CLONING AND EXPRESSION OF EQUINE NF-KB2

A Thesis

by

NEGIN MIRHOSSEINI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	Susan Payne
Committee Members,	C. Jane Welsh
	Waithaka Mwangi
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ABSTRACT

Cloning and Expression of Equine NF-κB2. (May 2008)

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Chair of Advisory Committee: Dr. Susan Payne

Equine infectious anemia virus (EIAV) is a macrophage-tropic retrovirus that causes persistent disease in horses and ponies. In addition to its structural proteins, EIAV encodes four regulatory/accessory genes, *tat, rev, ttm*, and *S2*. It has been documented EIAV *S2* gene expression is essential for disease expression of EIAV. Using a yeast two-hybrid assay, it was shown that S2 protein interacts with human NF-κB2. NF-κB2 plays a key role in the alternative or non-canonical NF-κB pathway. In order to determine if the interaction of S2 with NF-κB2 might be relevant to equine disease, a cDNA representing full length equine NF-κB2 was generated in our laboratory using PCR and rapid amplification of cDNA ends. To our knowledge this is the first time that equine NF-κB2 cDNAs have been recovered and characterized. The sequence of equine NF-κB2 was 95% homologous to human overall, however a major difference was found in the ankyrin repeat region where protein-protein interactions occur. Two splice variants of equine NF-κB2 were found that correspond to splice variants of human NF-κB2. We tested the interaction of EIAV S2 and equine NF-KB2 using the yeast two hybrid system (Y2H) and co-immunoprecipitation. Unfortunately we were not able to detect an interaction between EIAV S2 and equine NF-κB2 in either system. Despite this

result, NF-κB2 is an important component in the immune response so we examined its expression in equine macrophages. Moreover we were interested to know if EIAV might affect expression levels of equine NF-κB2, as NF-κB2 is a target of other viruses. Hence, the expression level of equine NF-κB2 was measured in uninfected and infected primary equine monocyte- derived macrophage (eMDM). Using quantitative PCR we determined that equine NF-κB2 gene expression is decreased in eMDM after 3 days post plating, about the time that monocytes start to differentiate into mature macrophages. However EIAV infection of eMDM upregulated the expression level of NF-κB2.

DEDICATION

To my family for their support, encouragement and unbounded love for me throughout my life.

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CHAPTER I

INTRODUCTION

Equine Infectious Anemia Virus (EIAV)

Equine infectious anemia (EIA) was first described in France in 1843. In 1904, it was determined that the disease was associated with a filterable agent that causes infection in horses and ponies. This was the first time that an animal disease was identified with a viral etiology (Leroux et al., 2004). The viral agent of EIA, equine infectious anemia virus (EIAV) is a member of the family Retroviridae. EIAV is a macrophage-tropic retrovirus (Leroux et al., 2004) and it has been shown that monocyte maturation has a key role in the regulation of EIAV expression. Peripheral blood monocytes allow viral entry and reverse transcription of viral RNA but do not allow EIAV replication (Maury, 1994). It has been shown equine lentivirus receptor-1 (ELR1), related to the family of TNF receptor proteins, serves as the EIAV receptor. ELR1 is expressed in various equine cells permissive for EIAV entry *in vitro*, including monocytes and macrophages (Zhang et al., 2005).

EIAV is transmitted mechanically by either an insect vector or an infected needle. EIAV causes a persistent infection which is typically classified in three defined stages: acute, chronic, and long-term asymptomatic (Leroux et al., 2001). Initial exposure of an animal to a virulent strain usually results in an acute disease (Leroux et al., 2004; Maury, 1998). The acute stage of disease usually resolves within a few days

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This dissertation follows the style and format of Virology.

and later the animal may experience the chronic stage of disease, characterized by the recurrence of clinical symptoms associated with cycles of viremia with fever, anemia, edema, thrombocytopenia, and various wasting syndromes (Leroux et al., 2004; Leroux et al., 2001; Payne et al., 1994). Some studies have shown that the asymptomatic stage of infection is due to control of virus replication by host immune response rather than virus attenuation (Hammond et al., 2000). This makes EIAV a practical model to examine the natural immunological control of lentivirus replication and disease (Hammond et al., 2000; Oaks et al, 1998).

EIAV Genome and Viral Proteins

The EIAV genome is the smallest and simplest of the Lentiviral genomes. This makes EIAV a useful model system to examine the contribution of specific viral genes in lentivirus replication, persistence, and pathogenesis (Leroux et al., 2004). Like other retroviruses, the EIAV genome contains two copies of single-stranded RNA which is capped and polyadenylated (Strauss et al., 2002). The 5′ and 3′ ends of genome contains the long terminal repeats (LTRs) which are composed of three segments namely U3 (unique sequence at 3′end), R (repeat sequence) and U5 (unique sequence at 5′end). LTRs are necessary for transcriptional initiation and polyadenylation of the viral RNA (Muary, 1998). The U3 region contains several elements important for viral transcription (Leroux et al., 2004; Payne et al., 1999). Studies have been shown that the U3 *ets* sites are essential in the regulation of EIAV transcription in macrophage (Maury, 1994). Similar work has revealed the fact that both envelope and long terminal repeat (LTR) influence cell tropism and virulence (Maury et al., 2005).

Similar to other retroviruses, EIAV uses *gag*, *pol* and *env* genes to encode structural proteins which are common to all retroviruses (Fig. 1A). In addition, four regulatory accessory proteins are encoded by virus. These proteins are Tat, Rev, Ttm, and S2 (Leroux et al., 2004; Beisel et al., 1993; Fagerness et al., 2006) (Fig. 1A). The locations of different gene products in the virion are illustrated in Fig. 1B.

Gag Gene

EIAV gag (group-specific antigen) encodes a large Gag- precursor ($Pr55^{gag}$) protein, which is translated from the full-length viral messenger RNA (Leroux et al., 2004). The Gag polyprotein has a key role in assembly and budding of retroviruses from host cells. Upon or immediately after budding of virus particles from host cells, the $Pr55^{gag}$ is cleaved by viral protease into four major internal structural proteins: the membrane–interacting matrix (MA), the capsid (CA), the RNA binding nucleocapsid (NC) P11, and P9 (Leroux et al., 2004; Hatanaka et al., 2002; Freed, 1998). The general structure of the main Gag proteins (MA, CA, and NC) in the Gag precursor and in the virion is highly conserved among retroviruses (Freed, 1998). MA, which forms the N-terminal domain of the Pr^{55}Gag, is associated with the inner face of the envelope and it may have a role in carrying the viral genome into the nucleus after infection (Leroux et al., 2004; Hatanaka et al., 2002; Freed, 1998). In the mature virion, capsid forms a shell around the NC/RNA complex (Freed, 1998). The CA domain of Pr55Gag plays an important role in virus assembly and the mature CA protein is involved in early postentry steps. In the virion, NC forms a core which is tightly associated with the viral

RNA (Fig. 1B). The NC domains of retroviral Gag proteins were known early on as playing a key role in RNA binding and encapsidation (Freed, 1998).

Pol Gene

Pol is located between *gag* and *tat* (Fig. 1A). The *pol* open frame overlaps with C-terminus of gag by 251 bases (Stephens et al., 1986). Along with *gag* products, *pol* products are translated from full length mRNA. These proteins carry out a variety of enzymatic activities such as reverse transcription and RNA degradation (RT-RNaseH), integration (IN), dUTP hydrolysis, and proteolysis (p12). (Leroux et al., 2004; Strauss et al., 2002).

Reverse transcriptase-RNaseH is important for the synthesis of viral DNA. Once the EIAV enters the cell, reverse transcription takes place in a subviral particle in order to generate a full length, linear double stranded (ds) DNA. Integrase is essential for insertion of the viral genome (dsDNA) into the host's chromosome through a single recombinational event (Strauss et al., 2002).

Viral dUTPase activity hydrolyzes dUTP to dUMP to provide a substrate for biosynthesis of dTTP which reduces the chances of misincorporation of dUTP into DNA. EIAV dUTPase is required for efficient viral replication in the non-dividing equine macrophages, where cellular dUTPase is expressed at low levels, but is dispensable in permissive cell lines where cellular dUTPase is abundantly expressed (Shao et al., 1997; Threadgill et al., 1993).

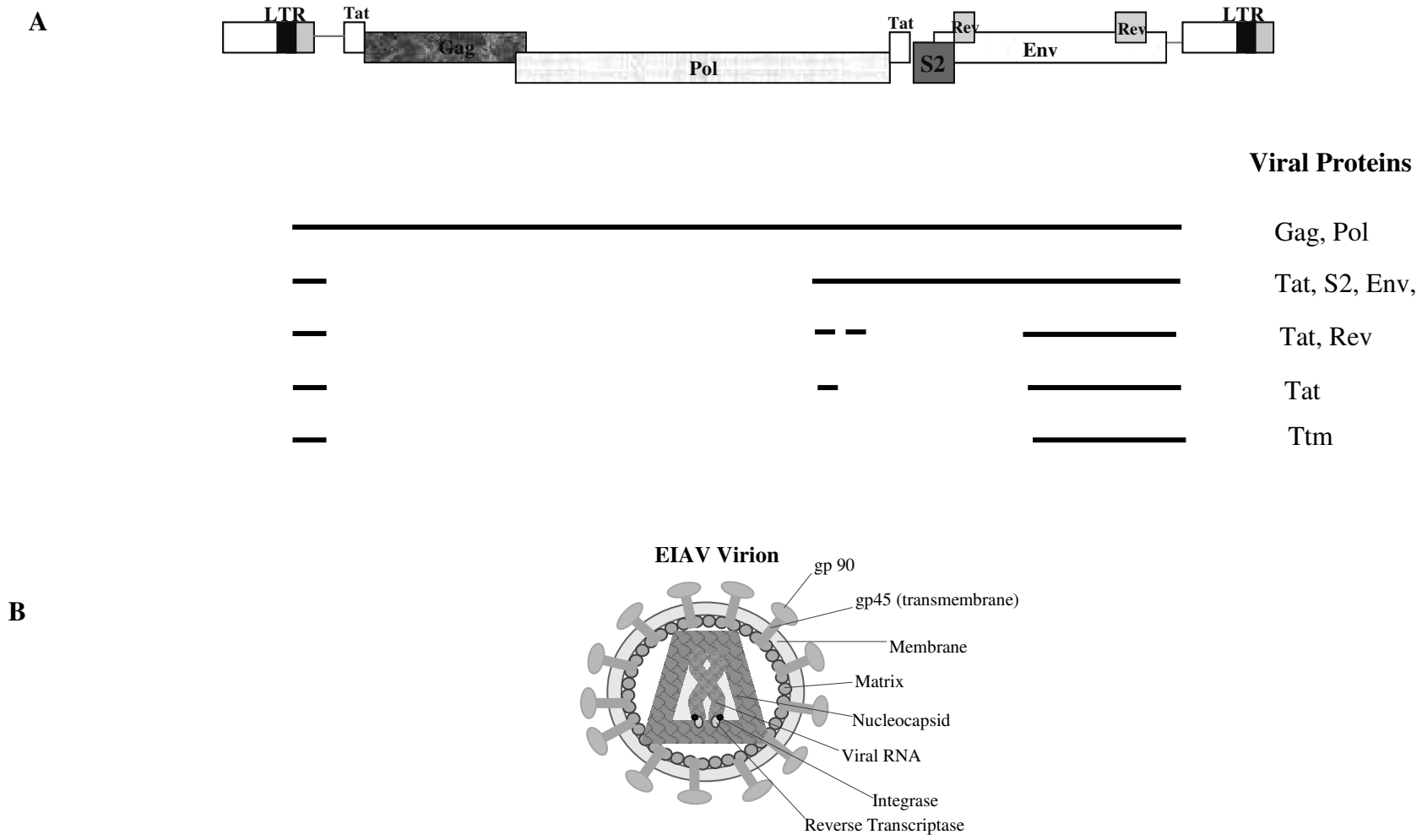


Fig. 1. Genome and mature virion of EIAV. (A) Viral genome schematic with open reading frames indicated by boxes. (B) Cartoon of EIAV virion showing the location of the structural proteins.

Viral protease (p12) is needed for the processing of $Pr55^{gag}$ which accurse after during assembly of progeny virions (Strauss et al., 2002).

Env Gene

The major open reading frame for *env* starts at nucleotide 1253 and continues for 862 codons. *Env* encodes the surface (SU) or gp90 and transmembrane (TM) or gp45 glycoproteins, which are incorporated into virions during virus budding from the plasma membrane (Leroux et al., 2004; Freed, 1998). Envelope glycoproteins of EIAV are a target for neutralizing antibody. It has been suggested that mutation in the envelope gene is a way for the virus to escape from immune responses, resulting in viral replication and periodic clinical cycles (Pang et al., 1997).

