLUC-T3 with β-Tubulin 3’ sequences only; C) Internal control construct pC5'-Renilla-C3’ with Renilla driven by B. bovis calmodulin 5’ sequences with Plasmodium faciparum calmodulin 3’ sequences.
Figure 2-3. Comparison of promoter activities using transient transfection. Luciferase expression is driven by 5’ regulatory sequences of calmodulin or ves1α gene at LAT in both orientations. The plasmid constructs are compared with a promoterless control as well as pEF1αIG containing a demonstrated promoter EF-1α IG Fragment B. Firefly luciferase activity was normalized to the level of Renilla luciferase activity obtained by co-transfection of a Renilla internal control construct pC5’-Renilla-C3’ at a molar ratio of 3:1. Error bars represent standard deviation of triplicate samples. The experiment and result is repeated at least 3 times. The analysis of variance is given in Table 2-1.
Figure 2-4. Comparison of lysis methods for detection of luciferase activity in *B. bovis* extracts. *B. bovis* C9.1 parasites are transfected with pC5’F-LUC and pC5’-Renilla-C3’ at a molar ratio of 3:2. Equal amount of transfected parasites from a single electroporation is lysed with either NH₄Cl-Tris or saponin lysis for comparison. Luciferase activity is measured and compared with the same amount of mock transfected parasites lysed with NH₄Cl-Tris method. Error bars represent standard deviation of triplicative samples.
Figure 2-5. Time course of luciferase expression in *B. bovis* parasites transfected with plasmid pLAT_Vα5‘. *B. bovis* C9.1 parasites were transfected with 11.5 pmol of firefly luciferase reporter plasmids Vα5‘ sequences. Luciferase activity was determined at 1, 2, 3, 4, 5, 6, 7, 8, 10, 24, 48, 72, 96 h after transfection. Firefly luciferase activity in parasite extracts was measured and normalized to the level of *R. reniformis* luciferase activity obtained by co-transfection of 7.7 pmol of pC5’-Renilla-C3‘. Upper panel shows luminescence values of Firefly and *Renilla* respectively. Lower panel shows the ratio of Firefly to *Renilla* luminescence. Error bars represent standard deviation of triplicative samples.
Figure 2-6. Expression of EGFP in *B. bovis* C9.1 parasites transfected with pTubulin 5’-EGFP or pVα 5’-EGFP. A. Scaled schematic illustration of 5’ sequences of β-tubulin and ves1α at LAT in pEGFP constructs. B. Detection of EGFP by fluorescence microscopy. Upper panel: pTubulin5’-EGFP; Lower panel: pVα 5’-EGFP. From left to right: phase contrast, fluorescence, and merge of the two images.
Figure 2-7. Live-cell IFA of *B. bovis* C9.1 parasites transfected with pVα5’-EGFP. A) Illustration and detailed structure of ves1α promoter. B) and C) Immunofluorescent detection of antigens on the surface of *B. bovis*-infected erythrocytes by live-cell IFA using R6a-v1765 and Chicken anti-rabbit IgG-Alexa594. From left to right: phase contrast, fluorescence (red or green channel), and merge of the previous images. Arrowheads are used to indicate infected erythrocytes.
Figure 2-8. *In vitro* growth inhibition of *B. bovis* parasites as a function of various drug concentrations, as assessed by tritiated hypoxanthine incorporation. From upper to lower: Puromycin, Blasticidin, and Pyrimethamine. Each compound was present in triplicate columns. Data was processed in OriginPro 8. The averaged data were then fitted through nonlinear regression with a sigmoidal inhibition curve (nHill=1; bottom asymptote fixed at 0).
Figure 2-9. Schematic representation of the strategy proposed to demonstrate induced switching from the LAT to another ves locus, and thus the capacity for in situ switching. Schematic representation of A) pDS-BSD and what the genomic locus look like before transfection; B) pBSD-Promiscous-targeting transfection construct C) Expected integration in the genomic locus after successful pDS-BSD integration.
Figure 2-10. Schematic representation of the original ef-1α locus before integration A); pgfpbsd-ef with size of fragments B); and what the genomic locus looks like after expected integration C).
Figure 2-11. Expression of GFP-BSD fusion protein. A) Parasites growing well at 22.2µM of blastcidin are able to glow green fluorescence constantly. B) Western blot analysis of lysates of the stably transfected parasite line, control non-selected C9.1 parasites using mouse anti-gfp serum as indicated at the bottom. Size markers in kD, are indicated on the left.
Figure 2-12. Southern blot analysis on the Stably Transfected parasite genomic DNA (STF); C9.1 genomic DNA, transfection plasmid pGFP-BSD and plasmid pBluescript. The DNAs are double digested with BglII and NotI (+), or undigested (-). The Southern blots were hybridized with α-[32P]-labeled pGFP-BSD and pBluescript probes.
Figure 2-13. Southern blot analysis on the Stably Transfected parasite genomic DNA (STF); C9.1 genomic DNA, transfection plasmid pGFP-BSD, and plasmid pBluescript. A) The DNAs are double digested with BglII and NotI (+), or undigested (-). The Southern blots were hybridized with α[^32P]-labeled rap1-3’ or ef-1αA orf 5’ probes. B). Schematic representation of rap1 locus and ef-1α locus with expected integration in the genome of C9.1 parasites.
CHAPTER 3
DISSECTION OF THE BIDIRECTIONAL PROMOTER STRUCTURE EMPLOYED IN THE
BABESIA BOVIS VES MULTIGENE FAMILY

Abstract

*Babesia bovis* is an intraerythrocytic protozoal parasite that maintains persistent infection in its vertebrate host by at least two mechanisms: cytoadhesion and antigenic variation. Both phenomena are mediated by variant erythrocyte surface antigen-1 (VESA1) protein, which is encoded by the *ves* multigene family. The *ves* genes have been found to be organized largely as closely-juxtaposed, divergently-oriented gene pairs. The similar organization and the presence of a highly conserved intergenic region (Igr) structure among all the *ves* genes identified suggested some biological significance of the 434 bp Igr. Because of our need to understand functional control of the Igr, we took advantage of the newly established transient transfection system for *B. bovis* to characterize the LAT Igr. We have identified a cluster of promoters within the 434 bp Igr, and revealed possible enhancing activity embedded within the exon/introns of the apposing gene. Preliminary results showed the inversion of certain intron element greatly reduced the promoter activity of regulatory sequences it lies in. The Igr of two transcriptionally silent donor *ves* gene pairs were found to display strong promoter activities when driving exogenous genes episomally, at levels comparable to that of the LAT Igr. These observations provide the first information available regarding the structure of babesial promoters, and provide additional evidence for the potential of silent *ves* gene pairs to become transcriptionally activated, becoming the new LAT.

