GENERATION OF A CYTOMEGALOVIRUS EXPRESSING INTERLEUKIN-12 TO STUDY VIRAL DISSEMINATION AND IMMUNOGENICITY IN THE GUINEA PIG MODEL

An Undergraduate Research Scholars Thesis

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ABSTRACT

Generation of a Cytomegalovirus Expressing Interleukin-12 to Study Viral Dissemination and Immunogenicity in the Guinea Pig Model. (May 2014)

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Congenital cytomegalovirus (CMV) is a leading cause of mental retardation and deafness in newborns; therefore, the development of a vaccine for the virus is of great importance. The guinea pig is the only small animal model for the study of congenital CMV infection and since HCMV and other CMV are highly specific to particular host species, guinea pig CMV (GPCMV) must be used for congenital CMV infection studies. Interleuking-12 (IL-12) is a proinflammatory cytokine dimer that stimulates T-cell activity during the immune response. The subunits of IL-12, especially the IL-12p40 (IL-12B) subunit, have potential for use as adjuvants in CMV vaccines to stimulate the T-cell response. The purpose of this project is to use molecular cloning strategies to create recombinant guinea pig CMV (GPCMV) strains expressing the guinea pig ortholog of IL-12B. This will allow the investigation of the effect of the IL-12B subunit on CMV pathogenicity in the guinea pig animal model as well as the impact of this cytokine on the resulting T-cell immune response to GPCMV infection.

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CHAPTER I

INTRODUCTION

The *Herpesviridae* virus family is composed of enveloped, icosahedral, double-stranded DNA viruses classified in three subfamilies: alpha, beta, and gamma [Knipe & Howley, 2013]. Herpesviruses are characterized by their ability to establish latency during infection, remaining in the host's system for the duration of the organism's life. During latency, the virus's genome is maintained in virtually dormant episomes after primary infection until circumstances are optimal for reactivation of infection [Knipe & Howley, 2013]. There are eight different human herpesviruses (HHV1-8) that cause a variety of diseases and establish latency in different parts of the body.

Cytomegalovirus (CMV) is a betaherpesvirus and like most herpesviruses, it has a relatively large genome; the human cytomegalovirus (HHV-5, HCMV) genome is the largest of any herpesvirus at 236,000 bp (Figure 1). It is spread by direct contact with body secretions such as saliva and sexual contact [Knipe & Howley, 2013]. It disseminates quickly in the host and establishes latency, but is usually asymptomatic because the host immune system suppresses the infection to low levels [Knipe & Howley, 2013]. However, the virus is opportunistic and when the host immune system is compromised for any reason, such as in transplant patients and HIV-infected individuals, it can cause a variety of life-threatening end organ diseases [Knipe & Howley, 2013]. It is also the most common congenital viral infection and the leading cause of neurological sequelae, mental retardation, and deafness in newborns [Knipe & Howley, 2013]. The rate of infected newborns is about 1%, though it varies by region and population and can range up to 2% [Kenneson & Cannon, 2007]; this transmission rate can increase due to a variety of risk factors, such as HIV exposure and premature delivery [Kenneson & Cannon, 2007].

Transmission rates from mothers with a primary HCMV infection is about 32%, while congenital infection from a maternal latent infection is much lower [Kenneson & Cannon, 2007]. Approximately 10% of congenitally infected infants exhibit symptoms [Kenneson & Cannon, 2007]; of those infants, 90% suffer from mental, hearing, visual, or motor disabilities apparent at birth, though some of these same symptoms may manifest later in life in initially asymptomatic children [Ghekiere et al. 2012]. This means that over 5000 children born every year suffer from serious medical conditions due to congenital HCMV infection, creating a long-term public health concern (Figure 2). A review panel at the Institute of Medicine declared that a vaccine to protect pregnant women from HCMV infection in order to prevent congenital infection was second in priority only to an HIV vaccine in the field of infectious disease [Stratton et al. 2000]. The same study estimated that an effective HCMV vaccine would save up to $4 billion annually in the U.S.

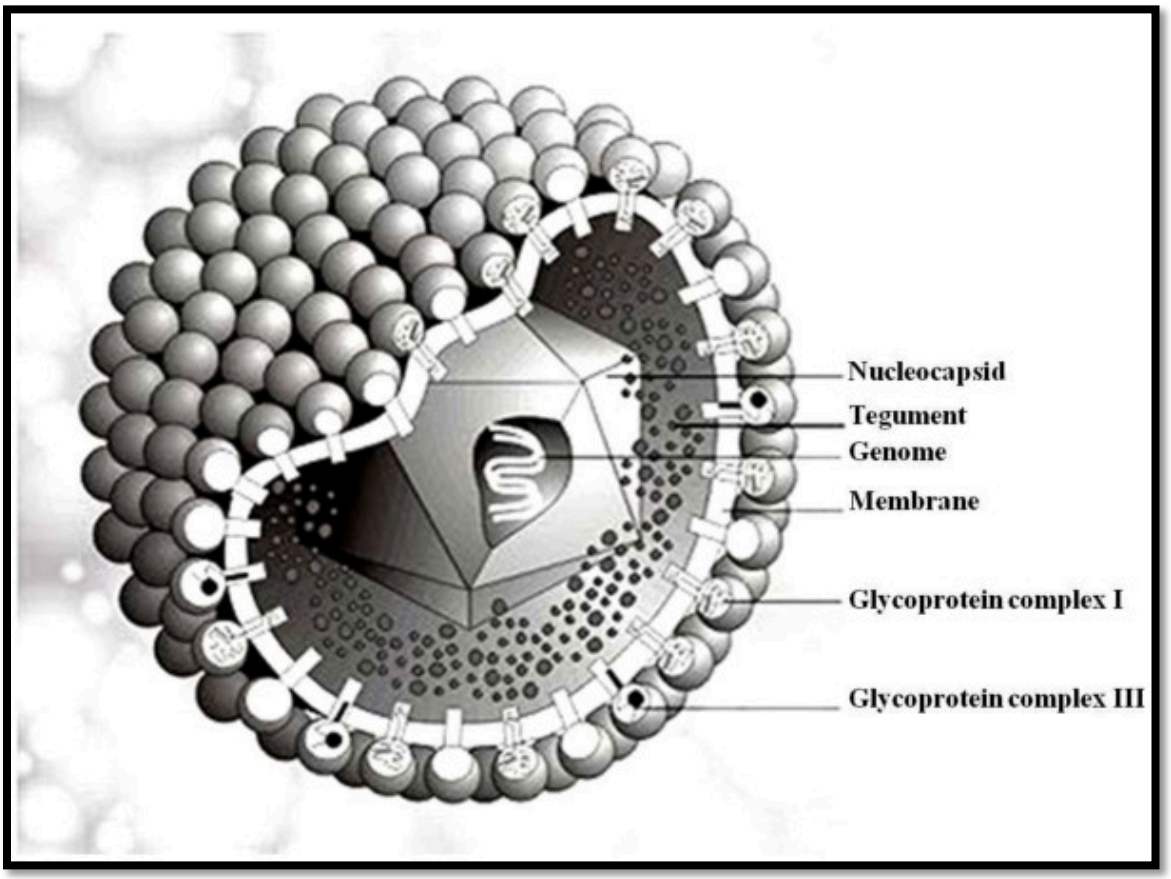

Figure 1. The general structure of the HCMV virion. [Reddehase, 2006]

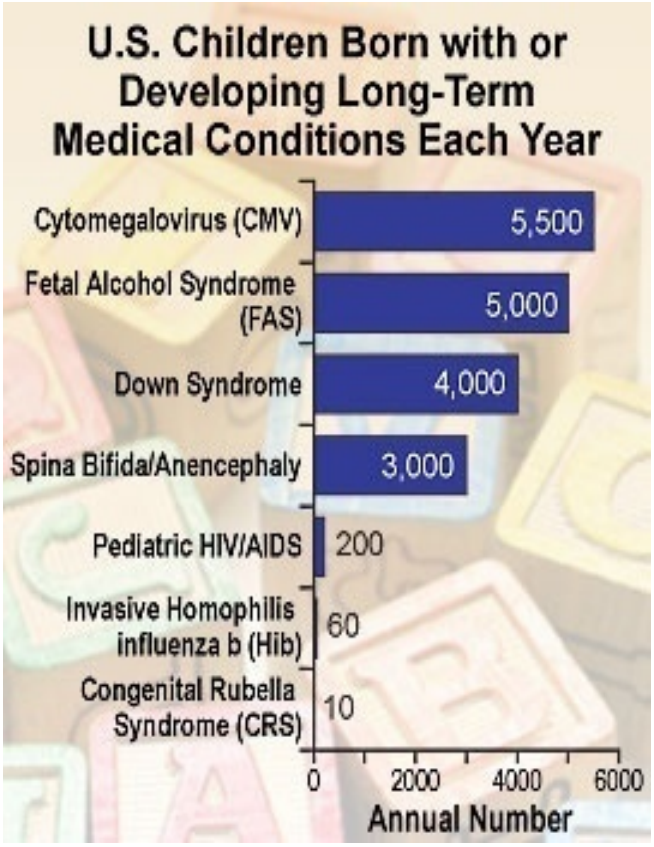

Figure 2. **HCMV epidemiology statistics.** Over 5000 children born each year suffer from long-term medical conditions because of congenital HCMV infection, more than any other condition.
Source: Center for Disease Control

However, efforts to produce an effective HCMV vaccine have been only partially successful. Over the past several decades, much time and effort has been expended in trying to develop various vaccine strategies, such as protein subunit, aphavirus replicons, and attenuated live vaccines [Lilja & Mason, 2012]. Many have focused on the neutralizing antibody response to the gB fusion protein [Lilja & Mason, 2012]. In fact, phase 2 clinical trials have been conducted to test the efficacy of a purified gB vaccine combined with the MF59 adjuvant, but the vaccine was found to be only 50% effective in protecting against primary HCMV infection [Pass et al. 2009]. Other studies have explored using T-cell targets such as the protein pp65 with some success in mounting a T-cell response, and though several vaccines have undergone clinical trails, no vaccine has shown enough clinical protection from HCMV to be approved for use [Lilja & Mason, 2012]. Further research is necessary to increase the efficacy of current vaccine strategies or develop more effective ones. A robust T-cell response to viral antigens is important for controlling life-long infections in convalescent HCMV patients. The endeavor to develop an effective vaccine not only includes the study of the antibody response and T-cell response to viral antigens; various adjuvants are being considered

in an effort to increase the efficacy of the vaccine [Chen et al. 2013]. Additionally, though the most common type of vaccine stimulates a humoral (antibody-mediated) immune response, stimulating the specific T-cell response to infection may also have great potential as a vaccine strategy.

The multicomponent immune response to HCMV infection is complex and incompletely understood. The primary targets for neutralizing antibodies are the viral envelope glycoproteins gB and gH and antibodies against various other glycoproteins and glycoprotein complexes have been detected [Knipe & Howley, 2013]. However, it appears that the antibody response is not enough to protect against HCMV infection, because seropositive individuals can often be reinfected [Ross et al. 2010]. The cytotoxic T-cell response appears to be more crucial to HCMV protection, as evidenced by the severe HCMV secondary infections that occur in T-cell deficient AIDS patients [Lilja & Mason, 2012]. The highly abundant tegument protein pp65 is a common $CD8^+$ T-cell target, as is the IE1 protein, which is expressed soon after infection [Gibson et al. 2004], but there may be other uncharacterized T-cell targets. Stimulating and maintaining a T-cell response to HCMV infection will likely be an important part of any successful vaccine strategy, and continued research is being conducted to develop delivery systems and adjuvants to do so [Lilja & Mason, 2012].