Tat Gene

Tat, located between *pol* and *env,* encodes a protein with both structural and functional homology to the Tat protein of HIV-1. Tat protein is a monomeric protein of 75 amino acids which is encoded by at least three alternatively spliced transcripts (Willbold et al., 1994); Rosin-Arbesfeld et al., 1993) (Fig. 1A). Tat is a transactivator of transcription of the long terminal repeat (LTR) region that is required for viral expression (Maury et al., 1994). Interactions between Tat and trans-activation responsive (TAR) elements, in the form of RNA stem-loops, enhance viral gene expression (Arbesfeld et al., 1998; Derse et al., 1991).

Rev Gene

Rev encodes a protein similar to HIV Rev, which regulates viral gene expression. Rev mediates mRNA transport from the nucleus to the cytoplasm through a Rev responsive element (RRE) positioned near the end of *env* (Leroux et al., 2004; Fagerness et al., 2006). Moreover, Rev variation may affect virus expression and long-term viral persistence during the long-term infection (Belshan et al., 1998).

Ttm Gene

Ttm encodes a novel Tat-TM fusion protein, a 27-kDa accessory protein, which is encoded by the first exon of tat and portion of the 3′-terminus of the transmembrane (TM) protein-coding region (Fagerness et al., 2006; Beisel et al., 1993). The carboxy terminus of lentiviral TM proteins has important roles in viral infectivity, growth kinetics, and cytopathology, suggesting that Ttm plays an important role in the EIAV life cycle (Beisel et al., 1993).

S2 Gene

The EIAV S2 gene, located between the second exon of *tat* and *env*, and overlapping the N-terminus of the *env*, encodes a 65 amino acid protein (Leroux et al., 2004; Yoon et al., 2000). The S2 protein contains 3 amino-acid motifs which are highly conserved among different isolates of EIAV. These motifs are GLFG (putative nucleoporin motif), PXXP (putative SH3 domain binding motif), and RRKQETKK (putative nuclear localization sequence) (Li et al., 2000) (Fig.2).

```
CONSENSUS           MGLFGKGVTWSASHSMGGSQGESQPLLPNSQKNLSVRR--TQCFNLIVIIMTVRTAWQNRRKQETKK
AF247394            MGLFGKGVTWSASHSMGGSQGESQPLLPNSQKNPSMRK--TQCFNLIVIIMTVRTAWQNRRKQETKK
EIAV19/17           MGVFGKGVTWSASHSMGESQGESQPLLPNSQKNLSVRR--TQCFNLIVIIMTVRTAWQNRRKQETKK
AB008197strainV     TELFGKGVTWSASHSMGGSQGESQPLLPNSQKNLSVRG--IQCFNLIVIMMTVRTAWQNRRKQETKK
AB008196Zhenget     MGLFGKGVTWSASHSMGGSQGESQPLLPNSQKNLSVRG--IQCFNLIV-MMTV--AWQNRRKQETKK
AF327878Tdonkey     MGLFGKGVTWSALHSVGVSQGEYQPLSPNKQNQQTHRKEIIWYINPIVIMIAIKKKWQRQETQDTKKK
AF327877Liaonin     MGLFGKGVTWSALHSMGVSQGEYQPLSPNKQNQQTHKKGITWYINPIVIMTAIKQKWQRQETQDTKKK
```

Fig. 2. Amino acid alignment of the S2 protein of different strains. Conserved domains are: nucleoporin (brown), N-myristylation (red), phosphorylation (green), SH3-binding (blue), and Nuclear localization (pink).

There is evidence showing that S2 is mainly localized in the cytoplasm and is coprecipitated with the EIAV Gag precursor (Yoon et al., 2000). Li et al. (1998) investigated the role of S2 in virus replication in vitro using an infectious molecular viral clone, designated by $EIAV_{UK}$. By using various $EIAV_{UK}$ mutants lacking S2 and examining the replication kinetics of these mutants in equine macrophage cells, they have concluded that the EIAV S2 gene is not essential for viral infection and replication in target cells in vitro. However, other studies have illustrated that S2 gene expression is essential for viral replication and disease expression of EIAV in infected horses (Fagerness et al., 2006; Li et al., 2000). In order to show that S2 is required for disease expression, Fuller and his colleagues (Fagerness et al., 2006) inoculated ponies with either wild type EIAV ($EIAV_{17}$), which is highly virulent, or with the mutant EIAV ($EIAV_{17\Delta S2}$) in which S2 was deleted. The $EIAV_{17}$-infected ponies showed severe febrile episodes with 40.5 to 41 °C body temperatures by 2 weeks post-infection. In addition to febrile episodes, these ponies also experienced severe thrombocytopenia. In contrast, the $EIAV_{17\Delta S2}$ –infected ponies did not experience a febrile episode or platelet drop within 2 weeks post-infection. No clinical signs were observed in these ponies through a 180-day observation period (Fagerness et al., 2006).

How S2 functions to modulate replication and virulence is unknown. In order to address this question, we investigated the interaction of S2 with host cellular proteins. A commercially available human spleen cDNA library was used in a yeast two hybrid screen to elucidate any possible interactions between cellular proteins and S2. Our laboratory results have shown that S2 interacts with three different human proteins (L. Covaleda, unpublished results). These are human OS-9, which is the product of a gene frequently amplified in osteosarcoma (Kimura et al., 1998), human Tat-binding protein-1 (TBP-1), which is a component of the 19S regulatory complex of the proteasome (Corn et al., 2003), and Nuclear Factor Kappa B p100/p52 (NF-κB2), which is a transcription factor known to play a role in the regulation of immune and inflammatory responses.

Nuclear Factor Kappa B (NF-κB)

Even though it has been more than two decades since the discovery of NF-κB, it is still an exciting area of research and study (Baldwin, 2001). NF-κB or Nuclear Factor Kappa B is a transcription factor which plays a key role in regulating immune and inflammatory responses in both innate and adaptive immunity (Baldwin, 2001; Pomerantz et al., 2002; Beinke et al., 2004). NF-κB is involved in a variety of viral diseases. Studies show that the NF-κB pathway is an attractive target for many pathogenic viruses such as HIV-1, the human T-cell leukemia virus HTLV-1, influenza virus, hepatitis B and C viruses, and herpesviruses. These viruses are able to hijack the NF-κB pathway and control it to their own benefit (Santoro et al., 2003; Tato et al., 2002). Typically, mammalian cells express five different NF-κB proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). These proteins share 300 amino acids

within their N-terminal Rel homology domain (RDH) that facilitates their binding to the specific DNA sequence named the κB site (Heusch et al., 1999; Xiao et al., 2006). There are two pathways that lead to activation of NF-κB: The canonical and non-canonical pathways.

NF-κB1/p50 is involved in the canonical pathway. This pathway, which has been also known as the classical pathway, is activated by a large variety of stimuli such as mitogens, cytokines (for example, tumor necrosis factor alpha (TNF-α) and interleukin one (IL-1)), microbial components (for instance, lipopolysaccharide (LPS)), and DNA damage. Upon stimulation, a specific IκB kinase (IKK) is activated. The activation of IKK initiates phosphorylation of IκB proteins which triggers their ubiquitination. After degradation of IκB by the 26S proteasome, the NF-κB dimer is released and translocated into the nucleus to induce gene expression (Xiao et al., 2006) (Fig. 3A).

NF-κB2 encodes p52 and p100. The expression of p52 is normally at low levels in many cells. However p52 is characteristically expressed at higher levels in dendritic cells, T cells, and macrophages (Heusch et al., 1999). While the classical pathway of NF-κB activation is mainly based on degradation of IκB proteins, the alternative pathway of NF-κB activation is based on p100 processing (Xiao et al., 2006). Unprocessed p100 acts as an IκB protein due to a cluster of ankyrin repeats in its C-terminal domain. Therefore, the processing of p100 to p52 regulates the alternative NF-κB activation pathway (Xiao et al., 2006). Principally, the processing of p100 to p52 is upon the activation of "NF-κB binding kinase" (NIK). NIK, then activates IκB kinase 1 (IKK1) and recruits it into the p100 to trigger ubiquitination of p100. Subsequently,

ubiquitinated p100 is processed to p52 by the 26S proteasome (Xiao et al., 2006). After that, p52 is translocated into the nucleus in association with other Rel subunits to activate a variety of genes (Beinke et al., 2004) (Fig. 3B).

Many studies indicated that the alternative NF-κB pathway controls the expression of the genes involved in B-cell proliferation and function, medullary thymic epithelial cell development, humoral immune responsiveness, and splenic structural design as well as cell proliferation and survival (Pomerantz et al., 2002; Tato et al., 2002; Zhang et al., 2006; Xiao et al., 2006). There are a number of receptors that are involved in activation of the alternative pathway, such as: BAFF, a B-cell activating factor, CD40L which stimulates B-cell proliferation and secretion of all immunoglobulin isotypes in the presence of cytokines, RANKL, a receptor activator of NF-κB ligand, lymphotoxin-β receptor (LTβR), and LTαR.

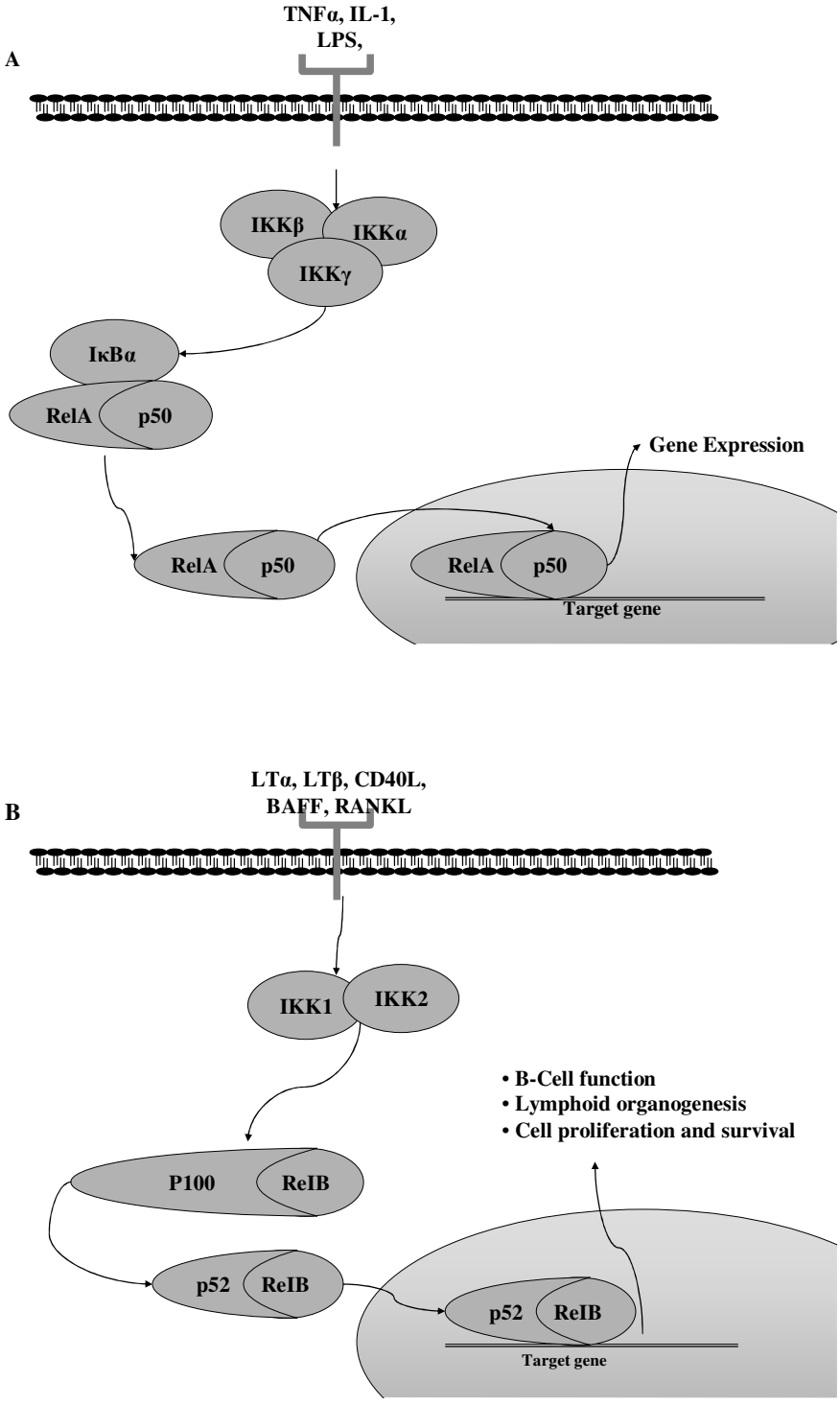


Fig. 3. NF-κB pathway. (A) The canonical (classical) NF-κB pathway. (B) The Non-canonical (alternative) NF-κB Pathway.