Introduction

At least two strategies are employed by *B. bovis* to survive immune defenses (Allred and Al-Khedery, 2004) of the host and promote long-term persistent infection (Allred et al., 1994; Calder et al., 1996; Callow, 1963.; Mahoney et al., 1973). One strategy is the adhesion of *B.
bovis-infected erythrocytes to bovine endothelial cells, resulting in sequestration of mature parasites in capillaries and post-capillary venules (Callow, 1963.; Wright, 1972; Wright and Goodger, 1979). The second significant strategy is the capability of the parasite to undergo clonal antigenic variation. Both of these two phenotypes are embodied within the VESA1 protein, a size-polymorphic, parasite-derived protein doublet “variant erythrocyte surface Ag 1” expressed on the surface of infected red blood cells. VESA1 has been identified as a key component for both cytoadhesion and antigenic variation (Allred et al., 1994; O'Connor et al., 1997).

Our understanding of the mechanisms underlying antigenic variation of VESA1 protein in B. bovis has benefited from the characterization of the genomic locus of active ves transcription (LAT). The LAT is organized as a quasi-palindromic structure containing two closely related but structurally different ves genes encoding the VESA1a and 1b subunits (Al-Khedery and Allred, 2006; Allred et al., 2000). The two genes are in a closely-juxtaposed, divergent orientation, with a short intergenic region (Igr) of only 433 bp (Allred and Al-Khedery, 2006). Because of this uniquely compact structure the ves1α and ves1β genes have overlapping 5’UTRs. It is therefore not unreasonable to propose that there are regulatory elements embedded within the intergenic region. Evidence supporting this idea comes from the identification of the transcription start site for the ves1α gene embedded within the Igr (Allred et al., 2000).

Evidence collected so far suggests that a majority of ves genes share structure and organization similar to that of the tightly-juxtaposed quasipalindromic ves gene pair of the current LAT. Alignment of the intergenic regions located between several ves genes has revealed that these 400-450 bp intergenic regions are highly conserved in organization and much of their sequence (Allred and Al-Khedery, 2006). This suggests equivalent potential of intergenic upstream sequences in each of these gene pairs to function as a promoter. As to which ves genes
may become the LAT, the choices may be limited. When a bulk parasite population was
analyzed by RT-PCR using universal primers flanking a highly variable region (Zupanska et al.,
2009), 78 out of 84 sequences analyzed strongly support that a single ves locus is
transcriptionally active at one time, which was the known LAT. One was a match for another
gDNA locus. The rest did not match any known genomic locus. This suggested the possibility
that in situ switching of transcription could occur from LAT to a different gDNA locus.
However, this question still remains to be answered.

So far, nothing is known regarding promoter structure in Babesia spp. parasites. The most
straightforward approach to perform an initial dissection of promoter structure is to determine
the ability of various portions of 5’ sequences to drive the transcription of exogenous reporter
genes in vivo. Recently, we have developed the ability to transf ect parasites transiently in a
robust and reproducible manner, with exogenous genes. Taking advantage of this technique to
observe the promoter activities of a variety of Igr and nearby sequences, we have identified a
cluster of promoter activities within the 434 bp Igr of the LAT. Possible cis-enhancing activity
has been observed. These observations provided the first information available on promoter
structure in a babesial parasite.

Material and Methods

Parasites

B. bovis parasites of the clonal line, C9.1, were maintained in vitro under microaerophilous
conditions, as described (Allred et al., 1994).

Cloning of Constructs with LAT Intergenic Region Regulatory Sequences

A total of 6 plasmid constructs were constructed in this group. Plasmid pLUC-T3, which
was described in the previous section, was used as template. Without adding regulatory
sequences, pLUC-T3 was also used as a promoterless negative control. The 434 bp LAT
intergenic region was amplified by PCR from phagemid 6-1 (Allred and Al-Khedery, 2006) using oligonucleotides (XW79: 5’-CG[GGATCC]TATGTTACCACCCTTTGTTT-3’) and (XW80: 5’-CG[GGATCC]TGTCAGTGCTTCTAGGAGTACT-3’). The resulting PCR product was digested by BamHI, and cloned into the BamHI site of pLUC-T3. As only plasmid with reversely inserted 5’ sequences was recovered, which was named pLAT_Ig_Vβ5’, an ApaI restriction site was then engineered into 5’ end of the insert using (XW95: 5’-AGG[GGGCCC]TATGTTACCACCCTTTGTTT-3’). This facilitated the strategy of oriented insertion of the digested PCR product into pLUC-T3 doubly digested with ApaI plus BamHI to get the plasmid with 5’ sequences inserted in forward direction. This construct was consequently named pLAT_Ig_Vα5’ (Figure 3-1).

Fragments containing one half of the Igr were amplified from pLAT_Ig_Vα5’ with creation of an ApaI site at the 5’ end and BamHI site at the 3’ end. The amplicon was again digested and cloned into pLUC-T3 doubly digested with ApaI plus BamHI, by directional insertion. Sequences to create the 233 bp of pLAT_halfIg#1 were amplified from pLAT_Ig_Vα5’ using (XW106: 5’-AGG[GGGCCC]AGATTCCGTATAAGCAATTC) and (XW80: 5’-CG[GGATCC] TGTCAGTGCTTCTAGGAGTACT-3’). Sequences for the 200 bp of pLAT_halfIg#2 were amplified from pLAT_Ig_Vα5’ using (XW95: 5’-AGG[GGGCCC]TATGTTACCACCCTTTGTTT-3’) and (XW107: 5’- CG[GGATCC] TACTGGATAATCCATATTCTTAC-3’). The 200 bp of pLAT-half-Ig#3 was amplified from pLAT_Ig_Vα5’ using (XW108: 5’-AGG[GGGCCC]TACTGGATAATCCATATTCTTAC-3’) and (XW79: 5’-CG[GGATCC]TATGTTACCACCCTTTGTTT-3’). Finally, the 233 bp of pLAT-half-Ig#4 was amplified from the pLAT_Ig_Vα5’ using (XW109: 5’-AGG[GGGCCC]TGTCAGTGCTTCTAGGAGTACT-3’) and (XW110: 5’-
CGC[GGATCC]AGATTCCGTATAAGCAATTC-3’). Again, digested PCR products were inserted into *Apa*I plus *BamHI* doubly digested pLUC-T3. The position of each relative to the full Igr, and its orientation relative to the luciferase gene upon insertion, are shown in Figure 3-1.