Interleukin-12 (IL-12) is an important proinflammatory cytokine produced by dendritic cells and phagocytes in response to recognition of pathogens by Toll-like receptors and other receptors [Chang & Radbuch, 2007]. Like all cytokines, this protein dimer is a secreted glycoprotein that interacts with receptors on nearby cells to regulate their gene expression and behavior [Shrum,

1996]. Interleukins in particular play an important role in regulating immunity, including stimulating T-cell proliferation and inducing fever [Shrum, 1996]. Interleukin-12 plays several roles in the immune response; it activates natural killer cells [Brunda, 1994], induces interferon-γ production, and is thought to be imperative for the development and proliferation of the T_h1 T-cell immune response by stimulating production of IFN-γ [Chang & Radbuch, 2007]. Effectively, it acts as a link between the innate and adaptive immune system [Trinchieri, 2003].

Mature T_h1 regulatory cells induced by IL-12 produce Interleukin-10 (IL-10) some time after the immune response is induced, providing a negative feedback mechanism to regulate the immune response [Chang & Radbuch, 2007]. This cytokine can limit the T-cell response during CMV or other viral infections, though it also stimulates the Natural Killer (NK) cell response to infection [Stacey et al. 2011]. It has been found that HCMV encoded an IL-10 homolog that exhibits the immunosuppressive functions of IL-10, but not its immunostimulatory activities [Kotenko et al. 2000]. This ability allows the virus to suppress IL-12 activity and avoid the T-cell mediated immune response [Kotenko et al. 2000]. Upregulating IL-12 expression or downregulating IL-10 expression may counter this evasion tactic.

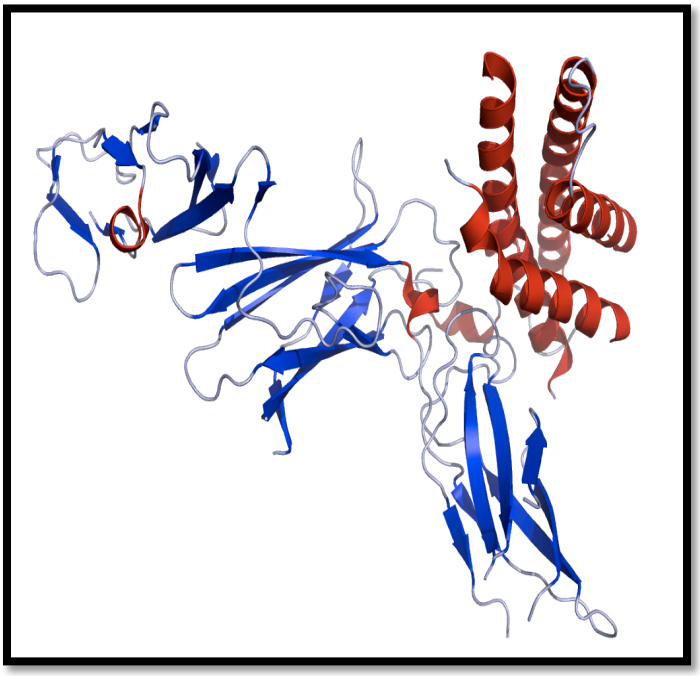

Figure 3. Structure of the human IL-12 dimer. The blue subunit is IL-12B and the red subunit is IL-12A. Source: Protein Database, rcsb.org

IL-12 consists of two subunits, designated p35 (IL-12A) and p40 (IL-12B); the p40 unit is shared with IL-23, but the p35 unit is unique to IL-12 (Figure 3) [Chen et al. 2013]. The cytokine has been shown to act as an effective adjuvant in vaccination for rhesus macaque CMV [Jacobson et al. 2006], but use of both subunits simultaneously may have cytotoxic effects in humans and has actually suppressed the immune response in mice [Leonard et al. 1997, Orange et al. 1995]. It has been suggested that the two subunits play different roles in the immune response [Osario & Ghiasi, 2005] and when administered individually, both subunits have been shown to have the ability to stimulate an immune response. However, there is some controversy about which subunit can more effectively do so. One study showed that IL-12A increased the efficacy of the immune response against HSV-1 in a recombinant viral vaccine [Osario & Ghiasi, 2005], but a study by Chen [Chen, et al. 2013] indicated that IL-12B is more important in stimulating an immune response to *Chlamydia muridarum* and a study conducted by Khader et al. showed that IL-12B is essential for immunity to *Mycobacterium tuberculosis* [Khader et al. 2006]. The subunits of this potent cytokine may be useful as adjuvants for a recombinant CMV vaccine, but further research is necessary to determine the full extent of the relative effects of each subunit.

As with many other infectious diseases, the use of animal models has proven invaluable in the study of HCMV. Guinea pigs offer the only practical animal model for congenital CMV infection studies. Guinea pig CMV (GPCMV) produces many of the same defects in newborn pups as appear in infected newborn humans [Hashimoto et al. 2013]. HCMV and GPCMV are very similar; most HCMV genes have homologs in GPCMV (Figure 4).

Importantly, many guinea pig cytokines have been cloned into cDNA and characterized at Texas A&M and other institutions, including the guinea pig ortholog of IL-12 [Shiratori et al. 2001]. The protein sequence and structure of guinea pig IL-12 (gpIL-12) are similar to that of human IL-12 and the cytokine performs the same functions [Shiratori et al. 2001]. This makes GPCMV an important model in which to study the effect of IL-12 on the host immune response to CMV infection.

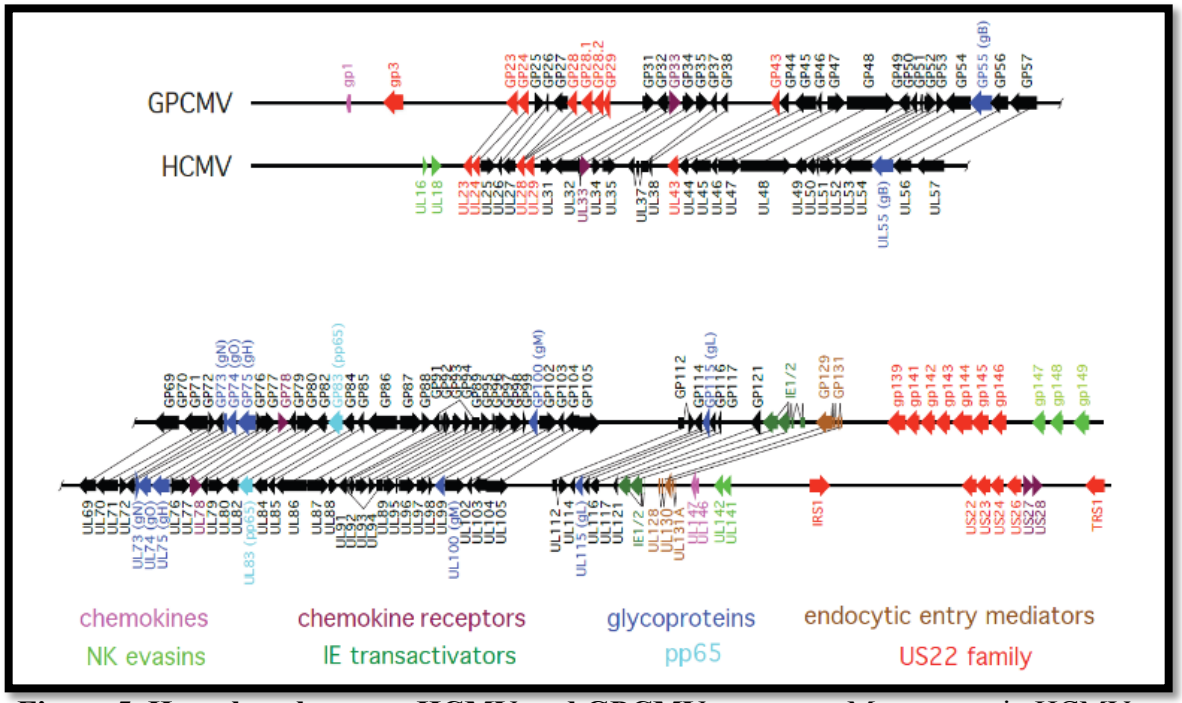

Figure 5. Homology between HCMV and GPCMV genomes. Most genes in HCMV have a homolog in GPCMV [McGregor et al. 2013].

Though there is some controversy over which component of IL-12 is most important to the immune response during infection, a majority of the literature indicates that IL-12B may be more effective at stimulating the T-cell response [Chen et al. 2013, Khader et al. 2006]. With that in mind, the goal of this project was to create a recombinant GPCMV virus encoding gpIL-12B in order to investigate the effect of IL-12B on the T-cell response during CMV infection in future experiments. Elucidating the role of IL-12B in this context will inform the possibility of its use as a vaccine adjuvant to stimulate the T-cell response.

IL-12A

Score	Expect	Method	Identities	Positives	Gaps
514 bits(1323)	0.0	Compositional matrix adjust.	259/330(78%)	284/330(86%)	6/330(1%)

```
Guinea pig  1    MCHRQLISSWLSLVLLASPLLAMWELKKDVYVVELDWHTDAPGETVVLTCNTAEEDGITW  60
            MCH+QL+ SW SLV LASPL+A+WELKKDVYVVELDW+ DAPGE VVLTC+T EEDGITW
Homology
Human       1    MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITW  60

            61   TSDRKSDILGSGKTLTIQVKEFEDAGGYTCHKGGEVLSRSQLLLHKKEDEIWSTDILKEQ  120
            T D+ S++LGSGKTLTIQVKEF DAG YTCHKGGEVLS S LLLHKKED IWSTDILK+Q
            61   TLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQ  120

            121  KGSNGKTFLKCEARSYSGRFTCWWLTAFGTDVKFSVKGSRGSSDPSGVTCG----EAERV  176
            K    KTFL+CEA++YSGRFTCWWLT   TD+ FSVK SRGSSDP GVTCG     AERV
            121  KEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAERV  180

            177  SGDNQEYKYSVECQEDSACPTAEESLPIEVVVDAIHKFKYENYTSSFYIRDIIKPDPPKN  236
             GDN+EY+YSVECQEDSACP AEESLPIEV+VDA+HK KYENYTSSF+IRDIIKPDPPKN
            181  RGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKN  240

            237  LQLKPSVNSQQVEVSWEYPDTWSTPHSYFSLTFLVQTHGKNKNRREKKYELFTDKTSATV  296
            LQLKP  NS+QVEVSWEYPDTWSTPHSYFSLTF VQ  GK+K  REKK  +FTDKTSATV
            241  LQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK--REKKDRVFTDKTSATV  298

            297  SCHKISKVEVRARDRYYSSSWSEWASVSCS  326
             C K + + VRA+DRYYSSSWSEWASV CS
            299  ICRKNASISVRAQDRYYSSSWSEWASVPCS  328
```

IL-12B

Score	Expect	Method	Identities	Positives	Gaps
306 bits(784)	2e-109	Compositional matrix adjust.	154/202(76%)	170/202(84%)	2/202(0%)

```
Guinea pig  54   SLARNLPVATPDPGMFP--CLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKD  111
            SLARNLP A+P P   P  C +HSQ LLRAV++ L KA Q L  Y CT EEIDHEDITKD
Homology
Human       20   SLARNLPRASPAPVTEPVQCFNHSQTLLRAVNSELHKAIQMLAVYSCTPEEIDHEDITKD  79

            112  KTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVE  171
            KTSTV+AC+PLEL KNESCL S   SF TNGSCLAS KTSFMMALCL+SIYEDLK+YQ+E
            80   KTSTVKACVPLELVKNESCLASGHISFTTNGSCLASGKTSFMMALCLNSIYEDLKLYQLE  139

            172  FKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCI  231
            FK MNA+LLMDP+RQIFLDQNML+ IDEL+QALN +  TVPQK SLEEPDFYK K+KLCI
            140  FKNMNAQLLMDPQRQIFLDQNMLSAIDELIQALNGSDVTVPQKLSLEEPDFYKIKMKLCI  199

            232  LLHAFRIRAVTIDRVMSYLNAS  253
            LLHAFRIRAVTIDRVMSYL +S
            200  LLHAFRIRAVTIDRVMSYLTSS  221
```

Figure 6. Human and guinea pig IL-12 protein alignments. A BLAST alignment comparing the protein sequences of human and guinea pig IL-12 subunits. IL-12A (top) shows 78% identity (identical amino acids) and 86% positivity (similar amino acids). IL-12B (bottom) shows 76% identity and 84% positivity.