Interestingly, some viral proteins are also able to induce the alternative pathway. These viruses hijack the host's alternative NF-κB pathway for their replication and this may influence their oncogenic properties (Dejardin, 2006). For example, human T-cell leukemia virus type I (HTLV-I), Epstein-Barr virus (EBV), and Kaposi′s sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) encode the viral proteins Tax, LMP1, and K13/v-FLIP, respectively that can process p100 and activate the alternative NF-κB pathway. Tax directly binds to the p100 and recruits IKK1 to p100 to trigger p100 processing. LMP1, encoded by EBV, mimics CD40 signaling in B cells and activates the NF-κB2 pathway. The mechanism of activation of NF-κB by K13/v-FLIP, encoded by KSHV/HHV-8 is similar to Tax (Xiao et al., 2006; Dejardin, 2006).

Moreover, there are some other diseases that are also related to deregulated activation of the alternative NF-κB pathway. For example, development of various lymphomas/leukemia such as cutaneous T-cell lymphomas, B-cell non-Hodgkin lymphomas, chronic lymphocytic leukemia and myelomas are due to aberrant activation of the alternative NF-κB pathway. In addition, there are some autoimmune diseases that are linked to activation of the alternative NF-κB pathway, such as systemic lupus erythematosus, Sjogren′s syndrome, rheumatoid arthritis and autoimmune diabetes (Xiao et al., 2006).

CHAPTER II

CLONING EQUINE NF-KB2

Introduction

Use of the yeast two hybrid system to reveal host cell proteins interacting with EIAV S2 led to the identification of human NF-κB2 (L. covaleda, unpublished data). To follow up on this finding and determine if interaction of S2 and NF-κB2 was important for EIAV pathogenesis, equine NF-κB2 was cloned.

The general methodology used was to generate a fragment of equine NF-κB2 by PCR using degenerate oligonucleotides representing conserved regions of the NF-κB2 gene. Rapid amplification of cDNA ends (RACE) was then used to obtain the 5′ and 3′ ends of the equine NF-κB2 cDNA. The RACE system is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3′ or the 5′ end of the mRNA (Zhang et al, 2006). Finally, an overlap PCR extension strategy was used to piece together a full-length equine NF-κB2 cDNA.

Materials and Methods

Isolation of white blood cells and establishment of equine monocyte derived macrophage (eMDM) cell cultures

Whole blood in the volume of 500 ml was collected from EIAV-negative horses into an anticoagulant citrate dextrose solution blood collection bottle (The Metrix

Company, Dubuque, Iowa 52002, USA). To obtain the buffy coats blood was centrifuged at 700 ×g for 20 min. Then eMDM were separated by density gradient centrifugation using HybriMax histopaque (d = 1.077 g/cm^3) cushion (Sigma, St. Louis, MO). The eMDM were washed four times with Dulbecco's Ca^{2+}- and Mg^{2+}-free PBS (Sigma, St. Louis, MO), 5% adult horse serum (endotoxin and EIAV tested, Invitrogen/Gibco, Carlsbad, CA) and 1% penicillin–streptomycin solution (Sigma, St. Louis, MO). The eMDM were seeded in HYQ MEM Alpha Modification 1X (Cat. Sh3026565.01), containing 1% (v/v) penicillin and streptomycin, 10% equine bovine serum, 1% NEAA (non-essential amino acids), 25 mM HEPES, and 1% L- Glutamine. Then the cells were incubated at 37°C in a 5% CO_2 atmosphere incubator.

Generating the internal fragment of eq.NF-κB2

A fragment of NF-κB2 was initially cloned as follows: after 2 to 3 days, when monocytes had differentiated to the macrophage, the cells were harvested and washed with PBS, and then resuspended in sufficient amount of lysate buffer containing 1X Halt Protease. Afterwards, total RNA was extracted from eMDM cells using the Qiagen RNeasy Mini kit (Cat. No. 74104). First strand cDNA was synthesized from total RNA with Reverse Transcriptase II (Invitrogen) and an oligo dT primer. Two PCR primers, MH NF-κB forward: 5′-CGATTTCGATATGGCTGTGA-3′ and MH NF-κB reverse: 5′-GGTGRRCMCAGCTTCTCTG-3′, designed based on homologies among human p49/p100 (accession #: NM_002502), mouse NF-κB2 (accession #: NM_019408), and canis NF-κB2 (accession #: NW_876285) sequences were used to generate a 2498 bp

internal fragment of equine NF-κB2. The internal fragment was cloned into the TOPO® vector (Invitrogen TOPO TA Cloning Kit for Sequencing) and sequenced.

Rapid amplification of cDNA ends (RACE)

In order to clone full length NF-κB2, we performed RACE using Invitrogen™ 5´ RACE System for Rapid Amplification of cDNA Ends, Version 2.0. In brief, three specific primers: GSP1 (5′-GTCACAAAGCAGATAA-3′), GSP2 (5′-GGGAGATAACTGGCTTCAGGGGCAGGGAGAA-3′), and GPS3 (5′-CCCTCCTCCTTGACCTACAACCCCTACCAGT-3′) were synthesized based on the cDNA sequence obtained by sequencing the products of the internal amplification. For each 5′- (primers GSP1 and GSP2) and 3′-RACE (primer GSP3), the cDNA was synthesized and amplified according to the manufacturer's protocol. The conditions for amplifying the 5′ end included an initial denaturation at 94 °C for 3 minutes, thirty cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 minute, followed by a final extension at 72 °C for 7 minutes. PCR conditions for amplifying the 3′ end were as follows: one initial denaturation at 94 °C for 3 minutes, thirty cycles at 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 1 minute and 40 sec, followed by a final extension at 72 °C for 5 minutes. PCR products of 5′- and 3′-RACE were visualized after electrophoresis in a 0.9% agarose gel containing ethidium bromide. 5′ and 3′ products were cloned into a TOPO vector (Invitrogen) and sequenced.

Generating full length equine NF-κB2 cDNAs using overlap PCR extension

After obtaining the 5′ and 3′ ends of equine NF-κB2, these two fragments were combined with an internal fragment by overlap PCR extension to obtain a full length cDNA clone. A short internal fragment (SIF), designed to overlap the 5′ and 3′ end segments by 71 and 65 nucleotides, respectively, to enhance the extension process, was made using Short NF-KB forward (5′-CCTGCGCTTCTCTGCCTTCCTT-3′) and Short NF-KB reverse (5′-CCTCCCGGGTAGCAGCCCATT-3′) primers. PCR conditions used to generate the SIF were as follows: Initial denaturation at 94 °C for 3 minutes, thirty cycles at 94 °C for 30 sec, 59 °C for 30 sec, and 72 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes. The SIF, 5′ end, and 3′ end were first amplified by PCR and each fragment was gel purified. A master mix reaction without any primer was made for joining the 5′ end and internal fragment together. The PCR conditions for this step were as follows: initial denaturation at 94 °C for 3 minutes, ten cycles at 94 °C for 30 sec, 72 °C for 30 sec, and 72 °C for 4 minutes, followed by a final extension at 72 °C for 7 minutes. The following primers were then added to amplify the joined fragment: 5′ end primer Forward (5′-CCCAGAGACATG GAGAGTTGCTA-3′) and Short NFKB reverse (5′-CTCCCGGGTAGCAGCCCATT-3′). The PCR conditions were as follows:

initial denaturation at 94 °C for 3 minutes, thirty cycles at 94 °C for 30 sec, 64 °C for 30 sec, and 72 °C for 3 minutes, followed by a final extension at 72 °C for 7 minutes. A PCR master mix reaction without any primer was then used to join the 3′ end to generate full length equine NF-κB2. The PCR conditions for this step were as previously described. The primers: 5′ end primer Forward (5′-CCCAGAGACATGGAGAGTTGCTA-3′) and 3′ race inner reverse (5′-AGAAAGCTGGGTGGCTGCTGAGTGGGTGACAGTT-3′) were then added to PCR reaction in order to amplify the joined piece. The PCR conditions for this step were the same as amplifying the 5′ end and internal fragment, except that the extension time was increased to 4 minutes. Full length equine NF-κB2 cDNA was visualized after electrophoresis in a 0.8% agarose gel containing ethidium bromide, and gel purified. Full length equine NF-κB2 was later cloned into a TOPO vector (Invitrogen), sequenced and compared to human NF-κB2.

The summary of all primers and PCR conditions for different steps for generating the full length equine NF-κB2 are shown in Tables 1 and 2, respectively.

Table 1
Primers to generate full length NF-κB2

Primer	Sequence
GSP1	5′-GTCACAAAGCAGATAA-3′
GSP2	5′-GGGAGATAACTGGCTTCAGGGGCAGGGAGAA-3′
GSP3	5′-CCCTCCTCCTTGACCTACAACCCCTACCAGT-3′
Short NF-κB forward	5′-CCTGCGCTTCTCTGCCTTCCTT-3′
Short NFKB reverse	5′-CCTCCCGGGTAGCAGCCCATT-3′
5′ end forward	5′-CCCAGAGACATGGAGAGTTGCTA-3′
3′ race inner reverse	5′-**AGAAAGCTGGGT**GGCTGCTGAGTGGGTGACAGTT-3′

The 3′ race inner reverse primer has some extra nucleotides, which are not in the sequence of 3′ end (shown as bold letters). This oligo, a part of attB Primer Sequence, is necessary for inserting the PCR product into a pDONR^{TM} using recombination. This vector can be used in the yeast two hybrid system.

Table 2
PCR condition to generate full length equine NF-κB2

Conditions to amplify 5′ end of equine NFκB2 (744 bp)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	30 sec	
Annealing	55 °C	30 sec	30 X
Extension	72 °C	1 min	
Final Extension	72 °C	7 min	1
Conditions to amplify 3′ end of equine NFκB2 (1702 bp)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	30 sec	
Annealing	62 °C	30 sec	30 X
Extension	72 °C	1 min and 40 sec	
Final Extension	72 °C	5 min	1
Conditions to amplify short internal fragment (SIF) of equine NFκB2 (633 bp)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	30 sec	
Annealing	59 °C	30 sec	30 X
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	1
Conditions to sew SIF and the 5′ end (without primers)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	30 sec	
Annealing	72 °C	30 sec	10 X
Extension	72 °C	3 min	
Final Extension	72 °C	7 min	1
Conditions to amplify the sewed part (SIF + 5′ end) (1306 bp)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	30 sec	
Annealing	64 °C	30 sec	30 X
Extension	72 °C	3 min	
Final Extension	72 °C	7 min	1

Table 2 continued

Conditions to join the sewed part to the 3′ end in order to generate full length equine NF-κB2 (adding 4% DMSO as cosolvent, but no primer)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	45 sec	10 X
Annealing	72 °C	30 sec	
Extension	72 °C	4 min	
Final Extension	72 °C	7 min	1
Conditions to amplify full length equine NF-κB2 (2942 bp)			
Step	Temperature	Time	Cycle(s)
Initial Denaturation	94°C	3 min	1
Denaturation	94 °C	45 sec	
Annealing	64 °C	30 sec	30 X
Extension	72 °C	4 min	
Final Extension	72 °C	7 min	1

Results

Two splice variants of equine NF-κB2 were detected

An internal fragment of equine NF-κB2 was cloned using RT-PCR. The size of this fragment was 2498 nucleotides. Sequence analysis of several cDNA clones revealed two splice variants in this internal region of equine NF-κB2 (Fig. 4). The splice variants (called 3a and 8a) were identical to reported splice variants of human NF-κB2. In these splice variants, which occur at the C-terminus of NF-κB2, there is an insertion/deletion (indel) of a single alanine. Other differences between the two equine cDNAs are listed in Table 3.

Full length equine NF-κB2 was generated using RACE System and overlap extension PCR

The 5′ and 3′ ends of equine NF-κB2, which were 744 and 1702 nucleotides, respectively, were generated using RACE (Fig.5A, B). Sequencing of different 5′ end cDNAs revealed different alleles. In one sequence, named B, the 100^{th} amino acid is Aspartic acid (D) and in the other one, named, C, the 100^{th} amino acid is glycine (G) (Table 3). A 633 bp SIF was joined to the 5′, clone B, and 3′ ends (Fig. 5C). Joining of

the internal and 5′ end generated a 1306 nucleotide fragment (Fig. 5D). Finally joining of this fragment with the 3′ end generated full length equine NF-κB2 (Fig. 5E, F). The size of full length equine NF-κB2 was 2942 nucleotides. Fig. 6 shows the nucleotide and deduced amino acid sequences of full length equine NF-κB2. The spliced region is shown in grey shade.