**Cloning of Constructs with Additional Exons and/or Introns from the Apposing Gene**

A total of 9 plasmid constructs were included in this group. pLAT_Vα5’ was described in the previous section as well as pLAT_Ig_Vα5’ and pLAT_Ig_Vβ5’. The 239 bp of coding sequence of actin housekeeping gene, which are presumably non-regulatory, were amplified from genomic DNA with (XW127: 5’-CC[AAGCTT]CTATCCAGGCTGTGCTTTC-3’) and (XW128: 5’-CC[AAGCTT]TTCCTTAATGTCACGCACAATC-3’). The resulting amplicon was digested with *Hind*III and added to the *Hind*III site of pLAT_Ig_Vα5’ to generate “size control” plasmid pActin_Ig_Vα5’. Sequences for the creation of other plasmids were amplified from phagemid 3-2-1 (Allred and Al-Khedery, 2006). The 584 bp used to create pLAT_Vα5’toE1 were amplified with (XW124: 5’-

AG[GGGCCC]GGGGGCCAGAGTCTCTGTGGTGG-3’) and (XW80: 5’-

CG[GGATCC]TGTCAGTGCTTCTAGGAGTACT-3’). The 3’ antisense primer used in all ves1β5’ constructs was (XW79: 5’-CG[GGATCC]TATGTTACCACCCTTTGTGTTT-3’). It was paired with (XW125: 5’-AG[GGGCCC]CACCATCACCAGCTTTTTAC-3’) for the 557 bp of the pLAT_Vβ5’toE1 construct; with (XW111: 5’-

AG[GGGCCC]CTGTATCAGTGAATTCCCATAG-3’) for the 670 bp of the pLAT_Vβ5’toI1 construct; with (XW126: 5’- AG[GGGCCC]CATGGTACTCCAGTTGTACTG-3’) for the 734 bp of Vβ5’toE2 fragment; and with (XW112: 5’-

AG[GGGCCC]CTTGTTTTGAGAACTGTCAG-3’) for the 807 bp of the pLAT_Vβ5’toI2 construct. Consequently, PCR products were doubly-digested with *Apa*I plus *BamHI* and inserted into similarly digested pLUC-T3 plasmid. These constructs are shown in Figure 3-2.
Cloning of Constructs with Intronic Sequences Inverted

Three plasmid constructs were made in this group. The 43 bp of inverted intron 1 sequences of ves1β were amplified from pLAT_Vα5’ with (XW135: 5’-CC[AAGCTT]GTAAGTCAATAGCACTAAC-3’) and (XW136: 5’-CCA[ATGCAT]CTGTATAGCCATGTAAGAG-3’). Similarly, the 111 bp of inverted intron 1 sequences of ves1α were amplified from pLAT_Vβ5’toI1 using (XW137: 5’-CC[AAGCTT]GTACGTTGGCATAGAC-3’) and (XW138: 5’-CCA[ATGCAT]CTGTATCAGAGTGAATTC-3’). Finally, the 72 bp of inverted intron 2 sequences of ves1α were amplified from pLAT_Vβ5’toI2 using (XW140: 5’-CC[AAGCTT]GTAAGTACCTAGTAGTGGAG-3’) and (XW141: 5’-CCA[ATGCAT]CTATAGGACACCATGAG-3’). Consequently, PCR products were doubly-digested with HindIII plus NsiI and inserted into similarly digested pLUC-T3 plasmid. These constructs are named pLAT_Vα5’toE1+revI1, pLAT_Vβ5’toE1+revI1, pLAT_Vβ5’toE2+revI2, and shown in Figure 3-3.

Cloning of Intergenic Regions from Two Other ves Donor Loci

The 669 bp of the 5’ sequences of the ves1αC gene was amplified from cosmid 1E10 (Allred and Al-Khedery, 2006) by PCR, using primers (XW77: 5’-CGC[GGATCC]TCCTTGGCGTCATACAGTAG-3’) and (XW78: 5’-CGC[GGATCC]TGTCAGTGCCTTTAGGAGTACTC-3’), with creation of BamHI restriction sites on both ends. The amplicon was inserted into pLUC-T3, and correctly inserted plasmid was named p1E10.

Another 658 bp of 5’ sequences of ves1αA was amplified from cosmid S621(Allred and Al-Khedery, 2006) with (XW92: 5’-AGG[GGGCC]TGTCAGTGCCTTTAGGAGTACTC-3’), with creation of BamHI restriction sites on both ends. The amplicon was inserted into pLUC-T3, and correctly inserted plasmid was named p1E10.
(XW76: 5’- ACGTCGC[GGATCC]TATCAGTGCTTCTAGGAGTACTCAG-3’). The amplicon was inserted into pLUC-T3, and the construct was named pS621.

All plasmids were confirmed by digestion mapping and sequencing. DNA plasmids were prepared using Endotoxin-free Plasmid Purification Maxi Kit (Qiagen), following the manufacturer’s instructions.

**Transient Transfection and Luciferase Assay**

Transient transfection was performed as described (Suarez et al., 2004), with minor modifications. Briefly, *B. bovis* C9.1 parasites were cultivated to a ppe of 5 ~ 15%. For each transfection, 150 µl packed infected RBCs were electroporated with 11.5 pmol DNA in 250 µl DNA/ cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/ KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA, 5 mM MgCl₂, final pH 7.6) suspension at 1.25 kV/ 1.07 mF at 200 Ω (Gene Pulser II, BioRad, Hercules, CA) in a 2 mm gap cuvette (Fisher Biotech, Subiaco, WA). Parasites were transfected with each construct in three separate cuvettes. Each individual transfection also included 7.7 pmol pC5’-Renilla-C3’, a plasmid expressing *Renilla reniformis* luciferase using *B. bovis* calmodulin 5’ and *P. falciparum* calmodulin 3’ flanking sequences, as transfection efficiency and recovery control. Transfected RBCs were then diluted into 3 ml complete culture medium at a packed cell volume of 2.5%. DNA concentration in the cuvette was approximately 0.25 µg/µl. Time constants recorded were limited within the range from 0.26 ms to 0.38 ms. The medium was changed daily and 50 µl of packed RBCs were added the next day to support the growth of the parasites. At 24 h post-transfection, RBCs were collected and lysed by NH₄Cl-Tris lysis as described in (Suarez et al., 2004). Parasites were washed 2X with 1xVYM, and lysed in 100 µl passive lysis buffer (Promega, Madison, WI).