CHAPTER II

METHODS

Restriction digests, gel electrophoresis, & DNA isolation

To isolate DNA fragments, confirm cloning results, or analyze BACs, digestions were carried out with restriction enzymes in appropriate buffers according to manufacturer protocol. The digested DNA was applied to an agarose gel and the DNA fragments separated by gel electrophoresis. The GeneClean® II (Obiogene) kit was used to purify DNA fragments from the gels when necessary. Briefly, the gel slices were dissolved in 1.5 mL of 6 M sodium iodine solution and 15 μL glassmilk was added to bind DNA. The glassmilk beads were washed three times with the GeneClean® II NEW wash buffer and the DNA was eluted from the glassmilk in 20 μL DNase/RNase-free water by centrifugation in a microcentrifuge at 15,000 rpm.

Gene cloning and transformation

Vector cloning was carried out by using T4 ligase to ligate restriction-digested DNA fragments into vectors digested with a compatible restriction enzyme. The ligations were either performed in a 10X ligation buffer overnight at 14˚C or in a 2X Quick ligation buffer followed by a 15-minute incubation at room temperature, using amounts of vector proportional to the amount of insert DNA. The ligations were transformed into DH5α bacterial cells using a one-minute heat shock at 42˚C followed by a 30-minute incubation on ice and a one hour incubation in 1 mL LB media at 37˚C, shaking at 100 rpm. The transformations were grown on agar plates at 37˚C with appropriate antibiotic selection. Colonies were then inoculated into 2 mL LB cultures under antibiotic selection and grown overnight at 37˚C, the plasmid DNA purified with a Qiagen plasmid miniprep kit, and analyzed by restriction enzyme digest analysis.

Polymerase Chain Reaction (PCR)

DNA was amplified using *Vent* DNA polymerase. All PCR was carried out according to the parameters in Table 1. All oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX).

Number of Cycles	Temperature	Duration
1	97˚C	3 minutes
35	97˚C	30 seconds
	56˚C	30 Seconds
	72˚C	Variable: 30 seconds per 500 bases
1	72˚C	5 minutes

Table 1. Parameters for PCR Reactions. The same parameters were used for all PCR reactions, with 35 cycles and the extension stage varying according to the size of the template (30 seconds per 500 bases).

Generation of Electrocompetent Cells

An inducible recombination system (ET system) plasmid was transformed into DH10B bacterial cells containing the GPCMV bacterial artificial chromosome (BAC) plasmid and plated under chloramphenicol selection. The cells were cultured in 10 mL LB media overnight shaking at 37˚C under chloramphenicol selection (15 μg/mL) and then in 500 mL LB at 30˚C, shaking at 300 rpm for 4 hours under chloramphenicol selection. The culture was placed in a 37˚C water bath for fifteen minutes. L-Arabinose was added (0.1% final concentration) from a stock solution of 20% (w/v) to the culture and incubation in the 37˚C water bath continued for another 10 minutes. The culture was then incubated in an ice slurry for one hour to induce ET expression. The culture was centrifuged at 5000 rpm at 4˚C, the supernatant decanted, and the pellet resuspended in 500 mL ice cold distilled water. The solution was centrifuged at 6000 rpm at 4˚C

for 20 minutes, the supernatant decanted, and the pellet resuspended in 250 mL cold distilled water. The centrifugation was repeated twice, the pellet being first resuspended in 100 mL cold distilled water and then in 10 mL ice cold 10% glycerol. The suspension was aliquoted into chilled microcentrifuge tubes, centrifuged at 6000 rpm for 1 minute, the supernatant poured off, and the pellets resuspended in ten 300 μL aliquots of 10% glycerol. The cells were then stored at -80˚C until use.

BAC Recombination

Vectors containing the DNA constructs of interest were recombined with GPCMV BACs contained in electrocompetent cells. The DNA was mixed with the ET cells in a microcentrifuge tube on ice and then transferred to a chilled cuvette before electroporation. The cells were incubated at 37˚C for several hours before plating on agar plates under chloramphenicol selection (for the BACs) and the appropriate antibiotic for the construct recombined into the BACs.

Colonies were inoculated into 2 mL starter cultures and then 500 mL cultures of double antibiotic LB media (chloramphenicol and antibiotic marker selecting for introduced DNA) and the BACs purified by large scale prep with a Qiagen maxiprep protocol modified for BACs. Restriction enzyme digest analysis was used to analyze the restriction profile of the BACs in comparison to wildtype.

Guinea pig cell transfection and tissue culture

The purified BACs were transfected into guinea pig fibroblast lung cells (GPL; ATCC CCL 158) using Lipofectamine 2000 (Invitrogen) following manufacturer protocol. All transfection

procedures were conducted in a biosafety hood. In a total volume of 200 μL of OPTIMEM media (Invitrogen), 30 μL of the BAC DNA was mixed with 2 μL of the Lipofectamine and incubated at room temperature for 10 minutes. The BAC solution was then added to 6-well dishes containing GPL cells and 1 mL of OPTIMEM media. The GPL cells had already been washed with 2 mL of OPTIMEM. The dish was incubated at 37˚C for three hours, the wells washed with 2 mL PBS, and then covered with 4 mL of F-12 media (Invitrogen/Gibco-BRL) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 10,000 IU of penicillin/liter, 10 mg of streptomycin/liter (Gibco-BRL), and 7.5% NaHCO3 (Gibco-BRL). The dishes were kept at 37˚C and the F-12 media changed twice on the day after transfection and then once per day for four days after that.

The cultures were monitored by fluorescent microscopy for GFP expression and development of GFP-tagged GPCMV.

FLP-FRT Recombination

To remove an antibiotic marker flanked by FRT sites, FLP-FRT recombination was performed with the tetracycline-resistant 707-FLPe plasmid (Genebridge) according to manufacturer protocol. Briefly, the 707-FLPe plasmid was transformed into bacterial cells already containing a BAC as described, plated on LB agar plates under antibiotic selection (tetracycline, chloramphenicol, and the resistance marker for the recombined DNA), and grown for 24 – 48 hours at 30˚C. Colonies were inoculated from these plates into 1 mL LB cultures supplemented with chloramphenicol and tetracycline and incubated 2 – 3 hours at 30˚C and shaking at 300 rpm. The temperature was increased to 37˚C for 3 hours, still shaking at 300 rpm. An inoculation loop was used to streak out cultures on LB plates supplemented with chloramphenicol and

incubated at 37˚C overnight. Resulting colonies were patch plated first on plates supplemented with chloramphenicol and the antibiotic selecting for the DNA modification and then on plates containing only chloramphenicol. The plates were incubated overnight at 37˚C. Colonies were selected that showed growth on the chloramphenicol plates but not the plates supplemented with the antibiotic selecting for the DNA modification; these were cultured under chloramphenicol selection and purified by large scale prep.

Virus Titration & Growth Curve

Recombinant GPCMV virus-infected GPL cells and supernatant (input virus multiplicity of infection (MOI): 1 PFU/cell) were harvested from six-well dishes every day post infection for seven days and stored at -80˚C until use. The viral supernatant was serially diluted to a 10^{-6} dilution and 100 μL of each dilution used to infect GPL cells. After four days, viral plaques were counted and plaque-forming units (PFU) per mL calculated. The results were plotted (PFU/mL versus time) on a semi-logarithmic plot.

CHAPTER III

RESULTS

Recombination System Testing

To ensure that the BAC recombination system would work properly for our purposes, we created a GP75 knock out BAC. The GP75 gene encodes guinea pig gH, a glycoprotein presumed to be essential for cell entry in HCMV [Paradowska et al. 2014], making the knock-out lethal. We recombined a version of the GP75 with a portion of the gene deleted as an *NruI/EcoRV* fragment and replaced with a kanamycin resistance marker (Suppl. Fig 1). The restriction profile of the resulting BAC was analyzed by restriction digestion with *EcoRI* and *HindIII* (Figure 7). The BAC was transfected into GPL cells to ensure lethality of the knock out (Figure 8a). Then it was co-transfected with a plasmid expressing gH to rescue the phenotype, confirming the cause of lethality as the knocked out GP75 gene (Figure 8b).

Cloning Shuttle Vector Construction

Part of the coding sequence of the GP25/26 locus was amplified by PCR with *EcoRI* sites incorporated at the 5' and 3' ends (Suppl. Fig. 2) because there is an endogenous *BamHI* site in the intergenic region that can be used for cloning. These were used to insert the GP25/26 gene complex into the multiple cloning site (MCS) of the pUC19 vector, which contains an ampicillin resistance marker (Suppl. Fig. 3). A *HindIII* digestion was used to linearize the DNA so the clones containing the modified vector could be easily identified (Figure 9).

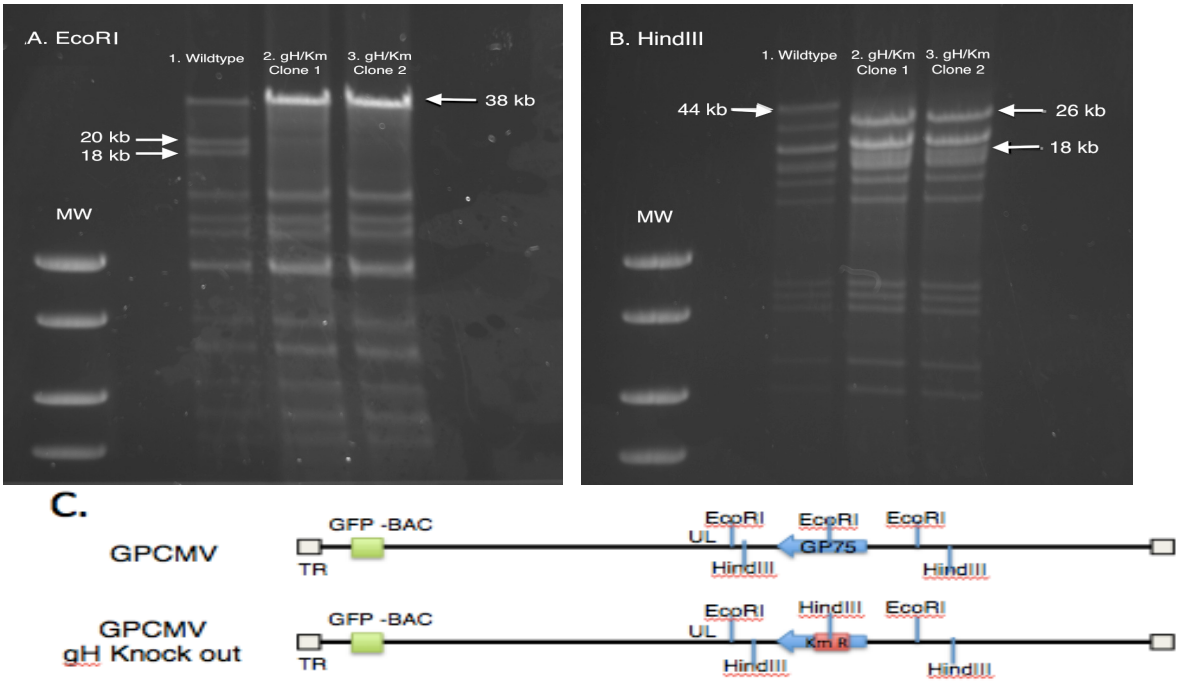

Figure 7. Restriction enzyme analysis of gH knock out recombinant BACs. (A) *EcoRI* digestion of wildtype GPCMV BAC (Lane 1) versus knock out recombinant BACs (Lanes 2 & 3). Due to the deletion in the gene, an *EcoRI* is missing in the recombinant BACs, causing the presence of a new 38 kb band instead of a 20 kb and an 18 kb band. (B) *HindIII* digestion of wildtype (Lane 1) versus recombinant BACs (Lanes 2 & 3). The introduction of a new *HindII* site in the kanamycin cassette causes two new bands at 26 kb and 18 kb instead of the large one at 44 kb. This result confirms the successful recombination of the modified GP75 gene construct. (C) GPCMV BAC maps. The top part of the figure shows the restriction profile of a wildtype GPCMV BAC. The lower part of the figure shows the modified profile, with a new *HindIII* site inserted and an *EcoRI* site deleted.