Equine NF-κB2 is about 95% and 92% is homologous to human and mouse NF-κB2, respectively

The predicted amino acid sequence of equine NF-κB2 was aligned with the human and mouse NF-κB2 using Biology Workbench 3.2 and the conserved region was about 95% homologous with human and 92% with mouse. Interestingly, the highest homology (100% identical) was in the IPT_NF-κB (DNA-binding) region which is shown by white letters highlighted with black in Fig. 7. Black letters highlighted with grey show the Rel homology domain which is common among all NF-κB proteins. The interaction domain of NF-κB, the ankyrin repeat region, is shown by white letters highlighted with grey. As it can be seen from Fig. 7, the major difference between human and equine NF-κB2 is in this region (90% identical).

Table 3
Amino acid differences in the sequence of different equine NF-κB2 clones

	Amino acid differences in the sequence			
Equine NF-κB2	**aa 100**	**aa 491**	**aa 711**	**aa 882**
Full length clone B (901 aa)	G	T	A	C
Full length clone C (901 aa)	D	T	A	C
Internal fragment clone 3a (755 aa)	N/A	T	A	C
Internal fragment clone 8a (754 aa)	N/A	A	-	G

Amino acid is designated by (aa), glycine by (G) aspartic acid by (D), threonine by (T), alanine by (A), and cysteine by (C).

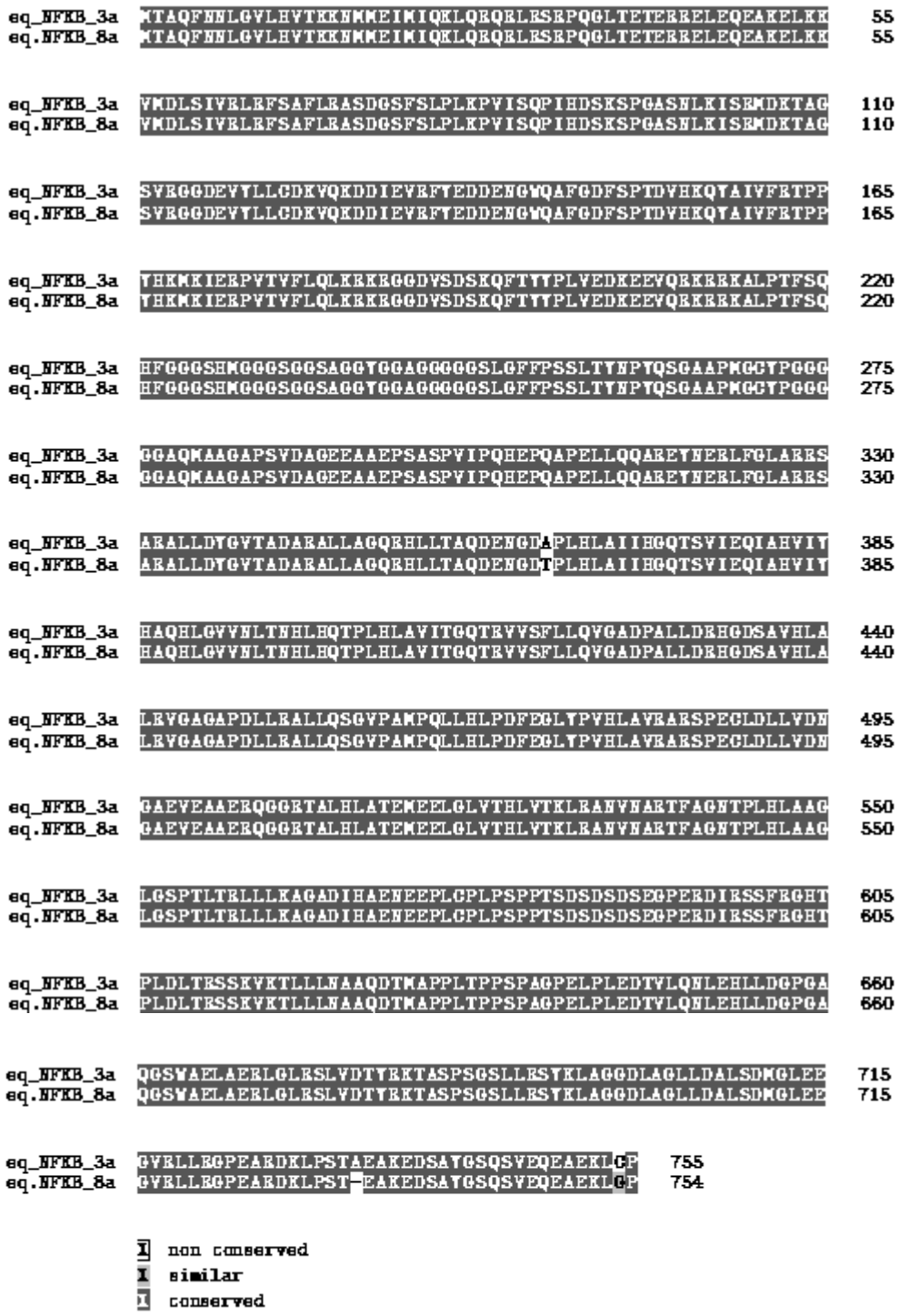


Fig. 4. Comparison of the splice variants of internal fragment of equine NF-κB2. Two splice variants in internal region of equine NF-κB2 occur at the C-terminus. In one splice, 3a, there is an addition of alanine (A).

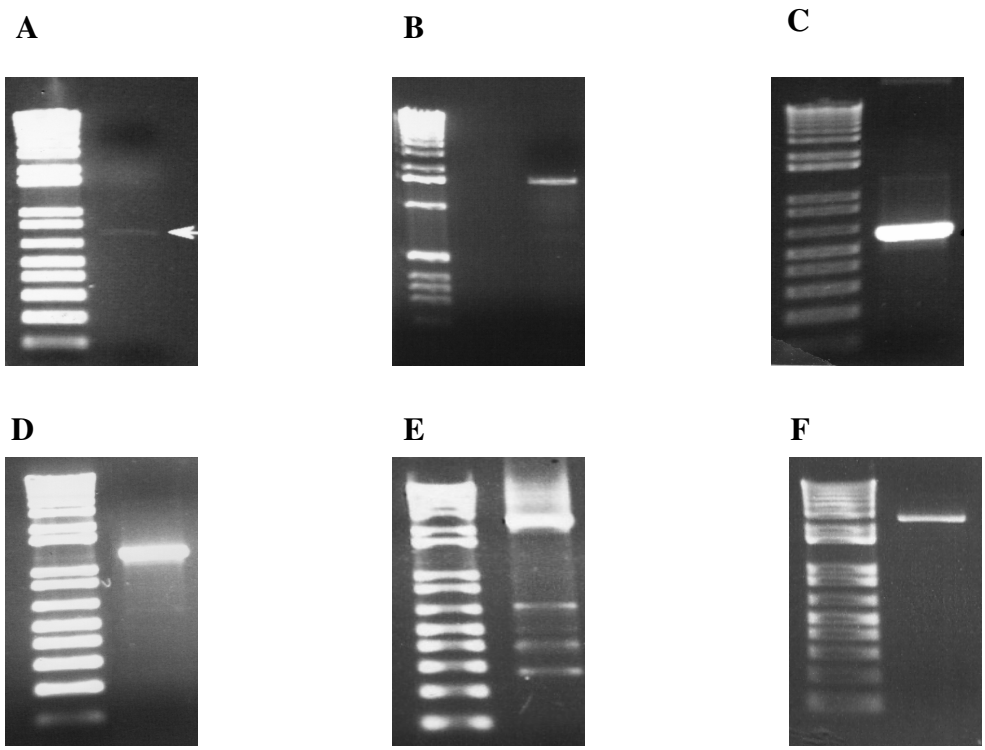


Fig. 5. Full length NF-κB2 was generated using rapid amplification of cDNA ends (RACE) and overlap extension PCR. 5′ and 3′ ends generated by RACE system have a size of 744 and 1702 bp nucleotides, panels A and B, respectively. A short internal fragment, 633 bp nucleotides, was generate to facilitate the sewing process, panel C. Short internal fragment was sewed to 5′ end to generate a 1306 nucleotide fragment, panel D. A full length cDNA of equine NF-κB with the size of 2942 nucleotides was generated by sewing the 1306 nucleotide fragment to 3′ end and gel purified later, panels E and F respectively.

```
      E  A  G  T  G  A  A  A  A  A  G  A  *  N  S  G  K  Q  N  P
2     gaagccggaaccggagccgccgccgcggcgggcgcctgaaactctgggaaacagaacccg  61
      A  G  A  T  R  Q  S  R  A  *  P  R  D  M  E  S  C  Y  D  P
62    gccggagccaccagacagagccgggcctagcccagagacatggagagttgctacgaccca  121
      G  L  D  G  I  I  D  Y  D  D  F  K  F  N  P  S  I  V  E  P
122   ggtctggatggtattattgactatgatgatttcaaattcaacccatccattgtggagccc  181
      K  E  P  A  P  E  T  A  D  G  P  Y  L  V  I  V  E  Q  P  K
182   aaggagccggccccagagacagctgatggcccctacttggtgatcgtcgaacagcctaag  241
      Q  R  G  F  R  F  R  Y  G  C  E  G  P  S  H  G  G  L  P  G
242   cagcgaggcttccgatttcgatatggctgtgaaggcccttcccatgggggactgccaggt  301
      A  S  S  E  K  G  R  K  T  Y  P  T  V  K  I  C  N  Y  E  G
302   gcctccagcgagaagggccggaagacttaccccactgtcaagatctgtaactacgaggga  361
      P  A  K  I  E  V  D  L  V  T  H  S  G  P  P  R  A  H  A  H
362   ccagccaagattgaggtggacctggtaacacacagtggcccgcctcgtgctcacgcccac  421
      S  L  V  G  K  Q  C  S  E  L  G  V  C  A  V  S  V  G  P  K
422   agcctggtgggcaagcagtgctcggagctgggggtctgcgccgtgtctgtgggacccaag  481
      D  M  T  A  Q  F  N  N  L  G  V  L  H  V  T  K  K  N  M  M
482   gacatgactgcccaatttaacaacctgggtgtcctgcatgtgaccaagaagaacatgatg  541
      E  I  M  I  Q  K  L  Q  R  Q  R  L  R  S  R  P  Q  G  L  T
542   gagattatgatacaaaaacttcagaggcagcgactccgctccaggccccagggccttacg  601
      E  T  E  R  R  E  L  E  Q  E  A  K  E  L  K  K  V  M  D  L
602   gagactgagcggcgggagctggagcaggaggccaaggagctgaagaaggtgatggacctg  661
      S  I  V  R  L  R  F  S  A  F  L  R  A  S  D  G  S  F  S  L
662   agcattgtgcgcctgcgcttctctgccttccttcgagccagtgacggctccttctccctg  721
      P  L  K  P  V  I  S  Q  P  I  H  D  S  K  S  P  G  A  S  N
722   cccctgaagccagttatctcccaacccatccacgacagcaagtctcctggggcctcgaac  781
      L  K  I  S  R  M  D  K  T  A  G  S  V  R  G  G  D  E  V  Y
782   ctgaagatttctcgaatggacaagacagctggctctgtgcgaggtggagatgaggtttat  841
      L  L  C  D  K  V  Q  K  D  D  I  E  V  R  F  Y  E  D  D  E
842   ctgctttgtgacaaggtgcagaaagatgacattgaggttcgattctacgaggatgatgag  901
      N  G  W  Q  A  F  G  D  F  S  P  T  D  V  H  K  Q  Y  A  I
902   aatggatggcaggcctttggggacttctctcccacagatgtccataaacagtatgccatt  961
      V  F  R  T  P  P  Y  H  K  M  K  I  E  R  P  V  T  V  F  L
962   gtgttccggacgcctccctatcacaagatgaagattgagcgtcctgtaaccgtattcctg  1021
      Q  L  K  R  K  R  G  G  D  V  S  D  S  K  Q  F  T  Y  Y  P
1022  caactgaaacgcaagcgtgggggggatgtctctgactccaaacagttcacctattaccct  1081
      L  V  E  D  K  E  E  V  Q  R  K  R  R  K  A  L  P  T  F  S
1082  ctggtagaagacaaggaggaggtgcagcgcaagcggaggaaagccttgcccaccttctct  1141
      Q  H  F  G  G  G  S  H  M  G  G  G  S  G  G  S  A  G  G  Y
1142  cagcatttcgggggtggctcccacatgggtggaggctccgggggctcagctgggggttat  1201
      G  G  A  G  G  G  G  G  S  L  G  F  F  P  S  S  L  T  Y  N
1202  ggaggagctggaggaggaggtggcagcctcggctttttcccctcctccttgacctacaac  1261
      P  Y  Q  S  G  A  A  P  M  G  C  Y  P  G  G  G  G  G  A  Q
1262  ccctaccagtccggcgcggccccaatgggctgctacccgggaggcgggggcggggcgcag  1321
      M  A  A  G  A  P  S  V  D  A  G  E  E  A  A  E  P  S  A  S
1322  atggccgccggggcgcctagtgtggatgctggggaggaagcggcagagccgagcgcgtcc  1381
      P  V  I  P  Q  H  E  P  Q  A  P  E  L  L  Q  Q  A  R  E  Y
```

Fig. 6. Nucleotide and deduced amino acid sequences of Equine NF-κB2. Full length equine NF-κB2 was cloned, and sequenced. The open reading frame is shown in black. The grey shaded amino acid shows the alternatively spliced region that results in an alanine indel. The stop codon is shown by *. The 4 bold italic highlighted amino acids at the middle and at the end show the start and end of N-terminus and C-terminus of internal fragment, respectively.