The luciferase assay was performed according to the manufacturer’s instructions (Promega, Madison, WI). Both Firefly and *Renilla reniformis* luciferase activities in 100 µl of
cleared extract were quantified on a MicroBeta Jet scintillation spectrometer following the manufacturer’s protocol (PerkinElmer; Waltham, MA). Briefly, a 24-well white plate protocol was set up to perform a 10-second measurement period for each reporter assay without setting premeasurement delay and mixing. Lysates from the same triplicative group were carefully laid out in the manner they were to be assayed simultaneously. Background lysate liquid luminescence was recorded three times, and the average reading in each well was subtracted from firefly luciferase activities to correct for this background. LARII reagent (100 µl) was dispensed by multichannel pipette to ensure simultaneous reaction of luciferin substrate with firefly luciferase and minimum time delay before measurement. After the first reading, 100 µl Stop&Glo reagent was added immediately, followed by a second measurement. Renilla luciferase reading from mock-transfected parasite lysates was subtracted from all Renilla luciferase readings. The level of firefly luciferase activity was normalized as a ratio the level of Renilla luciferase activity. These experiments with triplicate samples were repeated at least four times each, except those performed with inverted intron constructs, which were performed twice only.

**Biostatistical Analysis of Promoter Activities**

The normalized luciferase activities were plotted using Microsoft Excel as the mean ± standard deviation, calculated from the triplicate samples. Differences in promoter activities are determined by two-sided two sample t-test, using SPSS program (SPSS, Chicago, IL). Comparison and the calculated P values are listed in Table 3-1 to Table 3-4. If the P value is below the threshold value chosen for statistical significance (P=0.05), the two promoter activities are considered significantly different.
Results

Analysis of ves Igr Sequences

The Igr sequences of the LAT were examined for their ability to drive expression of exogenous gene. Template construct pLUC-T3 was previously made with 3’ β-tubulin flanking sequence cloned downstream of firefly luciferase (LUC) gene to provide the relevant transcription termination signals (X Wang, D.R Allred; unpublished data). New plasmids were constructed by insertion of the 434 bp Igr sequence in both directions upstream of the LUC gene in pLUC-T3. In order to test the minimal region necessary for functional promoter activity, expression plasmids possessing truncated Igr sequence were prepared to identify the minimal 5’ sequences that retained promoter activity. Four half Igr fragments were amplified from the 434 bp Igr and cloned to pLUC-T3. These six plasmids were introduced into B. bovis C9.1 parasites by electroporation, and firefly luciferase activity was measured 24 h post-transfection. To normalize the inherent variation in transfection efficiency, the level of firefly luciferase activity was normalized to the level of Renilla reniformis luciferase obtained by co-transfection of parasites with the plasmid pC5’-Renilla-C3’ as described in the previous section.

As shown in Figure 3-1 and Table 3-1, upon transfection, each of the six plasmids, containing a complete or half Igr respectively, produced luciferase activity regardless of insert orientation (Figure 3-1) (P<0.001). Transfection with pLAT_Ig_Vα5’ construct produced the significant luciferase activity among the six sequences analyzed (P=0.0001). This construct contained complete LAT Igr sequence, inserted in the “ves1α orientation”, i.e. with the LUC sequences on the same side of the Igr as the ves1α gene would normally be. Placement of both the 5’ and the 3’ half alone immediately upstream of the LUC gene produced significant LUC activity when expressed in vivo (P<=0.0001). When the three fragments were inserted in “ves1β orientation”, i.e. with the LUC sequences on the same side of the Igr as the ves1β gene would
normally be, there not much distinct differences in the levels of LUC expression were observed when comparing LAT_halfIg #3 and #4, or LAT_halfIg #4 and LAT_Ig_Vβ5' (P>0.05). The luciferase signals produced from each of the six plasmids were significantly different from those produced by pLUC-T3, (P<0.001), with which luciferase levels indistinguishable from background were detected.

**Analysis of ves Igr Flanking Sequences on Promoter Function**

In order to explore more of the promoter activities of ves genes, additional sequences were included in the construct to identify possible transcriptional control elements by examining for increases or reductions in expression levels. The pLAT_Vα5’ plasmid was used to compare with pLAT_Ig_Vα5’ construct containing full-length Igr. On the apposing side, the ves1β 5’ regulatory sequences through intron 1 of ves1α, as well as ves1β 5’ regulatory sequences through intron 2 of ves1α were amplified and cloned to pLUC-T3, creating plasmids “pLAT_Vβ5’toI1” and “pLAT_Vβ5’toI2”. As shown in Figure 3-2 and Table 3-2, upon transfection, the presence of ves1β exon 1 and intron 1 reproducibly resulted in 1.5 fold higher luciferase expression (P=0.0015). On the apposing side, the presence of the exon1/intron1 pair of ves1α resulted in significant increase in luciferase expression (P=0.0054), however, the presence of exon2/intron2 did not result in significant increase when compared with full length Igr or with exon1/intron1 pair only (P>0.05). Again, the luciferase signals produced from each of the five plasmids were significantly different from those produced by pLUC-T3, (P<0.05).

In order to test which part of the exon/intron pair resulted in the increase of luciferase expression, these sequences were further dissected. A new construct “pLAT_Vα5’toE1” was prepared, containing ves1α5’ regulatory sequences through exon1 only of ves1β. On the other side, the ves1β 5’ regulatory sequences through exon1 of ves1α, as well as ves1β 5’ regulatory sequences through exon2 of ves1α were amplified and cloned to pLUC-T3 to create
“pLAT_Vβ5’toE1” and “pLAT_Vβ5’toE2”. To control for any non-specific effects due to the length of apposing sequences, a size control construct was also assembled. Actin coding sequences 239 bp in length was added immediately upstream of Igr sequences of pLAT_Igr_Vα5' to form construct “pActin_Ig_Vα5’”. This control construct has a size comparable to that of pLAT_Vα5’. These new constructs were compared with the full-length Igr constructs, as well as those containing exon/intron pairs. As shown in Figure 3-2 and Table 3-2, upon transfection, the presence of actin coding sequences in a position 5’ of the pLAT_Ig_Vα5' Igr sequences did not alter the level of LUC activity with respect to the parental plasmid (P=0.409). The luciferase activities produced from pLAT_Vα5’toE1 were lower than from pLAT_Vα5’ (P=0.0095), but higher than from pLAT_Ig_Vα5’ (P=0.0354). On the apposing side, inclusion of sequences up to exon 1 of ves1α doubled the level of LUC expression with respect to the full-length Igr (P=0.0354), but addition of intron 1 of ves1α did not result in any further increases in LUC expression (P=0.1117). Similarly, inclusion of Exon 2 did not substantially increase the level of LUC expression compared to pLAT_Vβ5’toI1 (P=0.848). Addition of intron 2 of ves1α did not result in significant increase either, (P=0.9664). Overall, the luciferase signal produced by each of the nine plasmids was significantly different from that produced from the promoterless pLUC-T3 control (P<0.05).