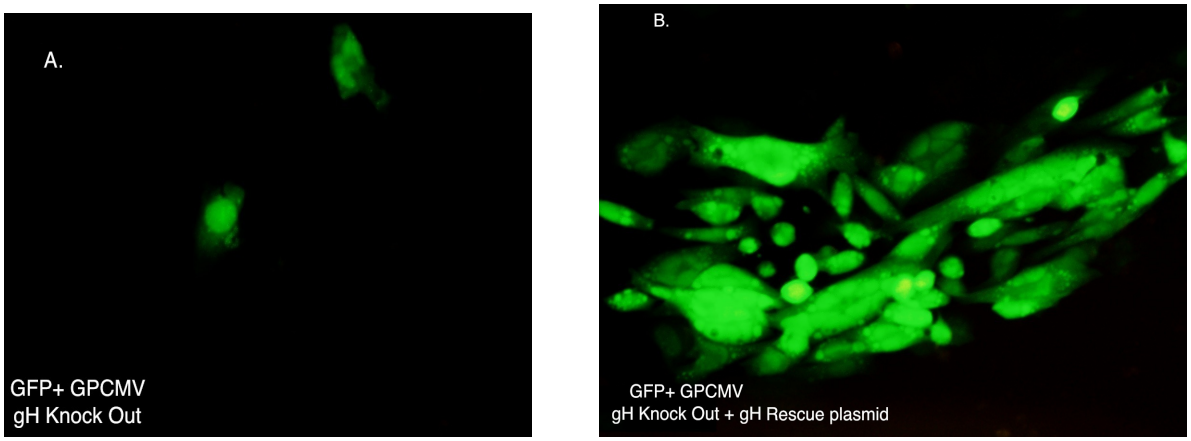

Figure 8. GPCMV gH knock out recombinant BAC transfections. (A) When transfected onto GPL fibroblast cells, the GFP signal could be detected in transfected cells, but the virus did not disseminate to other cells. (B) When the GPCMV gH knock out BAC was co-transfected with a plasmid carrying the wildtype GP75 locus, a rescued virus was generated through recombination that was able to disseminate through the monolayer.

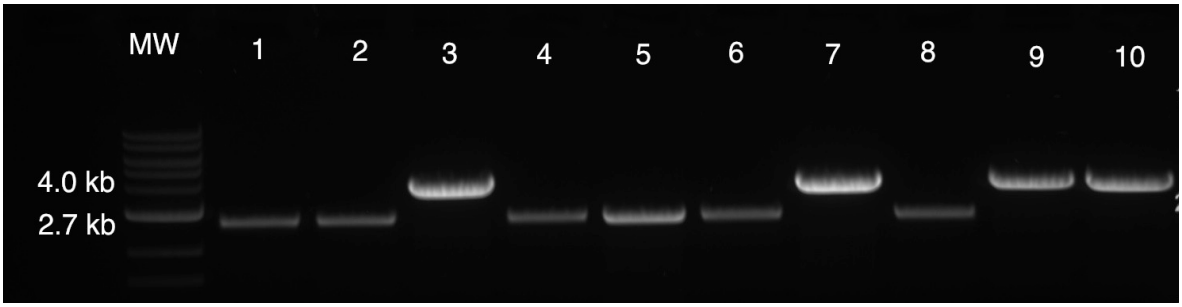

Figure 9. *HindIII* digestion of the pUC19/GP2526 vector. Ten clones were analyzed by *HindIII* digestion to determine whether the GP25/26 construct had been inserted into pUC19 vector. In clones 3, 7, 9, and 10, the 4.0 kb band size indicates the presence of the 1.3 kb insert into the 2.7 kb vector.

The SV40 promoter and polyadenylation sequences were digested out of a pSI vector (Suppl. Fig 5) as a *BglII*/*BamHI* fragment and inserted into the endogenous *BamHI* site between the GP25 and GP26 genes (Suppl. Fig. 4). Orientation of the insertion was determined by *HindIII* digestion (Figure 10). The intron downstream of the SV40 promoter and most of the MCS were collapsed by a *PstI/SalI* digestion and the SV40 multiple cloning site (MCS) modified to include an *XhoI* site (Suppl. Fig. 6).

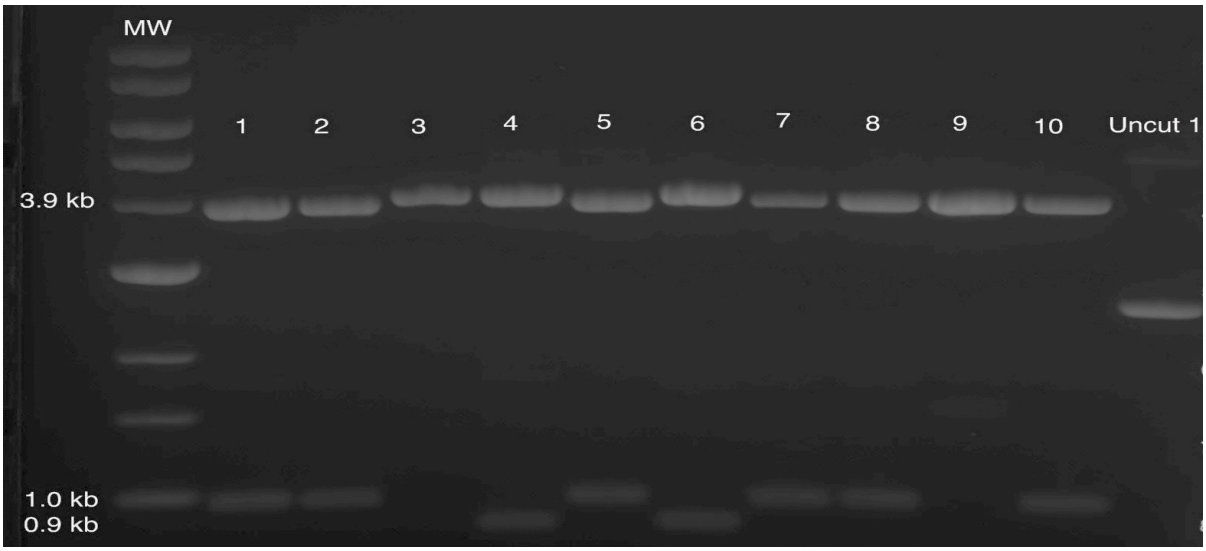

Figure 10. *HindIII* digestion of the pGP2526 + SV40 vector. There is a *HindIII* site immediately after the 0.4 kb SV40 promoter, allowing orientation of insertion to be determined. The clones that exhibit the 1.0 kb band (1, 2, 5, 7, 9, 10) are inserted so that expression will occur in the same direction as the GP25 gene. This was the orientation chosen for the next cloning experiments. Clones 4 and 6 exhibit a 0.9 kb band and have the SV40 construct inserted so that expression will occur in the same direction as the GP26 gene. Clone 3 does not contain the SV40 insert at all. The lane on the far right contains a sample of undigested vector (clone 1) for comparison.

Hygromycin Marker Cloning Strategy

The initial cloning strategy involved the use of a hygromycin marker to supply antibiotic selection that would stabilize the presence of the gpIL-12B gene in the virus. The gene for the resistance marker hygromycin was amplified by PCR. *BamHI* sites were incorporated into the 3' and 5' ends using primers and inserted into the *BamHI* site downstream of SV40 polyadenylation signal (Suppl. Fig. 7), allowing hygromycin selection of the construct. To ensure insertion and determine orientation of insertion, *BamHI* and *EcoRI* digests were performed (Figure 11).

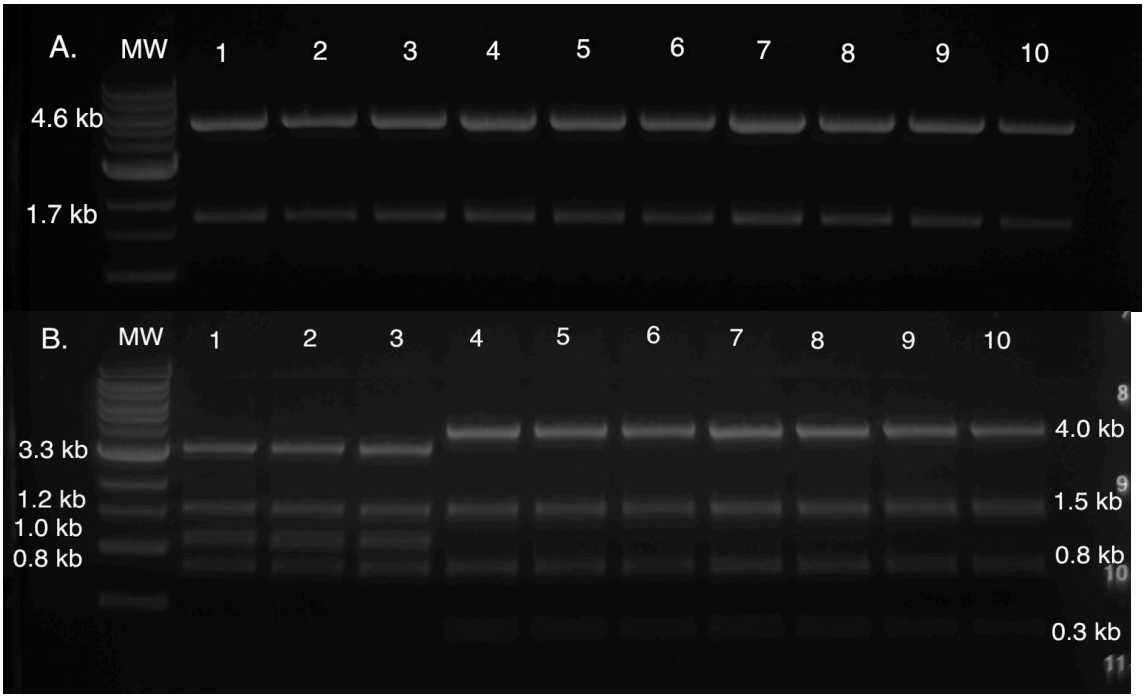

Figure 11. Restriction digests of the cloning vector containing hygromycin resistance marker. (A) *BamHI* digestion confirms insertion of hygromycin resistance cassette into all clones (1.7 kb band). (B) *EcoRI* digestion indicates which orientation the cassette inserted into the vector in each clone. In clones 1 – 3, the cassette inserted so that expression would occur in the same direction as the GP25 gene and clones 4 – 6 indicate that the cassette inserted so that expression would occur in the same direction as the GP26 gene. The first orientation (clones 1 – 3) was chosen for future cloning steps.

Generation of a Recombinant GPCMV Encoding a Hygromycin Resistance Marker

To ensure that it would not have an effect on virus growth kinetics, the cloning construct containing the hygromycin resistance cassette was recombined into a GPCMV BAC. The resulting BACs were analyzed by restriction enzyme digest analysis (Figure 12) and a full-length BAC was transfected into GPL cells, producing a replicating virus (Figure 13).

A multi-step growth analysis was performed to establish growth kinetics and the results are shown in Figure 14. The growth kinetics of the virus appear to follow a normal pattern, exhibiting characteristic growth followed by plateau at later timepoints when most of the cell monolayer has been destroyed by the virus. This indicates that the shuttle construct will not impair the dissemination of the virus in cell culture.