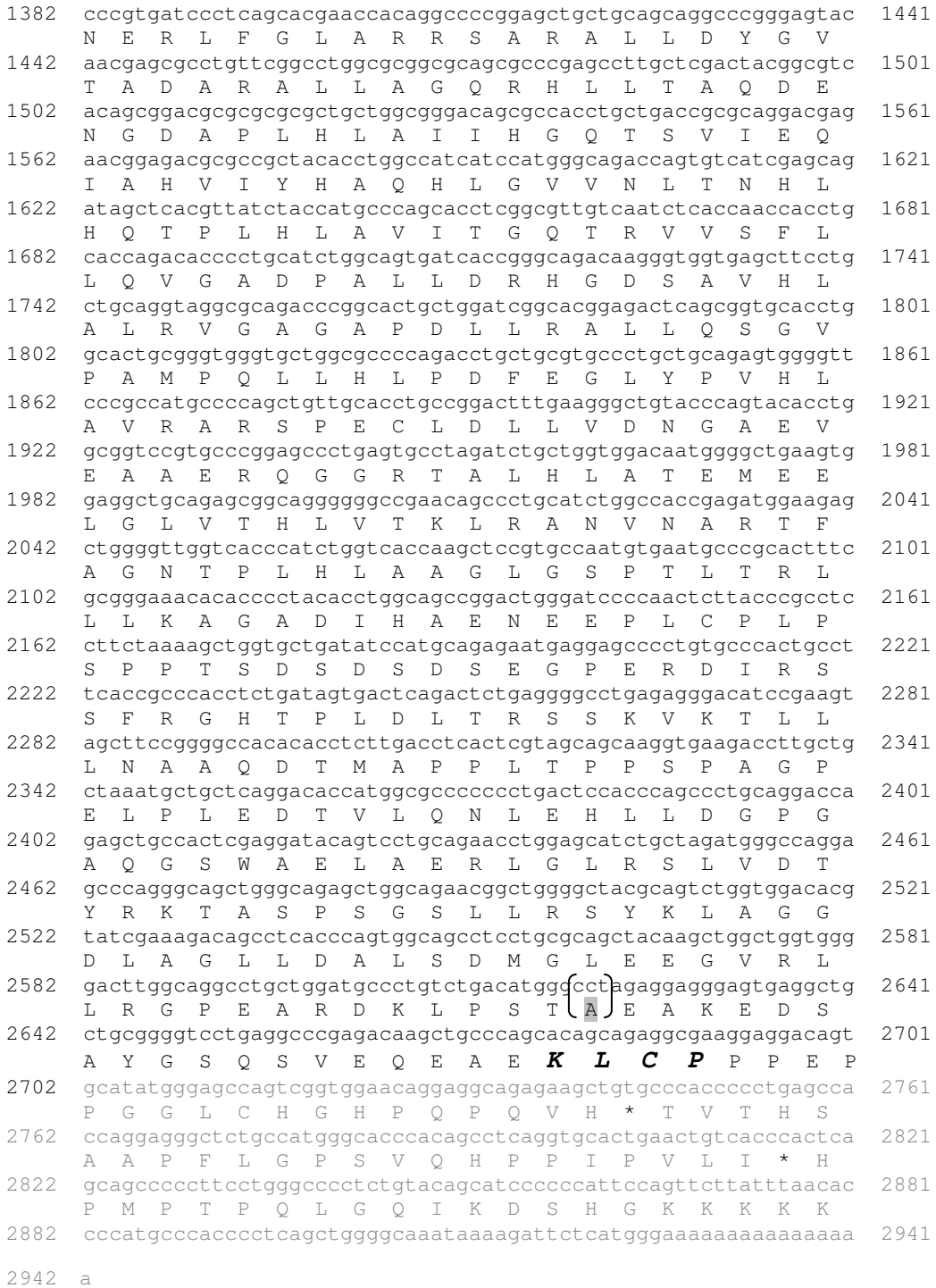


Figure 6, continued.

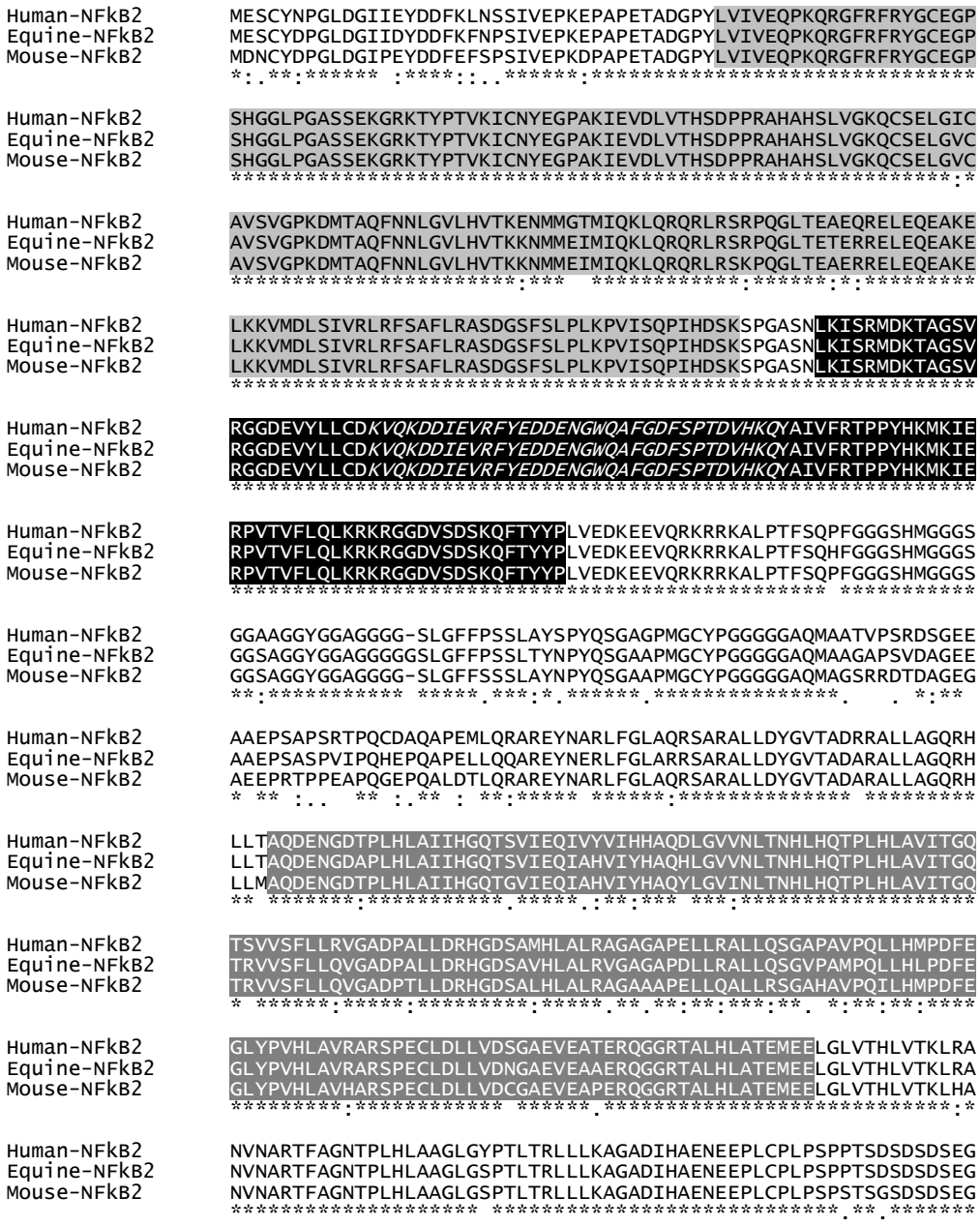


Fig. 7. Amino Acid Alignment of Equine, Human, and mouse NF-κB2. "*****" indicates single and fully conserved residue. "**:**" shows conservation of strong groups. "**.**" illustrates conservation of weak groups. " " demonstrates no consensus. Rel homology domain, IPT_NF-κB (DNA-binding) region, the ankyrin repeat region, ANK, region, are shown by black letters highlighted with grey, white letters highlighted with black, and white letters highlighted with grey, respectively.

```
Human-NFkB2       PEKDTRSSFRGHTPLDLTCSTKVKTLLLNAAQNTMEPPLTPPSPAGPGLSLGDTALQNLE
Equine-NFkB2      PERDIRSSFRGHTPLDLTRSSKVKTLLLNAAQDTMAPPLTPPSPAGPELPLEDTVLQNLE
Mouse-NFkB2       PERDTQRNFRGHTPLDLTCSTKVKTLLLNAAQNTTEPPLAPPSPAGPGLSLGDAALQNLE
                  **:* : .********** *:***********:*  ***:******* *.* *:.*****

Human-NFkB2       QLLDGPEAQGSWAELAERLGLRSLVDTYRQTTSPSGSLLRSYELAGGDLAGLLEALSDMG
Equine-NFkB2      HLLDGPGAQGSWAELAERLGLRSLVDTYRKTASPSGSLLRSYKLAGGDLAGLLDALSDMG
Mouse-NFkB2       QLLDGPEAQGSWAELAERLGLRSLVDTYRKTPSPSGSLLRSYKLAGGDLVGLLEALSDMG
                  :***** ********************:*.**********:******.***:******

Human-NFkB2       LEEGVRLLRGPETRDKLPSTTEVKEDSAYGSQSVEQKAEKLGPPPEPPGGLCHGHPQPQV
Equine-NFkB2      LEEGVRLLRGPEARDKLPSTAEAKEDSAYGSQSVEQEAEKLCPPPEPPGGLCHGHPQPQV
Mouse-NFkB2       LHEGVRLLKGPETRDKLPS-TEVKEDSAYGSQSVEQEAEKLCPPPEPPGGLCHGHPQPQV
                  *.******:***:****** :*.**************:**** ******************

Human-NFkB2       H
Equine-NFkB2      H
Mouse-NFkB2       H
                  *
```

Figure 7, continued.

CHAPTER III

PROBING THE INTERACTION BETWEEN EIAV S2 PROTEIN AND EQUINE NF-KB2

Introduction

Since EIAV S2 protein interacts with human NF-κB2, is it possible that it also interacts with equine NF-κB2? If EIAV S2 protein interacts with equine NF-κB2 there is a possibility that EIAV uses NF-κB2 pathway to evade the immune response and remain persistent in the infected equine. This might explain the role of S2 in EIAV pathogenesis**.** In order to investigate the interaction between EIAV S2 and NF-κB2 proteins, two different methods, the yeast two hybrid system and the co-immunoprecipitation process were used.

Identification of the interaction of two proteins (in our case equine NF-κB2 and EIAV S2) in the two hybrid system, which is an *in vivo* yeast-based system, is based on reconstitution of an active transcription factor. This active transcription factor is formed as a dimer between two fusion proteins, one of which contains a DNA-Binding Domain (DB) fused to the first protein of interest (DB-X; also known as the "bait") and the other, an Activation Domain (AD) fused to the second protein of interest (AD-Y; also known as the "prey" or "target protein"). Interaction between DB-X and AD-Y, through the X and Y domain, generates a functional transcription factor that activates reporter genes, which are present in the chromosome of yeast strain MaV203. Activation of reporter genes is driven by promoters containing the relevant DB binding sites (Fig. 8).