Effects of Intronic Sequences Inversion

Three more constructs were made in order to test for any effects that inversion of intronic sequences might have. These new constructs were compared with those constructs without introns and those with introns in their normal orientation. As shown in Figure 3-3 and Table 3-3, inversion of intron 1 of ves1β did not result in significant change in LUC expression when compared to pLAT_Vβ5’toI1 (P=0.8978). The luciferase expression is not significantly different from pLAT_Vα5’toE1 (P=0.0719). On the apposing side, inversion of intron 1 of
ves1α did not result in significant change in LUC expression when compared to pLAT_Vβ5’toE1 (P=0.7553) or pLAT_Vβ5’toI1 (P=0.2490). However, inversion of intron 2 of ves1α resulted in a substantial decrease in LUC expression when compared to both pLAT_Vβ5’toE2 and pLAT_Vβ5’toI2 (P=0.0031; P=0.0006). Intriguingly, the normalized luminescence is also significantly different from any of the other plasmids (P<0.01). Overall, the luciferase signal produced by each of these plasmids was significantly different from that produced from the pLUC-T3 control (P<0.01).

Comparison of Promoter Activities of Sequence Donor Loci with the LAT

In order to determine the competence of Igr sequences from sequence donor sites to serve a promoter function under in vivo conditions, the 658 bp of ves1αA 5’ sequences from cosmid S621, which is an α/α pair, as well as the 669 bp of ves1αC 5’ sequences from cosmid 1E10, which is an α/β pair, were amplified from corresponding cosmid template DNAs. These two sites are similar in size and organization to the 678 bp of ves1α gene 5’ sequences from the LAT, and are known sequence donors for observed segmental gene conversion events (Al-Khedery and Allred, 2006. The Igr sequences from 1E10 are similar to Vα5’ from the LAT in both the nature and composition of sequences apposing the reporter gene, as both are presumed ves1α promoters and both contain an exon 1/ intron 1 pair of an apposing ves1β gene. Alternatively, the Igr sequences from S621 are comparable to those from LAT in that both are ves1α promoters, but different in the composition of the sequences apposing the luciferase gene, as the S621 5’ sequences contains an exon1/intron 1 pair from a ves1α gene. In this sense, it is similar to Vβ5’.

As shown in Figure 3-4 and Table 3-4, LUC expression from Igr of 1E10 was roughly half that of LAT Vα5’ (P=0.0124), whereas LUC expression from Igr of S621 was about half that of LAT Vβ5’ (P=0.0349) and only one third of that of LAT Vα5’ (P=0.0077). However, all were
significantly greater than the promoterless control pLUC-T3 and drove appreciable levels of luciferase expression (P<0.05).

**Discussion and Conclusions**

Prior to this work, there was not much information available regarding gene regulatory sequences in *B. bovis*. Because of our interest in cytoadhesion and antigenic variation, both of which are mediated by the *ves* multi gene family (Allred et al., 2000). The newly developed transient transfection system was utilized to identify regulatory elements within this family. Finer dissection of the intergenic region of the LAT revealed a cluster of minimal promoters embedded within the short 434 bp intergenic region of the LAT, whereas the efficiency of the full-length Igr was not significantly different from the two half fragments in the same orientation in driving the expression of LUC gene. The Igr drives significant luciferase activity when present *in vivo* as an episome. There is no marked dependence on orientation. Previously, when *ves1α* transcripts derived from the LAT were analyzed, the putative transcriptional start site was identified by a cap-independent 5'- rapid amplification of cDNA ends (5'-RACE) method. The site of transcription initiation of the p9.6.2 cDNA mapped to the first base 3’ of an upstream pair of predicted hairpin/cruciform sequences in the *ves1β* side of the Igr (Allred et al., 2000). The presence of heavily overlapping 5’-UTR sequences on *ves1α* transcripts was confirmed by the use of a 5’-cap-dependent RACE procedure, and was also the case for C9.1*ves1β* transcripts (Al-Khedery and D. R. Allred, unpublished data). Based on these data, the 5’ sequences of the apposing genes may overlap by 110 bp or more. This organization and overlapping transfection start sites suggested that coordinate control of the transcription of both genes may occur. However, the results of live-cell immunofluorescence, performed with VESA1a and 1b-specific antibodies, suggest that co-localization of VESA1a and the VESA1b is not absolute, and mostly occurred in reactions with mature parasitized erythrocytes. Therefore, the temporal expression of
the two subunits is not necessarily equivalent, and the expression may not be tightly co-regulated (Y Xiao and D.R. Allred, submitted data).

Besides dissecting the promoter activity derived from Igr-only sequences, I also analyzed ves promoter activities with the inclusion of exon/intron pair(s). The experiments in Figure 3-2 revealed potential enhancing activity residing in the exon/intron pair in the opposing gene that may serve to regulate ves gene expression. To exclude a trivial explanation for the increased expression of LUC as being due simply to the increased template length, I inserted 239 bp of presumably non-regulatory β-actin coding sequences upstream of the Igr promoter of pLAT_Ig_Vα5’ to serve as a “size control”. As expected, the additional length had no effect on the expression of LUC gene (Figure 3-2, Table 3-2). Therefore, I conclude that the increase of LUC expression from pLAT_Ig_Vα5’ to pLAT_Vα5’tol1 resulted from regulatory effects imparted by the exon/intron pair.

Possible roles of the introns included in the constructs were analyzed by truncating the fragments at the distal end of each exon relative to Igr sequences. The results provided in Figure 3-2 suggested that there are possible enhancing activities embedded in intronic sequences of ves1β (P=0.0095) and in exon 1 of ves1α (P=0.0327). As potential enhancers should be orientation-dependent, each sequence was cloned in the reverse direction to determine whether this will result in elimination of enhancing activity. When ves1β intron 1 was reversely inserted, no change was observed in the luciferase expression, consistent with a potential enhancer function. In contrast, reversal of the ves1α intron 2 repeatedly caused a great reduction (approximately half) in luciferase expression (P=0.0031). Several potential explanations exist for this result. Firstly, it may be that normal promoter structure was disrupted by reversing sequences in the intron 2 of ves1α, and that the full promoter embedded in the 5’ sequences of ves genes is long, extending far beyond the intergenic region. Unfortunately, the effects of
inversion of intron 1 of ves1α varied in two experiments. It caused reduction in one experiment, but didn’t change much in LUC expression in the other. When the data from two experiments are pooled, it did not result in significant change (P=0.7553). Further analysis is needed to draw any conclusion. Intriguingly, despite the clear overall symmetry of the Igr, the complete promoter structure may be asymmetric, with minimal promoters centrally located, an auto enhancer on one side, and accessory elements on the other. The function(s) of any such accessory elements is not clear. An alternative explanation is that a separate promoter activity resides in intron 2 which, when reversed, drives transcription of antisense transcripts. Such antisense transcripts may interfere with ves promoter activity or with translation of transcripts containing ves 5’-UTR sequences. These possibilities remain to be explored.