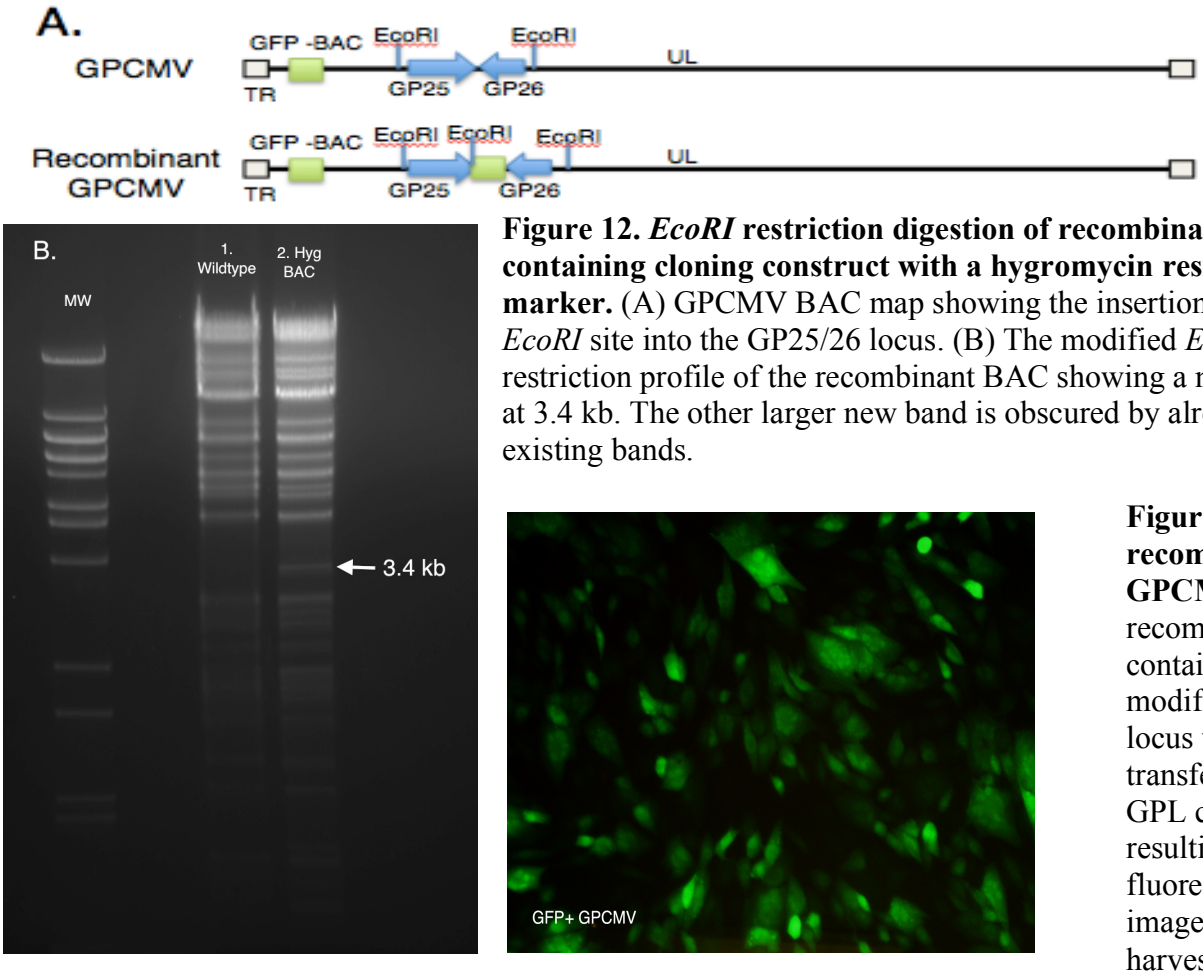

Figure 12. *EcoRI* restriction digestion of recombinant BAC containing cloning construct with a hygromycin resistance marker. (A) GPCMV BAC map showing the insertion of a new *EcoRI* site into the GP25/26 locus. (B) The modified *EcoRI* restriction profile of the recombinant BAC showing a new band at 3.4 kb. The other larger new band is obscured by already existing bands.

Figure 13. GFP+ recombinant GPCMV. The recombinant BAC containing the modified GP25/26 locus was transfected onto GPL cells and the resulting fluorescent virus imaged and harvested.

To ensure that the genes introduced into the GP25/26 locus would express properly, we treated a monolayer infected with the resulting recombinant virus with hygromycin at a concentration of 100 ng/mL two days after infection. This antibiotic inhibits protein synthesis and was expected to kill any eukaryotic cells not infected with the virus carrying the hygromycin resistance gene. Two days after treatment, much of the monolayer had been decimated but intact cells expressing the GFP signal indicating viral infection could still be detected. This indicates that the recombinant virus is causing expression of the hygromycin resistance gene (Figure 15).

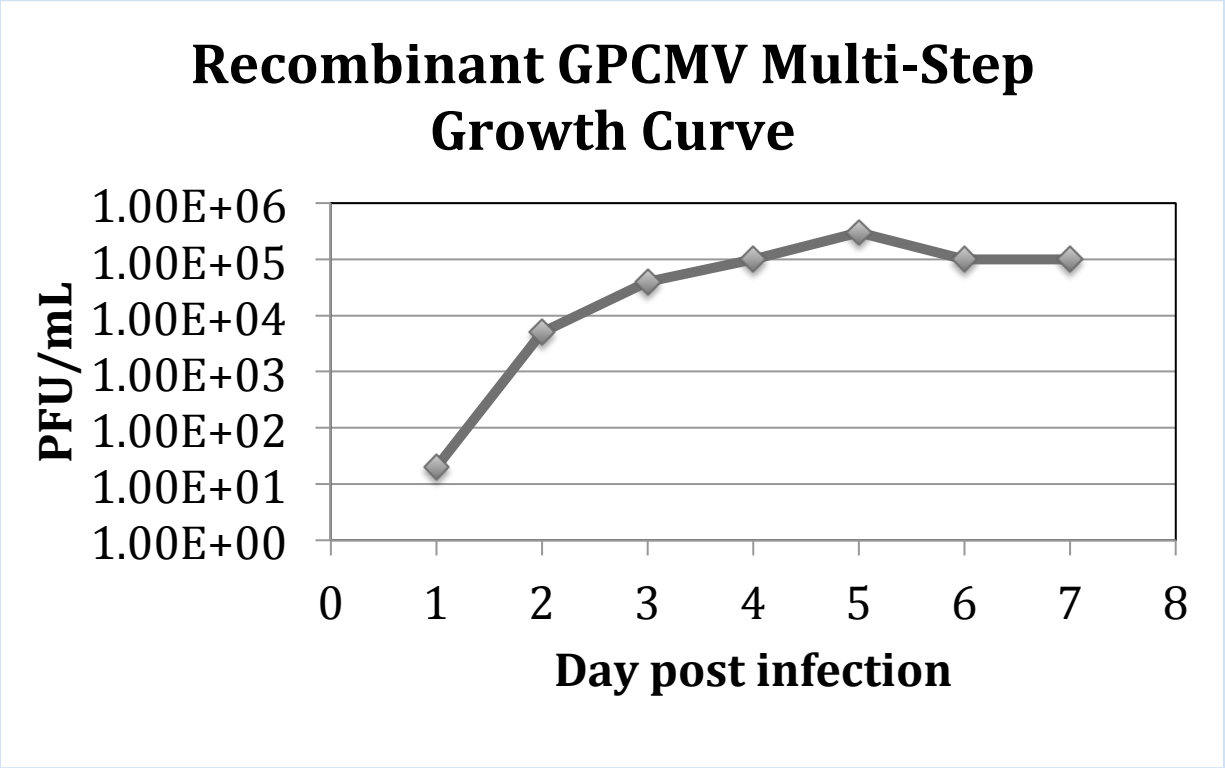

Figure 14. Growth kinetics of a recombinant GPCMV virus encoding a hygromycin resistance marker. The recombinant virus containing the modified GP25/26 locus was used to infect GPL cells and the supernatant and cell solution was harvested daily for seven days post infection. Titrations were performed and the resulting PFU/mL plotted on a semi-logarithmic plot. The growth kinetics exhibited by the recombinant virus appear normal relative to the characteristic growth of GPCMV.

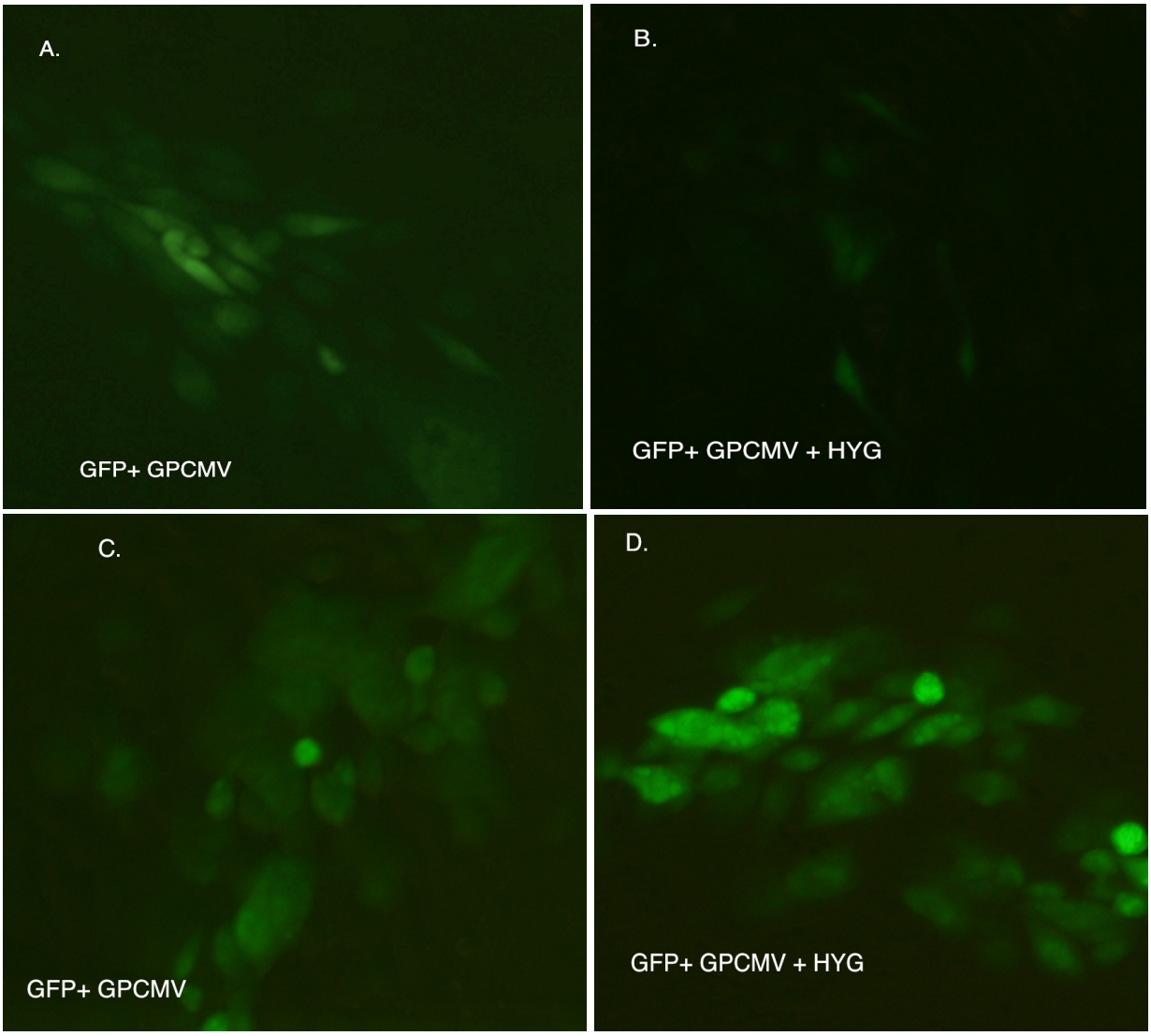

Figure 15. Recombinant GPCMV grown in the presence of hygromycin. (A) GPL cells infected with wildtype GPCMV. (B) When hygromycin (100 ng/mL) was added to the monolayer infected with the wildtype virus, the cells died and the GFP signal was almost completely lost. (C) GPL cells infected with recombinant GPCMV encoding the hygromycin resistance gene. (D) When hygromycin was added to the monolayer infected with the recombinant GPCMV, the infected cells survived and the GFP signal was fully preserved.