Interaction of the bait (X) and prey (Y) is elucidated by analysis of series of reporter genes. For example, in yeast which contains HIS3 as the reporter gene, interaction between bait and prey proteins can be shown by growth of cells on plates lacking histidine.

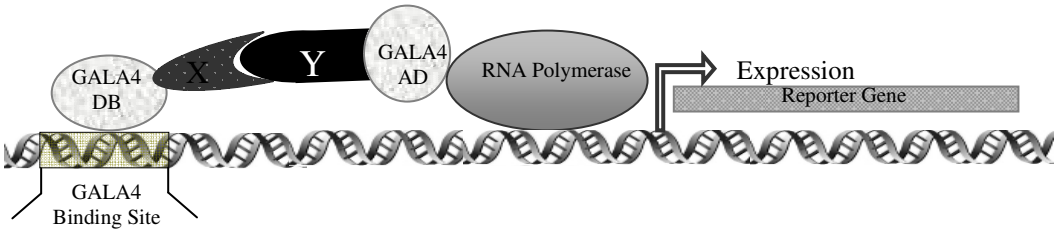


Fig. 8. The yeast two hybrid system. Interaction of two proteins generates a transcription factor that activates the reporter gene.

Co-immunoprecipitation (Co-IP) is another method that was used to probe possible interactions between EIAV S2 and eq.NF-κB2 proteins. This method recovers a protein together with its binding partner by using a specific antibody against one of the proteins, followed by recovery of the complex using protein G or A (depending on antibody species) attached to beads. Protein A and protein G are bacterial proteins that bind with high affinity to the Fc portion of various classes and subclasses of immunoglobulins from a variety of species. Then the result can be interpreted by western blot (Fig. 9).

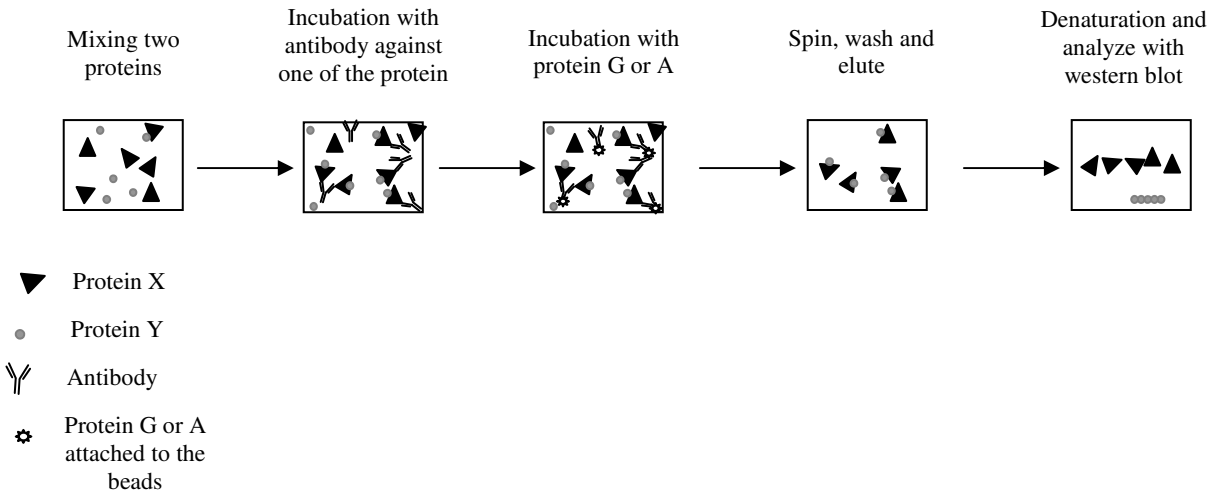


Fig. 9. Co-immunoprecipitation procedures.

Materials and Methods

Yeast Two Hybrid System (Y2H)

The bait plasmid was generated previously by cloning the S2 gene into pDEST 32 (L. covaleda, unpublished data). A set of equine NF-κB2 constructs were cloned by recombination into the pDEST 22 vector in order to make the prey plasmid as described in the Gateway™ Technology manual. In brief, primers containing the attB1 (5′-ACAAGTTTGTACAAAAAAGCAGGCTNN-3′) and attB2 (5′-ACCACTTTGTACAAGAAAGCTGGGTN-3′) sequences were generated to add attB1 and attB2 sites to the 5′ and 3′ ends, respectively, of following fragments of equine NF-κB2: the 2498 bp internal fragment of equine NF-κB2 (clone 3a); full length equine NF-κB2; and a fragment of equine NF-κB2 similar to the human NF-κB2 cDNA which was identified from the initial yeast two hybrid screening. A standard PCR was performed

for each fragment to prepare the attB-PCR product. The PCR products were gel purified and 5 µl of each purified DNA was cloned into the pDONR™201 vector, an entry vector, using BP Clonase™ Enzyme. The entry vectors, containing equine NF-κB2 genes, were transformed into competent cells (Top 10 cells) to generate the entry clones. The cells were plated on LB plates containing 50 µg/ml kanamycin and incubated for 24h at 37 °C. Then entry clones were isolated and the vectors were recovered from the competent cells using an Eppendorf, FastPlasmid™ Mini kit. Equine NF-κB2 genes were later transferred from Gateway® Entry Clones into pDEST™22 vectors using the LR recombination reaction to generate the destination vectors. The destination vectors were transformed into Top 10 cells and incubated at 37 °C for 24h. The vectors, containing the equine NF-κB2 gene, were purified from cells using the Eppendorf FastPlasmid™ Mini kit. These vectors, which are known as prey plasmids, were introduced into the yeast strain MaV203.

The bait and the prey plasmids were introduced into yeast strain MaV203 by transformation as follows: A colony of MaV203 was inoculated in 50 ml of YPAD, a rich, non-selective medium for the routine growth of yeast, and incubated at 30 °C until the culture reached the OD_{600} of 2.0. Then the culture medium was centrifuged at 3000 g for 5 minutes. The yeast were suspended in 25 ml ultrapure H_2O and centrifuged again. The yeast were then resuspended in 1 ml 100 mM Lithium Acetate (LiAc), and transferred to a 1.5 ml tube and then pelleted. Cells were resuspended in 400 µl of 100 mM LiAc and aliquoted into 50 µl samples for individual transformation. Cells were pelleted and resuspended in 240 µl 50% polyethylene glycol (PEG) 3350. Then the

following components were added to the suspension and mixed with it gently: 36 µl 1M LiAc, 25 µl boiled calf thymus DNA (2.5 mg/ml), 48 µl H_2O, and 0.1 µg/µl plasmids (prey and bait). The prey and bait plasmids are listed in Table 4. The suspension was incubated in water bath at 30 °C for 30 minutes followed by a heat shock at 40 °C for 20 minutes. Then cells were pelleted and resuspended in 200 µl H_2O. Each transformation was plated onto the selection plates containing uracil and histidine (SC-Leu-Trp+Ura+His). The plates were incubated for 60 to 72 hours at 30 °C, until the yeast colonies became visible. After incubation, four different colonies from each transformation were patched on a single SC-Leu-Trp+Ura+His plate (master plate) along with two colonies each from yeast control strain A-E. The plates were incubated at 30 °C for about 18 hours.

The master plate was replicated onto different testing plates listed in Table 5 as described in the instruction manual of ProQuest Two-Hybrid System with Gateway Technology. Table 5 shows the set of tests that were conducted in order to determine if there was any interaction between EIAV S2 and equine NF-κB2. Activation of the HIS3, URA3, and ß-Galactosidase reporter genes enables two-hybrid-dependent transcriptional

activation to be screened by cell growth on plates lacking histidine or uracil, and a change in the color of media from clear to yellow, respectively. Moreover, two hybrid-dependent induction of URA3 converts the compound 5-fluoroorotic acid (5FOA) to 5-fluorouracil, which is toxic. Therefore, cells in which the two proteins have interacted will grow on plates lacking uracil, and not on plates containing 5FOA.

Controls were performed as follows: To assess the self-activation of AD-NF-κB2, the yeast was transformed with pDBLeu plasmid and AD-NF-κB2. Yeast containing AD-NF-κB2 and pDBLeu grows in the selective media and FOA plates. AD-NF-κB2 and pDBLeu do not induce any reporter gene expression. Further, the strength of the interaction between two proteins was determined by using a set of control strains provided by the Gateway^{TM} system which contained plasmid pairs that express fusion proteins with a variety of interaction strengths.

Table 4
Transformation of MaV203 by different bait and prey plasmids

Transformation	Bait Plasmid	Prey Plasmid	Purpose
1	Control plasmid (pDBLeu)	pDEST ™22-eq. NF-κB2 (any fragment)	Self-activation control
2	pDEST ™32-S2	pDEST ™22-eq. internal fragment of NF-κB2	Interaction test for S2 and equine NF-κB2
3	pDEST ™32-S2	pDEST ™22- full length eq.NF-κB2	Interaction test for S2 and equine NF-κB2
4	pDEST ™32-S2	pDEST ™22- equine NF-KB similar to the human NF-κB2 cDNA, identified from Y2H, (using two different splice variants)	Interaction test for S2 and equine NF-κB2
5	pDEST ™32-S2	pDEST ™22-human NF-κB2 (the fragment that identified from the initial yeast two hybrid screening)	Positive control

Table 5
Set of tests for screening yeast two-hybrid-dependent transcription activation

Test	*HIS*3 induction	*URA*3 induction	*URA*3 induction	ß-Galactosidase induction
Assay	Histidine auxotrophy	5FOA sensitivity	Uracil auxotrophy	X-gal assay
Plates used	SC-Leu-Trp-His+3AT	SC-Leu-Trp+5FOA	SC-Leu-Trp-Ura	Yeast ß-Galactosidase assay kit
Concentrations	10 mM 3AT 25 mM 3AT	0.2% 5FOA	No Uracil	Not applicable

Co-immunoprecipitation (Co-IP)

To facilitate Co-IP assays, different versions of equine NF-κB2 were cloned into the pcDNATM 3.2/V5-DEST vector. The vector expresses a V5 tag sequence which can be fused to the gene of interest. We tagged full length equine NF-κB2, or a fragment of equine NF-κB2 similar to the human NF-κB2 cDNA which was identified from the initial yeast two hybrid screening (using two different splice variants). In order to clone the NF-κB2 into the vector, it was first cloned into an entry vector such as pDONR 201 vector. Afterwards, the Gateway LR clonase™ Enzyme Mix was used to introduce the NF-κB2 to the pcDNA™ 3.2/V5-DEST vector by recombination. The procedure for cloning is described in the Gateway™ Technology manual.

Chinese hamster (CHO-K1) ovary cells were transfected with GFP-S2 and equine NF-κB-V5 using Invitrogen Lipofectamin™ 2000. In brief, the procedure was as follows; CHO-K1 cell line was cultured in HYQ MEM Alpha Modification 1X (Cat. Sh3026565.01) containing 1% (v/v) penicillin, 10% fetal bovine serum (FBS), and 1% NEAA (non-essential amino acids), and incubated at 37°C in a 5% CO_2 atmosphere incubator. One day before transfection, 6×10^5 cells were seeded per well of a 6-well plate in 2ml of complete growth medium, and incubated at 37°C in a 5% CO_2 environment. Cells were incubated until they became 80% to 90% confluent, which generally took 18 to 24 hours. On the day of transfection, the complete growth medium was removed from the plates and replaced with 2 ml of complex of transfection medium without serum (Opti-MEM I Reduced Serum Medium) containing 5 µl of Lipofectamin™ 2000 and 4 µg DNA plasmid (GFP-S2 or NF-κB- V5). Then the cells

were incubated with this complex for 4 to 6 hours at 37°C in a 5% CO_2 environment. After the incubation time, the transfection medium was replaced by the original complete growth medium. After 24 to 48 hours, the cells were harvested and washed one time with PBS. To extract the protein, the cells were resuspended in the 500 µl lysate buffer containing 1X Halt Protease Inhibitor and incubated for 5 minutes on ice. In order to have only the lysate of CHO-K1 cells, as a control which will be explained later, one well of cells was kept untreated.

We also examined possible interaction of EIAV S2 with endogenous equine NF-κB2. Western blots using antibody to human NF-κB2 were performed to assay the lysate.