In B. bovis, few regulatory elements sufficient to drive gene expression have been characterized, and no information is available regarding their cognate DNA-binding proteins. Most transcription factors common to higher eukaryotes are not found in the Apicomplexan genome. However, an amplified family of Apicomplexan specific AP2 (ApiAP2) transcription factors has been identified by bioinformatic means as the primary DNA-binding domain present in all Apicomplexan parasites sequenced to date (Balaji et al., 2005). It has been shown that the DNA-binding domain sequences, and potentially their binding specificities, of orthologous pairs of AP2 domains are fundamentally conserved in six Plasmodium spp. and six other Apicomplexan species, including B. bovis (De Silva et al., 2008). DNA motifs specifically bound by AP2 domains of the PF14_0633 gene were predicted using protein binding microarray and FIRE algorithm computational analysis (De Silva et al., 2008; Elemento et al., 2007). The core nucleotides were determined to be CATGC or CGTGC. Interestingly, CGTGC was found in the exon 1 of ves1β. It is possible that transcription factors are recruited to this region and involved in regulation of the locus. The alignment of 25 intron 1 sequences from ves1α has revealed two
highly conserved DNA motifs within this short region. As shown in Figure 3-5, one motif is GTTACTGTAGACAT at the beginning of the intron 1. The second motif is GCGCGGCC, located close to the 3’ end of the intron 1. The GCGCGGCC sequence is a motif known as a “GC box” (Ohme-Takagi and Shinshi, 1995) and is found in both the Igr and intron 1 sequences, and may be recognized by AP2 transcription factors. This is consistent with the facilitated promoter activity observed in constructs containing these sequences in addition to Igr sequences. No clear DNA-binding protein motifs were identified in the ves1α intron 2.

In *P. falciparum*, silencing of var genes is associated with transcriptional activity of a second, intron- associated promoter found in each var gene, and silencing seems to depend upon a pairing of the two (Calderwood et al., 2003; Frank et al., 2006). However, more recent evidence suggests the intronic promoter may serve primarily to maintain silencing imposed upon the gene by sequences in the 3’ regulatory region (Muhle et al., 2009).

The last experiment showed all the 3 different ves loci (representing the α/β LAT, α/α S621, and α/β 1E10 loci) are capable of driving comparable levels of luciferase expression, revealing the potential for this gene family to undergo in situ switching. Figure 3-6 illustrates a hypothetical in situ switching event, which shows that silencing of the current LAT may be accompanied by the activation of a different ves locus, which would become the new LAT site. The overall similarities of the intergenic regions of the known transcribed LAT gene copy and the sequence donor gene copies do reflect functional similarities. There are 119 annotated ves genes in the *B. bovis* genome, over half of which are organized as divergent gene pairs (Brayton et al., 2007). Yet, analysis of ves transcripts in the C9.1 line parasite population strongly suggests that ves transcription occurs only at the known LAT and is mutually exclusive, with silencing of all ves loci but one (ie. the LAT) (Zupanska et al., 2009). It is possible that the LAT is the only ves locus with an operational promoter. However, our data showed that Igr sequences from non-
transcribed ves loci also display transcriptional competence, indicating that other loci have the potential to be transcribed in situ. The mechanism underlying this exclusion is not known. Previously, we anticipated that ves Igr-containing constructs, upon insertion into parasites, would rapidly become silenced by the machinery silencing the rest of the ves gene repertoire. However, the Igr from the LAT can drive significant expression of the EGFP gene when replicating in the parasite episomally, without any apparent effects on VESA1 expression (X Wang; D.R. Allred, unpublished data). This result indicated that the episomal ves promoter sequences failed to be recruited into, or affected by the silencing mechanism.

In conclusion, our results demonstrated that the ves Igr is a true bidirectional promoter. A cluster of minimal promoter activities are embedded within this area and the capacity to express exogenous genes in vivo is enhanced by regulatory elements embedded within flanking exonic or intronic sequences. It is unclear what effect these sequences might have on the gene being transcribed. ves Igr from non-transcribed loci also showed promoter activity, when inserted episomally, and thus their potential to be transcribed in situ. This result renders less likely a situation in which there exists only one or a few sites competent for transcription. This would open new avenues to study the mechanism of antigenic variation by disrupting or replacing the current LAT, and revealed potential for in situ switching of LAT.
<table>
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Table 3-2. Statistical analysis of promoter activities in Igr of LAT with additional exon(s) and intron(s).

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Table 3-3. Statistical analysis of promoter activities affected by intron inversion

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Table 3-4. Statistical analysis of promoter activities of Igrs from LAT as well as non-transcribed ves loci