Kanamycin Marker Cloning Strategy

It was unknown what long-term effect the hygromycin resistance marker might have on the stability of a recombinant virus in cell culture, so for the creation of a BAC expressing only gpIL-12B, a second cloning strategy was employed that does not require that an antibiotic

resistance marker to be present in the recombinant BAC. First, gpIL-12B was synthesized by PCR with primers that incorporate *XhoI* sites into the 3' and 5' ends. It was ligated into the pLitmus128 vector and transformed under kanamycin selection. The gpIL-12B shuttle vector was purified from culture by large-scale prep, the IL-12B gene was excised by *XhoI* digestion, and it was inserted into the *XhoI* site of the shuttle vector containing GP25/26 and the SV40 genes (Suppl. Fig. 8). An *XhoI* digestion was performed to establish insertion of the gpIL-12B gene and *EcoRI* and *HindIII* digestions were performed to establish orientation of insertion in each clone (Figure 16). It was important that the gene be inserted in the same orientation as the SV40 promoter and polyadenylation sequence.

A kanamycin FLP-FRT recombinase cassette was inserted as a *BamHI* fragment into the *BglII* site of the gene construct, between the SV40 promoter and the gpIL-12B sequence (Suppl. Fig. 9). Insertion and orientation of insertion was determined by digestion with *HindIII* (Figure 17). This construct was amplified by PCR, purified by gel electrophoresis, and recombined into a GPCMV BAC.

After recombination, a FLP-recombinase was used as described to excise the kanamycin resistance marker from the BAC, leaving only the FRT sites and gpIL-12B in the modified GP25/26 locus (Suppl. Fig. 10).

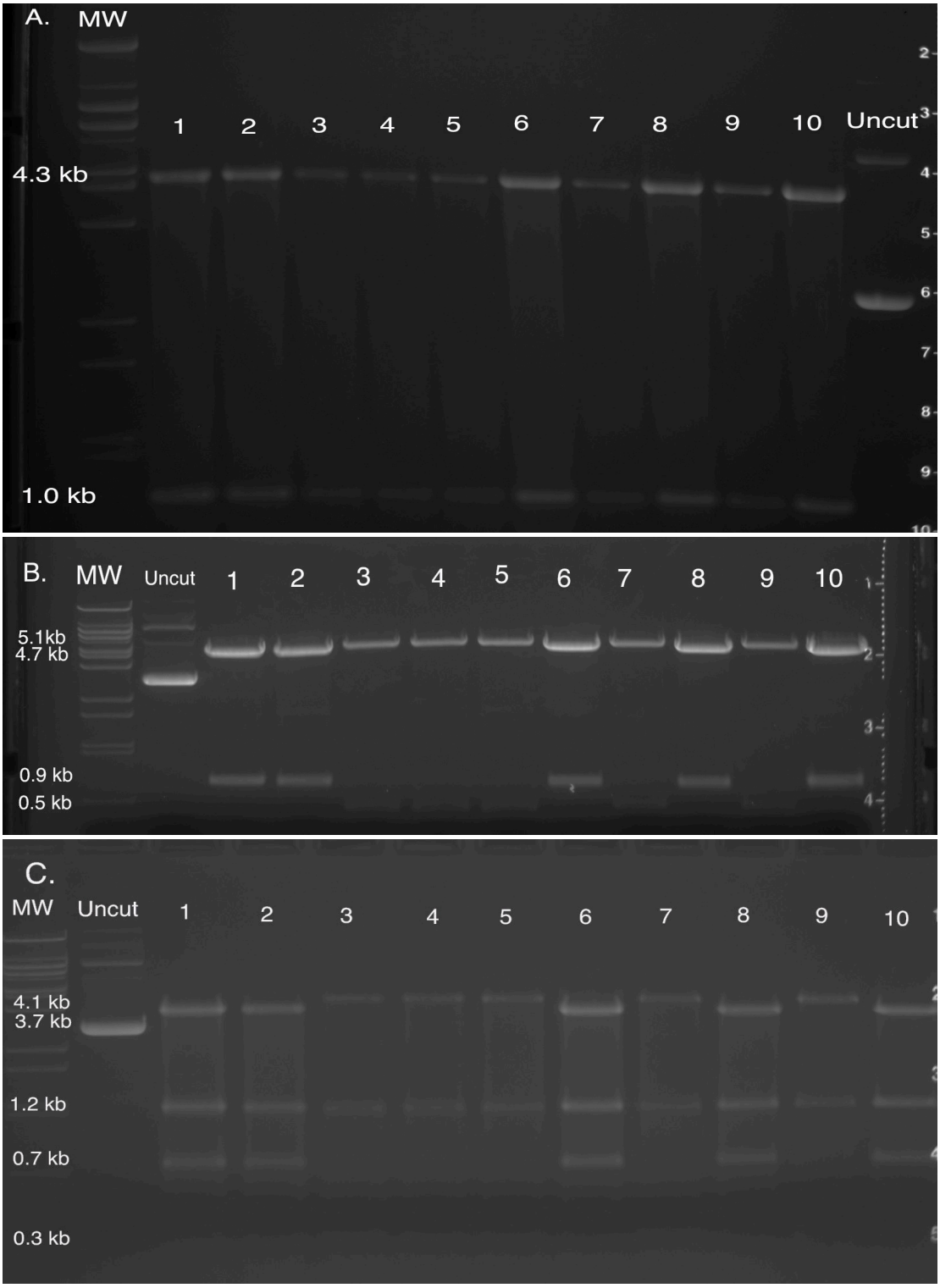

Figure 16. Restriction digestion of shuttle vector containing gpIL-12B. (A) XhoI digestion confirms that all 10 clones analyzed have the 1.0 kb gpIL-12B insert. (B) BamHI digestion showing that clones 1, 2, 6, 8, and 10 have gpIL-12B inserted in the same orientation as the SV40 promoter, with bands at 4.7 kb and 0.9 kb The restriction profile of clones 3, 4, 5, 7, and 9 (5.1 kb and 0.5 kb) indicate that gpIL-12B inserted in the opposite orientation. (C) An *EcoRI* digestion confirmed the results of (B). The correct orientation has a profile of 3.7 kb, 1.2 kb, and 0.7 kb.

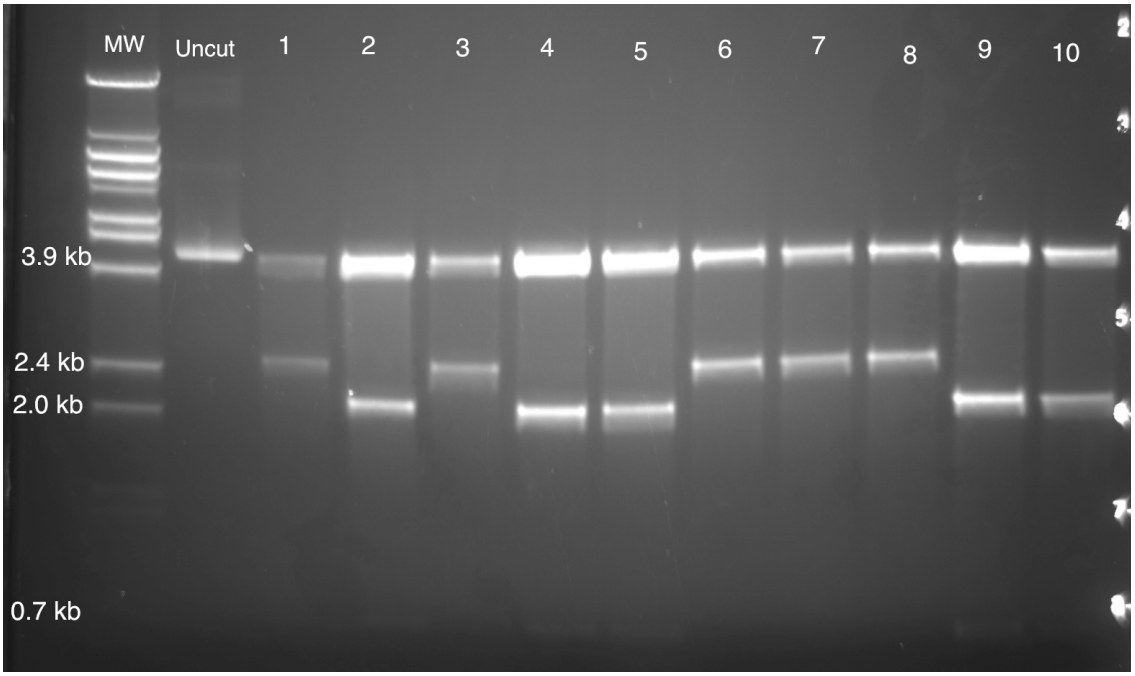

Figure 17. *HindIII* digestion of gpIL-12B cloning vector containing a kanamycin resistance marker. The restriction profiles indicate that all 10 clones contain the kanamycin resistance cassette, but only clones 2, 4, 5, 9, and 10 exhibit the profile (3.9 kb, 2.0 kb, and 0.7 kb) that indicate the cassette inserted in the same direction as the SV40 promoter. A clone with this orientation was chosen for recombination into a BAC.

Two bacterial clones containing the recombinant BAC were cultured and purified in a large scale prep. However, when digested with *EcoRI*, the resulting BACs did not appear to be full length (Figure 18). Minipreps of 10 other clones containing the recombinant BAC were performed and digested with *EcoRI*, confirming that none contained a full-length BAC. Recombination will have to be repeated or the strategy modified to obtain a full-length BAC containing IL-12B.

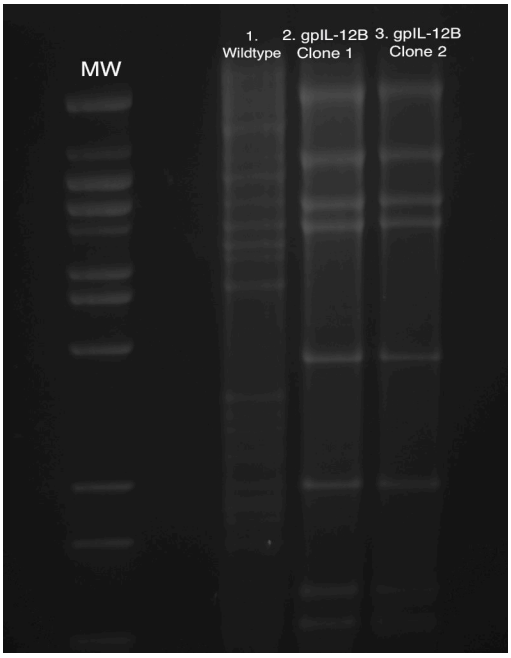

Figure 18. *EcoRI* restriction profile of recombinant GPCMV BAC containing gpIL-12B. The two clones containing recombinant BACs (Lane 2 & 3) show an unusual restriction profile that indicates that the BAC does not contain the entire GPCMV genome.

CONCLUSION

BAC Recombination System

The successful creation of a gH knock out BAC shows that the use of recombinant BACs can be used to study viral gene function and develop recombinant viruses. The recombinant knock out BAC expressed GFP but did not produce any infectious virus because it was missing an essential gene. To ensure no additional secondary mutations occurred that would be lethal to the BAC, the knock out BAC was co-transfected with a rescue plasmid that restored the GPCMV mutant to wildtype. This showed that the gH knock out alone was responsible for the impairment of the virus.

This result concurs with current evidence; recombinant BACs have been used extensively in the past to create recombinant virus. Creating a recombinant virus directly is very difficult, time-consuming, and often produces unexpected mutations in the viral genome. This method of generating recombinant virus is much quicker, easier, and more accurate [Dekhtiarenko et al. 2014]. It produces the modification desired without introducing any other mutations, making it the perfect technique for creating a recombinant virus expressing gpIL-12B.