Co-immunoprecipitation of NF-κB- V5 or endogenous NF-κB 2 with GFP-S2

In a 1.5 ml tube, an aliquot of NF-κB- V5 or endogenous NF-κB 2 and GFP-S2 were incubated with a binding buffer overnight on a rocker at 4 ^{o}C. Then the antibody for detecting one of the tags (mouse α-GFP or mouse α-V5) was added to the tube and incubated for two hours at room temperature or overnight at 4 ^{o}C with rotation. During this time, the antibody bound to the tag. Sixty µl immobilized protein G-agarose beads (Pierce) was added to immune complexes and incubated for 2 hours at room temperature with rotation. Then the beads were collected using a Pierce Handee^{TM} spin cup-paper filter (Prod # 69700) and washed 4 times with washing buffer (PBS, 8mM Na_2PO_3, 2mM K_2PO_3, 140mM NaCl, 10mM KCl, pH 7.4). After washing the beads, the elution buffer (0.1 M glycine pH 2-3) was added to the spin column. After 5 minutes of incubation at room temperature, the spin column was centrifuged. The eluted components were loaded

on 10% SDS-PAGE gels and transferred to nitrocellulose. Membranes were incubated with mouse anti-V5 or mouse anti-GFP antibodies, followed by incubation with anti-mouse HRP TrueBlot (ebioscience), to eliminate interference of immunoprecipitating immunoglobulin heavy and light chains. Immunoreactive bands were visualized by enhanced chemiluminescence (West Femto; Pierce).

A set of controls were performed for co-immunoprecipitation as follows: To ensure that both proteins partners were present in cell lysates, aliquots of cell lysates were run on SDS PAGE and probed by Western blot with appropriate antisera. To ensure proper washing of the beads (removal of unbound material), the last wash was analyzed by SDS PAGE and probed by Western blotting. To control for absence of non specific interactions, NF-κB p100 containing lysates were incubated with GFP alone, followed by a pull down with anti-GFP. NF-κB and GFP-S2 were also incubated with protein G alone in control reactions.

In order to analyze the presence of the proteins in the eluted fragment, the western blot technique was used. First, all samples were denatured by heating them for 5 minutes at 95^{o}C. Then the proteins were separated on 10% SDS-polyacrylamide gels and electroblotted to a nitrocellulose (NC) membrane. Then the membrane was blocked with 5% non-fat milk for 1 hour at room temperature. After blocking, the membrane was incubated with either anti- GFP or anti- V5, followed by peroxidase-conjugated secondary antibodies, and detected by chemiluminescence. Between the incubation with two antibodies and also before the detection, the membrane was washed 4 times as follows: once with Tris- buffered saline (TBS) for 5 minutes, two times with Tris-

buffered saline containing 0.05% Tween 20 (TBST), each time for 10 minutes, and finally with TBS for 5 minutes. Controls for western blots were equine NF-κB- V5 or endogenous equine NF-κB 2 and GFP-S2.

Results

Analysis of interaction between EIAV S2 and Equine NF-κB2 in yeast two hybrid system

To study the interaction of EIAV S2 and equine NF-κB2, pDEST ™ 32-S2 and pDEST™ 22-eq.NF-κB2 were introduced to yeast strain MaV203 by transformation as described in Table 4. Four different equine NF-κB2 constructs were used. Detailed results are shown in Table 6. None of the tested constructs was found to interact with EIAV S2. As shown in table 6, an interaction between EIAV S2 and human NF-κB2 was used as a positive control.

Analysis of interaction between EIAV S2 and Equine NF-κB2 using Co-immunoprecipitation (Co-IP)

As there was not any detectable interacting between EIAV S2 and equine NF-κB2 in the yeast two hybrid system, a second methods, co-immunoprecipitation, was used to detect EIAV S2/eq. NF-κB2 interaction. For this propose, we tried to immunoprecipitate GFP-S2 with NF-κB- V5 or with endogenous equine NF-κB2 as described in methodology. Examples of IP results using different versions of equine NF-κB2 in constructions with GFP-S2 are shown in Fig. 10. In all results shown, both GFP-S2 and NF-κB-V5 were detected in flow through samples and there was no detectable protein in terminal washes. Under all tested conditions, only GFP-S2 was detected

elution samples. We were not able to see any band in the elution samples that corresponds to the endogenous eq.NF-κB2 or eq.NF-κB-V5 (different sizes). The controls indicate that our Co-IP system did work correctly but that EIAV S2 and equine NF-κB2 did not interact with each other under the conditions used (Fig. 10).

Table 6
Result of the Interaction between EIAV S2 and equine NF-κB in the Yeast Two Hybrid System

Test	*HIS*3 induction	*URA*3 induction	*URA*3 induction	ß-Galactosidase induction
Plates used	SC-Leu-Trp-His+3AT	SC-Leu-Trp+5FOA	SC-Leu-Trp-Ura	Yeast ß-Galactosidase assay kit
Concentrations	10 mM 3AT 25 mM 3AT	0.2% 5FOA	No Uracil	Not applicable
Result of the Interaction between EIAV S2 and equine NF-κB2 (different fragment)*	No growth 	Growth 	No growth 	Negative 
Negative control (yeast strain A)	No growth 	Growth 	No growth 	Negative 
Strong Positive control (yeast strain E)	Growth 	No growth 	Growth 	positive 
Positive control (S2 and Hum. NF-κB2	Growth 	Growth 	No growth 	positive 

*Middle fragment (3a), Full length, similar to Human fragment which pulled of from Y2H (different splice variants).

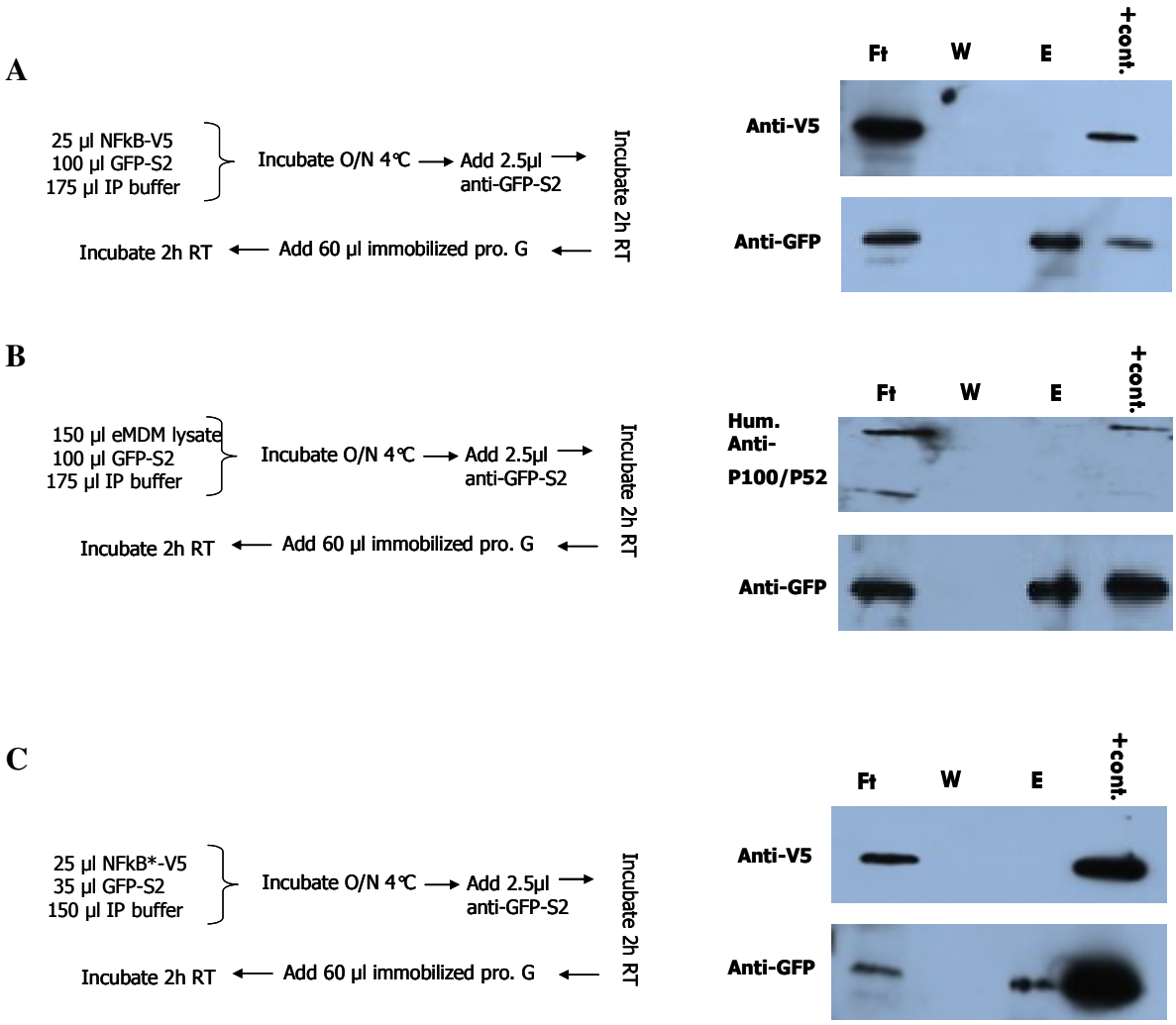


Fig. 10. Co-IP assay to probe the interaction of EIAV S2 and equine NF-κB2. The amount of each protein and the procedure for each Co-IP is illustrated beside each western blot. Flow through samples are designated by (Ft.), wash samples by (W), elute samples by (E) and positive control by (+ control). Full length of equine NF-κB2, endogenous equine NF-κB2, and a fragment of equine NF-κB2 similar to human NF-κB2 which was pulled from Y2H assay were used to probe the interaction with S2, panels A-C, respectively.

* Both splice variants were tested.

CHAPTER IV

EXPRESSION OF NF-KB2 IN EQUINE MACROPHAGE

Introduction

As NF-κB2 is an important component of immune response, and EIAV is a macrophage- tropic virus, it was important for us to examine NF-κB2 expression in eMDM. Moreover, we were interested to see if EIAV influences NF-κB2 expression in these cells. To analyze NF-κB2 gene expression, the northern blot, Reverse-Transcriptase-PCR (RT-PCR), and quantitative PCR methods were used.

Materials and Methods

Primary equine monocyte- dreived macrophages (eMDM) were established as described previously. The Qiagen Rneasy®® Mini kit was used to extract total RNA at various times post plating. The collected RNAs were used either for generating cDNA or for northern blots.

Northern Blot

A Northern blot assay was conducted to reveal possible splice variants of equine NF-κB2. In brief the procedures were as follows: RNA was isolated from eMDM from day one to day four post plating using Qiagen RNeasy® Mini kit, exactly according to the manufacturer's instructions. One microgram of each RNA sample in 12 µl of loading buffer (50 µl of formamide, 16.6 µl of 37% formaldehyde, 10 µl of 10X MOPS, 2 µl of 2.5% Bromphenol blue, 11.4 µl diethyl pyrocarbonate [DEPC]-treated double

distilled (dd) H_2O) was denatured at 65 °C for 15 minutes and chilled on ice for 1 minute. Then the denatured RNA samples were loaded on a 1% agarose gel containing 2% formaldehyde and electrophoretically separated at 50 V in 1X MOPS running buffer (10X MOPS: 0.4 M Morpholinopropanesulfonic acid (free acid); 0.1 M Na-acetate-3 x H2O; 10 mM EDTA; adjusted to pH 7.2 with NaOH) for about 3 hours. Prior to blotting, the gel was rinsed one time with ddH_2O and later rinsed for 2 times for 15 minutes each time in 20X SSC (0.3 M sodium chloride and 0.03 M sodium citrate). RNA was transferred overnight to a positively charged nylon membrane (BrightStar®-Plus, cat. # AM10102) by upward capillary action using 20X SSC. After blotting, membrane was air dried, and washed in 5X SSC which contained 0.5 % SDS at room temperature for 10 minutes. Then RNA was crosslinked to the nylon membrane with a UV cross-linker. The membrane was rinsed two times in 2X SSC + 0.1% SDS and was prehybridized with 8ml Ambion ultrasensitive hybridization buffer (ULTRAhyb® cat # 8669, 8670) for 4 hours at 50 °C with gentle agitation in a water bath shaker. Meanwhile, a biotinylated DNA probe was made from the 5′ end of equine NF-κB2 according to Ambion's BrightStar Psoralen-Biotin Labeling Kit (cat # 1480). The probe was first tested at different concentrations to ensure that it was detectable (Fig. 11). The probe then was diluted 10-fold with 10 mM EDTA and denatured at 90°C for 10 minutes. The denatured probe was added directly to the hybridization buffer. After prehybridization of the membrane, it was hybridized with probe/hybridization (240ng/4ml) mixture and incubated overnight. The membrane was then washed 2 times for 15 minutes each time in a 2X SSC containing 0.1% SDS at room temperature followed by 3 times for 30

minutes each at 60 °C in 0.1X SSC containing 0.1% SDS. Immunological detection of the probe was done exactly in the same manner as described in DIG High Prime DNA Labeling and Detection Starter Kit II protocol (cat. # 11585614910). The only difference was that Streptavidin-Alkaline Phosphatase was used instead of Anti-Digoxigenin-Alkaline Phosphatase Conjugate.