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Figure 3-1. A cluster of promoters are revealed in the IG region of LAT. *B. bovis* C9.1 parasites were transfected with 11.5pmol of firefly luciferase reporter plasmids containing a series of LATIG sequences. At 24h post-transfection, firefly luciferase activity in parasite extracts was measured and normalized to the level of *R. reniformis* luciferase activity obtained by co-transfection of 7.7pmol of pC5'-Renilla-C3’. Error bars represent standard deviation of triplicate samples. The lower panel illustrates the LATIG and LAT halfIG series sequences, which were cloned into 5’ of LUC gene in pLUC-T3 template, as shown on the right. The experiment and result is repeated at least 3 times. Independent two sample t-tests were performed and the calculated P values are given in Table 3-1.
Enhancing activities are revealed in the individual exon or intron sequences of the apposing genes. *B. bovis* C9.1 parasites were transfected with 11.5 pmol of firefly luciferase reporter plasmids containing a series of LAT regulatory sequences. At 24 h post-transfection, firefly luciferase activity in parasite extracts was measured and normalized to the level of *R. reniformis* luciferase activity obtained by co-transfection of 3.8 pmol of pC5'-Renilla-C3'. Error bars represent standard deviation of triplicative samples. The lower panel illustrates the series of LAT ves 5' sequences, which were cloned into 5' of LUC gene in pLUC-T3 template. The experiment and result is repeated at least 3 times. Independent two sample t-tests were performed and the calculated P values are given in Table 3-2.
Figure 3-3. Effects on luciferase expressions when introns are reversely inserted. *B. bovis* C9.1 parasites were transfected with 11.5 pmol of firefly luciferase reporter plasmids containing a series of LAT regulatory sequences. At 24 h post-transfection, firefly luciferase activity in parasite extracts was measured and normalized to the level of *R. reniformis* luciferase activity obtained by co-transfection of 7.7 pmol of pC5′-Renilla-C3′. Error bars represent standard deviation of triplicative samples except for the bar labeled #7, which represents standard deviation of six samples in one experiment. The lower panel illustrates the series of LAT ves 5′ sequences, which were cloned into 5′ of LUC gene in pLUC-T3 template. The experiment and result is repeated two times. Independent two sample t-tests were performed and the calculated P values are given in Table 3-3.
Figure 3-4. Comparable promoter activities revealed in donor ves IG region with LAT. A. *B. bovis* C9.1 parasites were transfected with 11.5 pmol of firefly luciferase reporter plasmids containing ves 5' sequences of 1E10 and S621 non-transcribed loci. At 48h post-transfection, firefly luciferase activity in parasite extracts was measured and normalized to the level of *R. reniformis* luciferase activity obtained by co-transfection of 3.8 pmol of pC5'-Renilla-C3'. Error bars represent standard deviation of triplicative samples. B. Illustration of Igr sequences of 1E10 and S621, which were cloned into 5' of LUC gene in pLUC-T3 template. The data shown is from one experiment with triplicative samples. The experiment and result is repeated at least three times. Independent two sample t-tests were performed and the calculated P values are given in Table 3-4.
Figure 3-5. Alignment of sequences of ves1α from LAT (accession number: DQ267461) from *B. bovis* C9.1 line, and 24 donor loci from the T2Bo isolate revealed two highly conserved DNA motifs (red bar).
Figure 3-6. Illustration of a possible *in situ* switching of transcription activity event in *B. bovis*.
CHAPTER 4
CONCLUSION

With this body of work in addition to published studies, the ability to transiently transflect *B. bovis* has been well established. This technology now provides the opportunity to determine how gene expression is developmentally controlled in *B. bovis*, as well as to identify important genetic elements for gene regulation. Several promoters are now available to drive transgene expression efficiently, and parameters for introducing DNA into the parasites have been improved. Promoter activity is now convincingly quantifiable with an efficient internal control, and is also visualized with the employment of a second reporter, EGFP.

Stable transfection has now been achieved in *B. bovis* a second time, previously reported by Suarez et al (Suarez and McElwain, 2009a), confirming the feasibility of this approach to the genetic manipulation of this parasite. Several selectable markers for *B. bovis* have been identified to provide tools for efficient selection and maintenance of the transfectants. Successful integration of the exogenous fusion gene has been confirmed, although gene targeting is not yet reproducible. Further investigation into the parameters for optimal gene targeting may provide the opportunity for functional analysis of proteins by gene disruption and gene replacement. Hence, the *in situ* switching mechanism behind antigenic variation of *B. bovis* may be addressed. Stable transfection will ultimately serve as a great tool to assist in dissecting and understanding the biology of how *B. bovis* mediates the phenotypic variation of surface protein to accomplish sequestration and immune evasion.

Investigation of promoter activity embodied in LAT sequences by transient transfection revealed a cluster of promoters embedded within the intergenic region, as well as possible enhancing activities residing in both exonic and intronic sequences in the opposing gene. At least two predicted DNA motifs specifically bound by AP2 domains were found to be located within
this region, and conserved sequences in intron 1 of the ves1β gene were identified. Further investigation is now possible to understand the regulation of the ves multigene family. In addition, investigation of promoter activity of ves Igr from other non-transcribed loci also showed their potential to be transcribed in situ. This allowed discrimination from a situation with only one or a few sites competent for transcription.

Now, the development of both transient and stable transfection technologies has been facilitated by the release of the full genome sequence of the Texas T2Bo isolate of B. bovis (Brayton et al., 2007). With the genomic sequences accessible to all, elucidation of the function of many other proteins and identification and validation of candidates that are most suitable for vaccine and drug target development may be accelerated by the application of this genetic tool. All the work that has been done, as well as the additional development of the genetic tools, will greatly improve the genetic manipulation of B. bovis, and significantly contribute to our understanding of the biology of the parasite.
## APPENDIX A
PRIMERS USED IN STUDY

Table A-1. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (Restriction sites are shown in brackets)</th>
<th>Accession Number and sequence number</th>
</tr>
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<tbody>
<tr>
<td>XW17: calmodulin 5’_5’HindIII</td>
<td>CC[AAGCTT]TACCGAGAAGAGCCTGCAAC</td>
<td>AAXT01000005: 583125-583144</td>
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<tr>
<td>XW18: calmodulin 5’_3’HindIII</td>
<td>CC[AAGCTT]GTATTTAATAATATTAATGGC TAACTG</td>
<td>AAXT01000005: 584492-584521</td>
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<tr>
<td>XW21: tubulin 5’_5’HindIII</td>
<td>CC[AAGCTT]GAAACTCGCATCGCTCTAAAC</td>
<td>AAXT01000001: 1031672-1031692</td>
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<tr>
<td>XW22: tubulin 5’_3’HindIII</td>
<td>CC[AAGCTT]CTATTTGTTACACTACAGATG AACATGAAC</td>
<td>AAXT01000001: 1033307-1033277</td>
</tr>
<tr>
<td>XW23: tubulin 3’_5’SacI</td>
<td>C[GAGCTC]ACATAGTATAACCTTTATGCAGAAGTC</td>
<td>AAXT01000001: 1034813-1034842</td>
</tr>
<tr>
<td>XW24: tubulin 3’_3’SacI</td>
<td>C[GAGCTC]AAGAGCGTAATATGCGCTTG</td>
<td>AAXT01000001: 1035786-1035805</td>
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<tr>
<td>XW25: ves IG_5’HindIII</td>
<td>GC[AAGCTT]GGAATCATACAGTAGGTCTTC</td>
<td>DQ267461: 8048-8069</td>
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<tr>
<td>XW26: ves IG_3’HindIII</td>
<td>GC[AAGCTT]TGTCAGTGCTTCTAGGAGTACT</td>
<td>DQ267461: 8701-8725</td>
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<tr>
<td>S621 IG_5’BamHI</td>
<td>ATCG[GAGCTC]TGTACAGGGGTGAGTC TAT</td>
<td>DQ267461: 3964-3983</td>
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<tr>
<td>S621 IG_5’ApaI</td>
<td>AGG[GAGCTC]TGTACAGGGGTGAGTCTAT</td>
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<td>S621 IG_3’BamHI</td>
<td>ACGTGC[GAGCTC]TATCAGTGCTTCTAGGAGTACT</td>
<td>DQ267461: 4621-4597</td>
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<tr>
<td>S621 IG_3’ApaI</td>
<td>AGTACAGGGGTGAGTAATGCTATG</td>
<td>DQ267461: 3964-3983</td>
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<tr>
<td>XW75: LAT_IG5’_BamHI</td>
<td>CGC[GAGCTC]TGCCTGCTTCTAGGAGTAC TC</td>
<td>DQ267461: 8292-8311</td>
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<td>XW76: LAT_IG5’_ApaI</td>
<td>AGG[GAGCTC]TATGTTACCACCCCTTTGTTT</td>
<td>DQ267461: 8292-8311</td>
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<tr>
<td>XW77: 1E10 IG_5’BamHI</td>
<td>CGC[GAGCTC]TGCCTGCTTCTAGGAGTAC TC</td>
<td>DQ267461: 8704-8725</td>
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<tr>
<td>XW78: 1E10 IG_3’BamHI</td>
<td>CGC[GAGCTC]TGCCTGCTTCTAGGAGTAC TC</td>
<td>DQ267461: 8498-8517</td>
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<td>XW79: LAT_halfIG#1_ApaI</td>
<td>AGG[GAGCTC]TATGTTACCACCCCTTTGTTT</td>
<td>DQ267461: 8498-8517</td>
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<tr>
<td>XW80: LAT_halfIG#2_BamHI</td>
<td>CGC[GAGCTC]TGCCTGCTTCTAGGAGTAC TC</td>
<td>DQ267461: 8498-8517</td>
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Table A-1. Continued