GP25/26 Modified Locus

Inserting DNA into the region between GP25 and GP25 should hypothetically not affect expression of the genes or the growth of the virus because the utilized *BamHI* site is in the intergenic region the genes co-terminate in this region. The results of inserting the hygromycin resistance cassette and SV40 genes confirm this result. The recombinant virus produced had normal growth kinetics, indicating that the added genes in the modified locus did not noticeably

impair the virus. Additionally, the hygromycin resistance gene inserted into the locus was expressed well enough to provide resistance to hygromycin treatment at a level that wound normal kill the cells. The locus and the gene construct recombined into it appear to be an optimal environment for introducing a new gene to the GPCMV genome.

Guinea pig IL-12B Cloning & Recombination

The guinea pig IL-12B was successfully cloned into the shuttle vector, but there was an unidentified error in recombination, causing the production of BACs that contain only part of the GPCMV genome. It is possible that the gene or the kanamycin resistance cassette causes instability of the genome in the BAC. As it only adds 1 kb to the size of the BAC, it seems more likely that there was an error in the recombination procedure. The recombination should be repeated and the results analyzed.

If full-length GPCMV BACs cannot be produced with the described cloning strategy, the strategy will be modified. The hygromycin marker cloning strategy could be completed. Though it is unknown what long-term effects the resistance marker might have on the GPCMV genome in cell culture, it does provide selection for the recombinant virus. This could ultimately stabilize the modified GP25/26 locus in tissue culture; it is at least clear that the cassette does not initially inhibit the virus. This could be advantageous, as deletion of non-essential genes is common in GPCMV and the presence of gpIL-12B is likely to be detrimental to infection i*n vivo* [Cui et al. 2009].

Future experiments

Once a recombinant GPCMV virus encoding gpIL-12B is successfully created and confirmed by sequencing, the expression of gpIL-12B must be confirmed using enzyme-linked immunosorbent assay (ELISA assays). The SV40 promoter is a medium-strength promoter [Yang et al. 2014]; reasonable levels of gpIL-12B expression are expected, but it is unknown what effect the genome environment of GPCMV might have on expression.

Once gpIL-12B expression has been confirmed and quantified, experiments in live guinea pigs will be conducted to characterize the immune response to the recombinant virus *in vivo.* It is expected that gpIL-12B expression will enhance the T-cell response against GPCMV infection. This will be tested using an enzyme-linked immunosorbent spot (ELISPOT) assay to quantify the specific T-cell response to different GPCMV antigens. It is likely that the response to pp65 will be enhanced, but it will be interesting to see if it enhances the T-cell response to other T-cell target antigens.

Final Conclusion

Though this study is still in progress, several goals have been achieved. A convenient and precise recombination system has been selected and tested, a shuttle vector containing a gene construct that does not impair GPCMV has been created, and gpIL-12B has been successfully cloned into that shuttle vector.

When completed, this study may confirm the possibility of utilizing gpIL-12B as a GPCMV vaccine adjuvant to enhance the T-cell response to infection, which in turn will inform the pursuit of creating effective HCMV vaccines.

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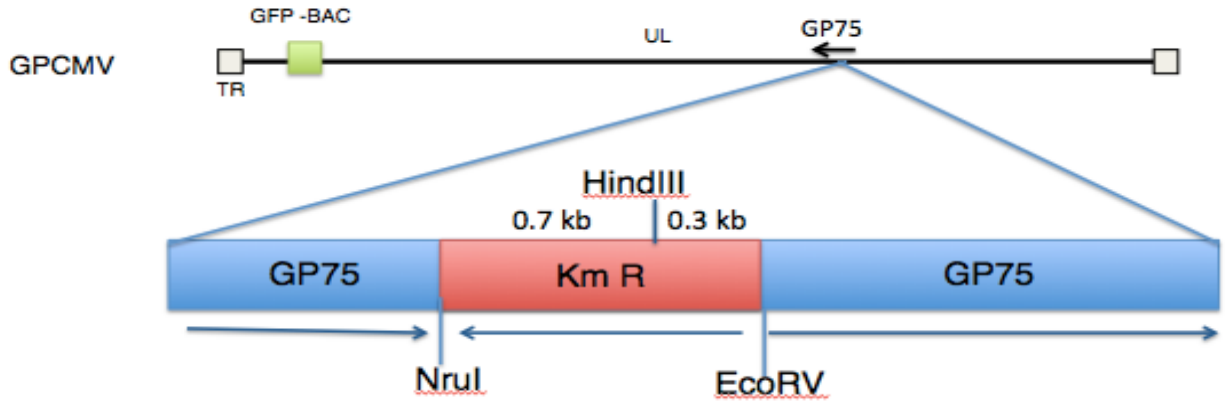
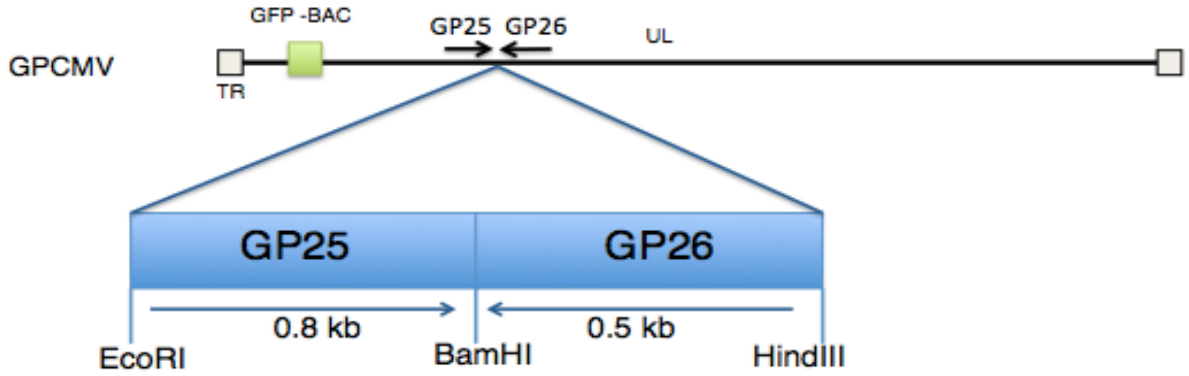
APPENDIX A

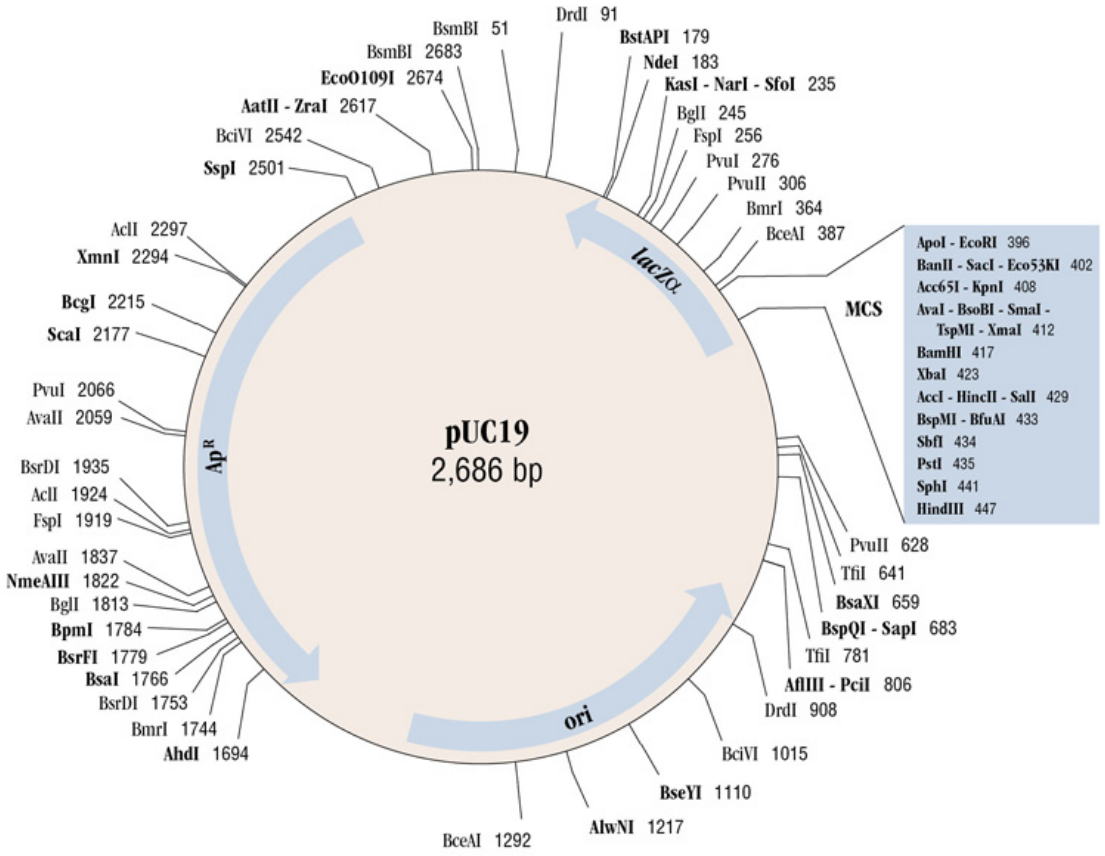
SUPPLEMENTARY FIGURES



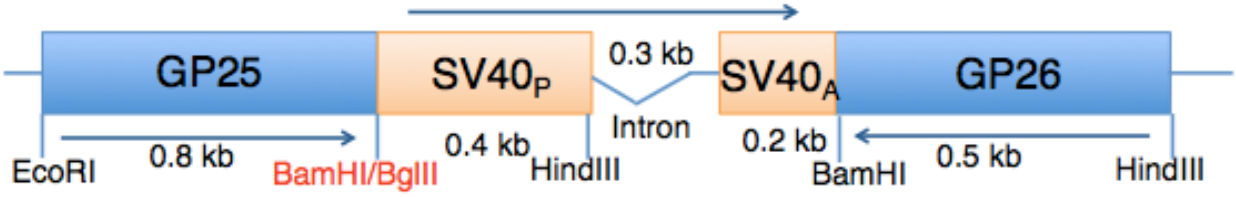
Supplementary Figure 1. Recombinant GP75 knock out BAC. The GP75 gene of GPCMV, which encodes the gH protein, was modified to contain a kanamycin resistance marker. In a shuttle vector, a portion of the gene was deleted using endogenous *NruI* and *EcoRV* sites within the gene. The kanamycin cassette was amplified as a *NruI/EcoRV* fragment by PCR and ligated into the digested vector. An *EcoRI* site was deleted and a *HindIII* site added, 0.7 kb from the start of the kanamycin cassette. The construct was recombined into the GPCMV BAC.



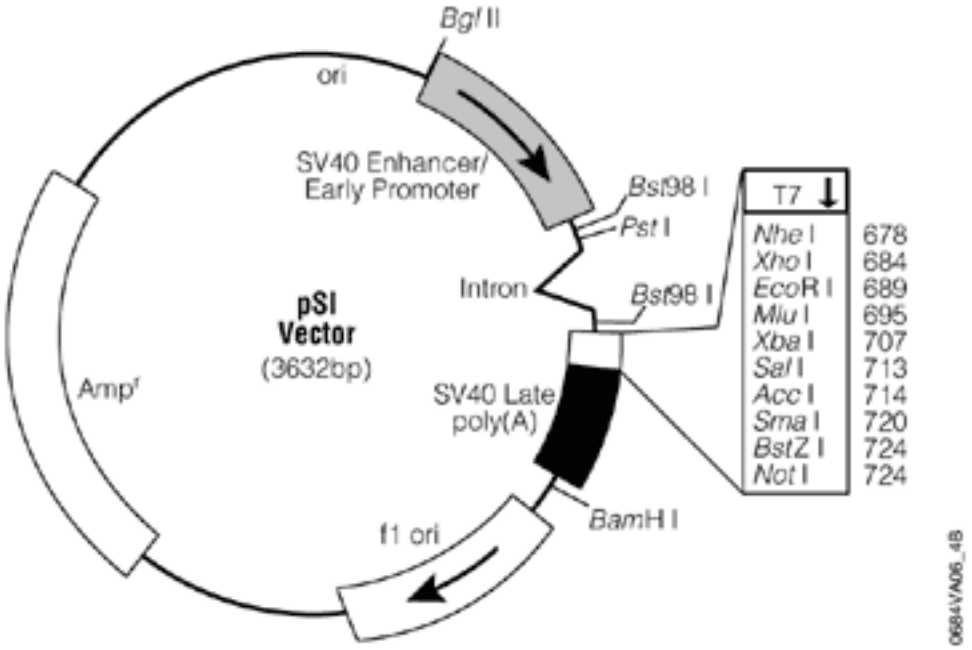
Supplementary Figure 2. Modified GP25/26 gene construct. A portion of the GP25/26 gene construct was amplified by PCR with the addition of an *EcoRI* site to the 5' end and a *HindIII* site to the 3' end of the construct. These sites were used to clone the construct into the pUC19 vector (Suppl. Fig. 3), displacing the multiple cloning site (MCS) and creating the pGP2526 vector. There is an endogenous *BamHI* site in the intergenic region. The construct is approximately 1.3 kb and the resulting modified vector is 4 kb.