PCR and quantitative real-time PCR

Standard PCR and quantitative PCR were used to check the expression of equine NF-κB2 in uninfected eMDM cells and in eMDM cells infected with EIAV 17b (wild type) or EIAV ΔS2 (S2 is knock out) virus. eMDM were established in 12-well plates at $4x10^5$ cells/well and incubated for 2 days at 37 °C in 5% CO_2. At 2 days post plating, EIAV 17b or EIAV ΔS2 (containing 5000 cpm of RT activity) were added to each well of eMDM. At 4, 24, 48, 72, and 120 hpi, eMDM were washed with PBS, harvested with 10mM EDTA/PBS, and total RNA was extracted by using the Qiagen RNeasy®® Mini kit. First strand cDNA was made for either standard PCR or quantitative PCR, from each set of total RNA, as described previously. Short NF-κB (forward and reverse) primers [Table 1] were used for standard PCR to amplify the cDNA. PCR conditions for amplifying equine NF-κB2 cDNA were as follows: one initial denaturation at 94 °C for 3 minutes, thirty cycles at 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 1 minute and 40 sec, followed by a final extension at 72 °C for 5 minutes. For quantitative PCR, applied Biosystem's Assaysby-Design^{SM} Service was used to generate a set of Custom TaqMan® Gene Expression Assays of primer and probe sets for equine NF-κB2 and 18S RNA. The sequences of the forward and reverse primers and the probe are listed in table

7. The PCR reactions containing Custom TaqMan® Gene Expression Assays Mix (containing primers and probe), TaqMan® Universal PCR Master Mix (containing Taq, dNTPs and buffer), No AmpErase® UNG (final concentration 1X), and cDNA in a volume of 20 µl were prepared and placed in 96-well plates. The samples were amplified in an automated fluorometer (ABI 7500 Sequence Detection System, Applied Biosystems) under the following conditions: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 minute at 60 °C. Comparative threshold cycle (Ct) method was used to analyze the raw data. The value for each sample from infected cells and uninfected cells was normalized using 18S, a housekeeping gene (Li et al., 2000).

Table 7
Primers and probes for quantitative PCR

Primer	Sequence
eNF-KBp100-49 forward	5′-TGTCTGTGGGACCCAAGGA-3′
eNF-KBp100-49 reverse	5′-CATGCAGGACACCCAGGTT-3′
eNF-KBp100-49 probe	5′-ATGACTGCCCAATTTAA-3′
18S forward	5′-AAACGGCTACCACATCCAA-3′
18S reverse	5′-TCGGGAGTGGGTAATTTGC-3′
18S probe	5′-AAGGCAGCAGGCGC-3′

Probes are labeled with 5′ 6FAM, fluorescent dye 6-carboxyfluorescein, and 3′MGBNFQ.

Results

Analysis of equine NF-κB2 transcripts by Northern blot

In order to directly analyze equine NF-κB2 transcripts, one µl aliquots of total RNA extracted from eMDM at one day to four days post-plating were purified and used for northern blotting. As seen in Fig. 11 one major transcript of approximately 4.5 kb was present in all samples. Expression of this transcript decreased over 4 days. The observed transcript was larger than expected (the expected size was about 3 kb) based on the recovery cDNAs by PCR. The blot shows no evidence of splice variants not detected by PCR.

EIAV infection up regulates expression of NF-κB2

Based on preliminary data suggesting an interaction between the EIAV S2 protein and human NF-κB2, eMDM cells were infected with EIAV17b or EIAVΔS2 to determine if infection had effect on NF-κB2 expression. Total RNA from infected and uninfected eMDM cells were collected at different days. First strand cDNA was made from each RNA and used for PCR or quantitative PCR. The results of a standard PCR assay suggested that in uninfected cells the expression of NF-κB2 is decreased after 3 days post plating, about the time that monocytes start to differentiate. Interestingly in EIAV infected eMDM the expression level of NF-κB2 appeared to be somewhat variable (Fig. 12, A). To validate these data, quantitative real-time PCR was used. The signal from 18S, a housekeeping gene, was used to quantify the relative of target gene (equine NF-κB2) in each sample (infected versus uninfected). By using the comparative

threshold cycle (Ct) method (Li et al., 2000) the raw data was analyzed and the value for each sample was normalized using 18S. Comparison the expression of NF-κB2 in infected versus uninfected cells suggests that EIAV induces the expression of equine NF-κB2 in the eMDM (Fig. 12, B). We did not detect a significant difference between NF-κB2 expression levels in cells infected with wild type versus S2 deleted virus.

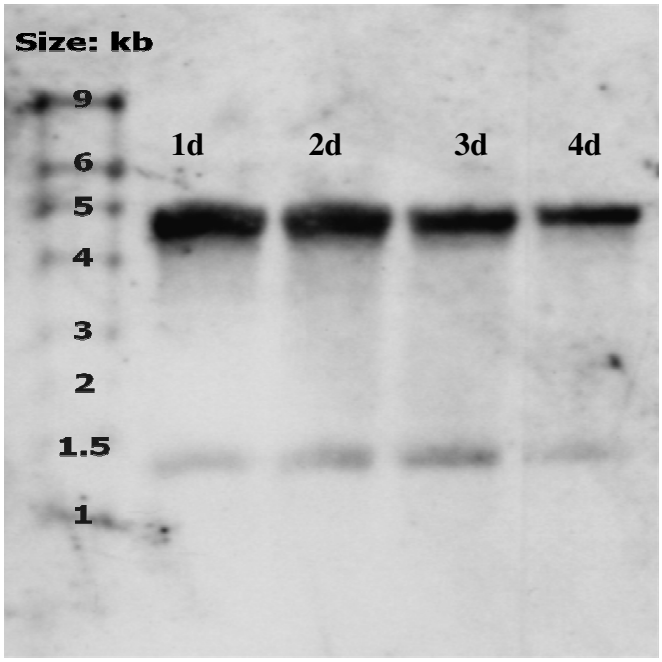
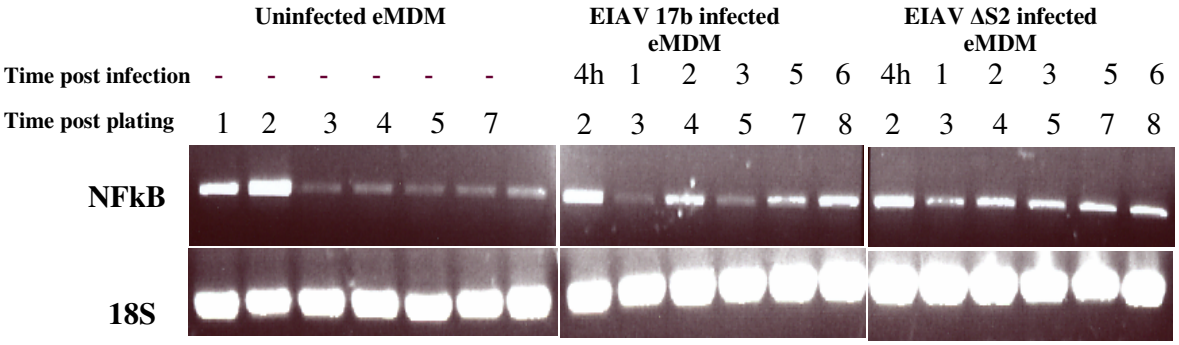


Fig. 11. Analysis of equine NF-κB2 transcripts by Northern blot. One μg aliquots of total RNA extracted from eMDM at one day to four days post-plating were purified and used for northern blot. As it can been in Fig. 12 one major transcript of approximately 4.5 kb was seen in all samples.

A



B

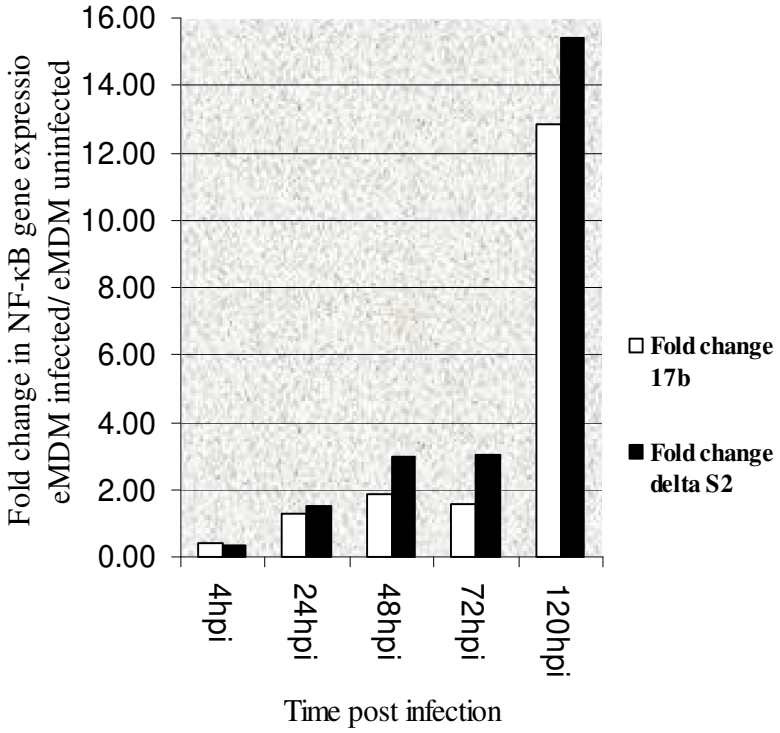


Fig. 12. EIAV infection up regulates expression of NF-κB2. (A) Results of standard PCR to detect NF-κB2 gene experssion. (B) Quantitative real time PCR was performed to quantify NF-κB2 gene expression. Values of each time point were normalized using 18S rRNA levels.

CHAPTER V

SUMMARY AND CONCLUSIONS

EIAV S2 gene expression is essential for viral replication and disease expression of EIAV in vivo (Fagerness et al., 2006; Li et al., 2000). One method for determining the role of S2 in pathogenesis is to identify the cellular proteins that interact with S2. In our laboratory, due to lack of an equine cDNA library, a commercially available human spleen cDNA library was used in a yeast two hybrid screen to elucidate any possible interactions between cellular proteins and S2. Interestingly, three potential proteins were found that interacted with EIAV S2. Nuclear Factor Kappa B p100/p52 (NF-κB2) was one those proteins. NF-κB2 has a significant role in the regulation of immune and inflammatory responses, is involved in a variety of viral diseases and also is a target for many pathogenic viruses such as HIV-1 (Santoro et al., 2003; Tato et al., 2002). Therefore, we thought it would be significant to check for any possible interaction between EIAV S2 and equine NF-κB2.

In order to investigate the interaction between S2 and equine NF-κB2, we cloned and sequenced equine NF-κB2, for the first time, in our laboratory. Two splice variants of equine NF-κB2 that were similar to reported human NF-κB2 splice variants were detected. There was about 95% amino acid sequence homology between equine and human NF-κB2. However, we found the greatest difference, with only 90% amino acid similarity, at the ankyrin repeat region (ANK), which is the location where interaction between two proteins takes place.

We tested for possible interaction between EIAV S2 and equine NF-κB2 using two different methods, the yeast two hybrid system and co-immunoprecipitation. Interestingly, neither the yeast two hybrid screening nor the co-immunoprecipitation method showed any interaction between EIAV S2 and equine NF-κB2. Since, human NF-κB2 and equine NF-κB2 are not 100% similar, there is a possibility that S2 interacts with the section of the human NF-κB2 which is not identical to the equine NF-κB2. Moreover the interaction between S2 and human NF-κB2 is a weak interaction (based on yeast two hybrid results). Therefore, there might be also an interaction between S2 and equine NF-κB2, however it is too weak to be detected by the yeast two hybrid system or the co-immunoprecipitation method. We attempted to check the interaction using the co-IP system with a cross linker (data not shown), however the system did not work as it should have worked and hence we could not get any useful results.

Although no interaction was detected between EIAV S2 and equine NF-κB2, we were interested to know how NF-κB2 is expressed in eMDM and whether EIAV has any effect on its expression. After analyzing the expression of equine NF-κB2 using northern blot or RT-PCR, we found that the greatest amount of equine NF-κB2 expression in eMDM cells was in the monocyte stage. As monocytes differentiated to macrophages, the level of NF-κB2 expression was reduced. However, in infected eMDM cells, the level of NF-κB2 expression in the macrophage stage remained at the same level as monocyte stage, which suggests that EIAV up regulates the expression of NF-κB2.

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