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<th>Name</th>
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<tr>
<td>XW108: LAT_halfIG#3_ApaI</td>
<td>AGG[GGGCC]TACTGGATAATCCATATTATT CTAC</td>
<td>DQ267461: 8468-8492</td>
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<td>XW109: LAT_halfIG#4_ApaI</td>
<td>AGG[GGGCC]TGTCAGTGCTTCTAGGAGTA CT</td>
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<tr>
<td>XW110: LAT_halfIG#4_BamHI</td>
<td>CGC [GGATCC]AGATTCCGTATAAGCAATTTC</td>
<td>DQ267461: 8498-8517</td>
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<tr>
<td>XW111: LAT_ves1βIntron 1_ApaI</td>
<td>AG[GGGCC]CTGTATCAGAGTGAATTCCATAG</td>
<td>DQ267461: 8960-8938</td>
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<tr>
<td>XW112: LAT_ves1βIntron 2_ApaI</td>
<td>AG[GGGCC]CTTGGTTTTGAGAACTGTCAG</td>
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<td>XW124: LAT_ves1βexon1_ApaI</td>
<td>AG[GGGCC]GGGGGCCAGAGTCCTTGTTTG</td>
<td>DQ267461: 8141-8161</td>
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<tr>
<td>XW125: LAT_ves1αexon1_ApaI</td>
<td>AG[GGGCC]CACCATCAACCAGCCTTTTACAG</td>
<td>DQ267461: 8849-8829</td>
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<tr>
<td>XW126: LAT_ves1αexon2_ApaI</td>
<td>AG[GGGCC]CATGGTACTCCAGTTGTACTG</td>
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<td>XW127: actinCDS_5'HindIII</td>
<td>CC[AAGCTT]CTATCCAGGCTGTGCTTTC</td>
<td>AAXT01000006: 89587-89605</td>
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<td>XW128: actinCDS_3'HindIII</td>
<td>CC[AAGCTT]TTCTTATGTCAAGCACAAT</td>
<td>AAXT01000006: 89367-89388</td>
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<td>XW135: ves1β Intron 1Rev5'_HindIII</td>
<td>CC[AAGCTT]GTAAGTCAATAGCACTAACCAG</td>
<td>DQ267461: 8140-8122</td>
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<td>XW136: ves1β Intron 1Rev3'_NsiI</td>
<td>CCA[ATGCAT]CTGTATAGCCCATGTAAGAG</td>
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<td>XW137: ves1αIntron 1 Rev5'_HindIII</td>
<td>CC[AAGCTT]GTACGGTGGCATAAGAC</td>
<td>DQ267461: 8850-8865</td>
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<tr>
<td>XW138: ves1αIntron 1 Rev3'_NsiI</td>
<td>CCA[ATGCAT]CTGTATAGCAGTTGAATTTC</td>
<td>DQ267461: 8960-8943</td>
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<tr>
<td>XW140: ves1αIntron 2 Rev5'_HindIII:</td>
<td>CCA[ATGCAT]GTAAGTGACCTTGGTGAATGAG</td>
<td>DQ267461: 9027-9046</td>
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<td>XW141: ves1αIntron 2 Rev3'_NsiI</td>
<td>CCA[ATGCAT]CTATAGGACACCATGAG</td>
<td>DQ267461: 9098-9082</td>
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APPENDIX B
STABILITY OF TRANSFORMABLE DNA

The stability of exogenously inserted DNA in bovine erythrocytes was previously tested (excellent technical support provided by Jeanne Blackwell). This was done by examination of the ability to recover circular, transformable DNA, and quantified based on the ability to transform *E. coli*. Recovery was quantified as the number of antibiotic-resistant colonies as a function of hours post-transfection. It was determined that, using the condition of 150 V, 1000 µF, and 70 Ω, it is possible to load bovine RBCs with exogenous DNA with minimal loss of parasite viability. The half-life (t₁/₂) of transformable DNA in RBCs was approximately 10 hours 15 min, according to the exponential curve that fitted to the data points (R² = 0.997).

Figure B-1. Transformed *E. coli* recovery as a function of hours post-transfection of bovine RBCs with transformable DNA. DNAs were loaded into bovine erythrocytes at the condition of 150 V, 1000 µF, and 70 Ω. DNAs were collected from wells at 24h, 48h, 72h time points post transfection and used to transform *E. Coli* by electroporation. (Experiment by Jeanne Blackwell)
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Xinyi Wang was born in 1982 in Shanghai. She was brought up in the vicinity of Fudan University, one of the most prestigious universities in China, and had a happy childhood. In 2000, she was admitted to Fudan University for her undergraduate education. 4 years later, Xinyi Wang earned her B.S. degree in Life Sciences Department from Fudan University, and left Shanghai for the first time. She came to the United States to continue her graduate study in the Interdisciplinary Program at College of Medicine of the University of Florida in 2004. While taking classes, she worked as a Research Assistant in a lab of the Department of Infectious Diseases under the supervision of Professor David R. Allred. She quite enjoyed the time she spent in the lab, working on transfection and genetic manipulation of Babesia bovis. Her research focused on the mechanism of antigenic variation in B. bovis.