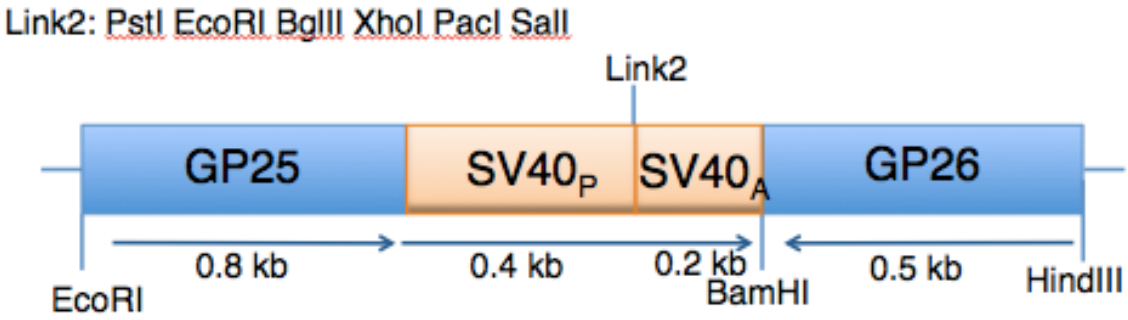
Supplementary Figure 3. pUC19 vector. Gene constructs were constructed in the pUC19 vector (NEB). The vector was digested with *EcoRI* and *HindIII* and GP25/26 construct ligated into the vector, displacing the MCS. The vector is approximately 2.7 kb in size. Source: New England Biolabs



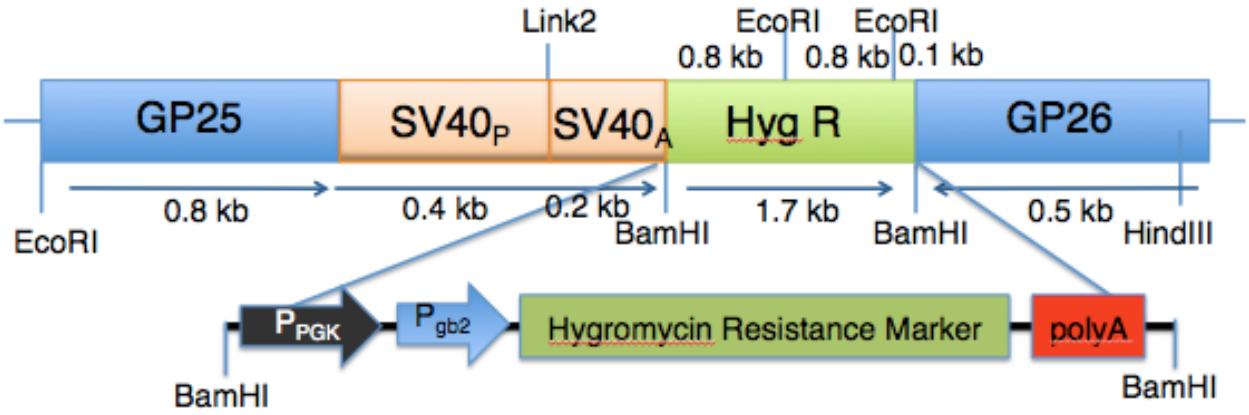
Supplementary Figure 4. pGP2526 containing SV40 promoter & polyadenylation sequence. The SV40 promoter, polyadenlyation signal, and all intervening sequences were digested out of the pSI vector (Suppl. Fig. 5) as a *BglII/BamHI* fragment and ligated into the pGP2526 vector *BamHI* site, destroying the upstream *BamHI* site. Restriction enzyme digestion with *HindIII* was used to confirm orientation of insertion. This construct was designated pGP2526pSI. The vector is approximately 4.9 kb in size.



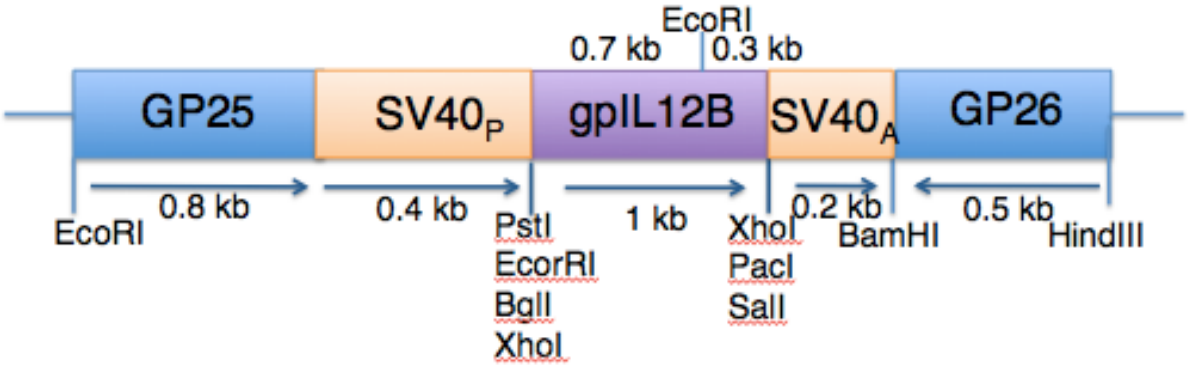
Supplementary Figure 5. pSI vector. The pSI vector contains the SV40 promoter and polyadenylation sequence, as well as the T7 MCS and associated sequences. This construct was digested out of the vector as a *BglII/BamHI* fragment for ligation into the pGP2526 vector.
Source: Promega



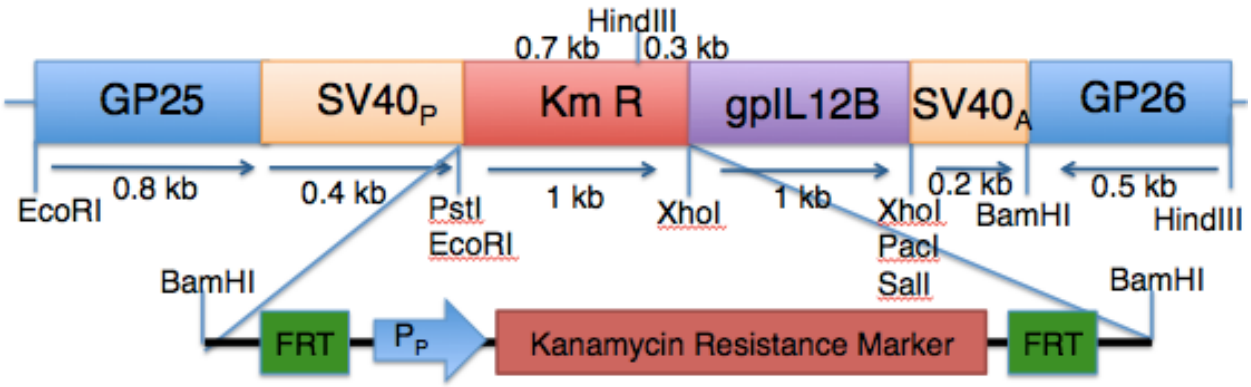
Supplementary Figure 6. pLink2 vector. The intron between the SV40 promoter and polyadenylation sequence was not needed for our experiments and was causing instability in our gene construct, so it was digested out as a *PstI/SalI* fragment, which also removed most of the T7 MCS. A modified linker designated Link2 was ligated in place of the original MCS, containing the restriction sites described above, as well as the remaining sites from the T7 MCS: *AccI, SmaI, BstZ I, NotI.* The vector size is approximately 4.6 kb.



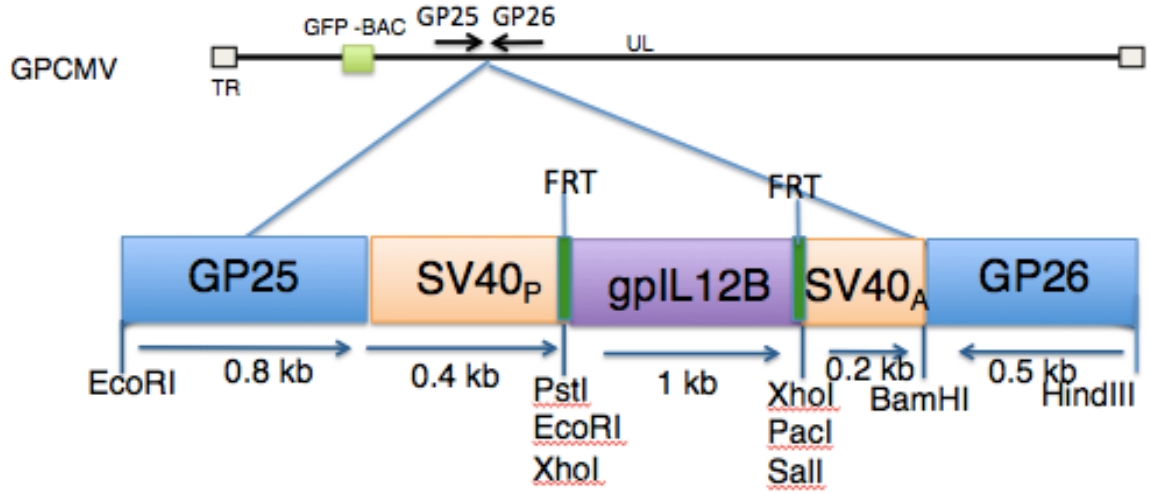
Supplementary Figure 7. pLink2/Hyg Vector. A hygromycin resistance marker was ligated into the *BamHI* site of the pLink2 vector. The hygromycin cassette (Genebridge) contains both a prokaryotic (P_{gb2}) and a eukaryotic promoter (P_{PGK}) and was modified to contain end *BamHI* sites. *EcoRI* digestion was used to confirm orientation of insertion. The vector size is 6.3 kb.



Supplementary Figure 8. pLink2/IL-12B vector. Guinea pig IL-12B was synthesized by PCR and ligated into the pLink2 vector as an *XhoI* fragment, disrupting the Link2 MCS as shown above. *EcoRI* digestion was used to determine orientation of insertion. The vector size is 5.6 kb.



Supplementary Figure 9. pLink2/IL12BKM$_{FRT}$ vector. A kanamycin resistance marker was amplified from the pACYC177 plasmid (NEB) as a *BamHI* fragment and ligated into the *BglII* site of the vector, destroying the site. The cassette contains a prokaryotic promoter and flanking flippase recognition target (FRT) sites for use in FLP-FRT recombination. *HindIII* digestion was used to determine orientation of insertion. The vector size is 6.6 kb.



Supplementary Figure 10. Recombinant GPCMV BAC containing IL-12B gene construct. The construct in Suppl. Fig. 9 was recombined into a GPCMV BAC using the Red/ET recombination system (Genebridge) and a recombinase (Flp) was used to remove the kanamycin cassette, leaving only the FRT sequences flanking the gpIL-12B gene.