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PERMISSIVENESS OF SELECTED CELL LINES TO EQUINE ARTERITIS VIRUS: ESTABLISHMENT, CHARACTERIZATION, AND SIGNIFICANCE OF PERSISTENT INFECTION IN HELA CELLS

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the College of Agriculture
at the University of Kentucky

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Lexington, Kentucky
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A major goal of this research was to evaluate a variety of cell lines for their permissiveness to equine arteritis virus (EAV) infection and then identify the mechanism that restricts EAV infection in certain cell lines. The cell lines BHK-21, RK-13, and C2C12 were found to support productive infection with EAV strain VBS53, whereas Hela, Hep-2, and L-M cell lines exhibited limited susceptibility to infection with this virus. In the course of the study, it was found that the Hela cell line became more susceptible to infection with EAV strain VBS53 after extended serial passage. The respective cell lines were referred to as Hela High (passage 170-221) and Hela Low (passage 95-115) lines. While the Hela High cell line was more susceptible than the Hela Low cell line, it was still considerably less susceptible than the BHK-21 cell line to EAV infection. Subsequent studies demonstrated that infection with EAV strain VBS53 was restricted at the entry step in Hela, Hep-2, and L-M cell lines.

The second major goal of this research was to establish an *in vitro* model of persistent EAV infection using cell culture and then use the persistently infected cultures as a tool to study virus-host cell interactions, and to investigate virus and host cell evolution. Persistent infection was successfully established in the Hela High cell line with the VBS53 strain of EAV. Properties of the persistently infected Hela High cell line were characterized. Virus evolution with respect to virus growth characteristics, ability of the virus to initiate secondary persistent infection, and genetic changes during persistent

EAV infection in Hela cells was investigated. Neutralization phenotypic changes of viruses were observed during the course of persistent EAV infection in Hela cells. Reverse genetics studies identified that amino acid 98 of the GP5 protein is a new neutralization determinant of EAV. Using an *in vitro* assay, it was found that EAV probably became progressively less virulent during the course of persistent infection in Hela cells. The potential changes in pathogenicity of EAV during persistent infection of Hela cells need to be verified by inoculation of horses.

KEYWORDS: Equine Arteritis Virus, Hela Cells, Persistent Infection, Neutralization Determinants, Virus Virulence

Jianqiang Zhang

October 31, 2005

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter One: General Introduction

I. INTRODUCTION

Equine viral arteritis (EVA) is a globally distributed infectious disease of equids caused by equine arteritis virus (EAV). EAV was first isolated from the lung of an aborted fetus in the course of an outbreak of respiratory disease and abortion in Standardbred horses at Bucyrus, Ohio, USA, in 1953 (Doll *et al.* 1957a; 1957b). The majority of horses develop a subclinical infection following natural exposure to EAV. Occasionally, clinical disease can occur: influenza-like illnesses in adult horses, abortion in pregnant mares, and interstitial pneumonia, or enteritis, or pneumonoenteritis in young foals (Glaser *et al.* 1997; Timoney and McCollum 1993). Up to 30% to 70% of stallions infected with EAV can subsequently become carriers which constantly shed the virus in semen (Timoney and McCollum 1993). Persistently infected stallions are the principal reservoir of EAV and are responsible for perpetuation and dissemination of EAV in equine populations (Timoney *et al.* 1987; Timoney and McCollum 1993). Carrier stallions are also thought to be a significant natural source of genetic and phenotypic diversity of EAV (Balasuriya *et al.* 1999a; 2004a; Hedges *et al.* 1999a).

Persistent infection of cell culture has been documented with many viruses. Such infections have been used to study virus-host cell interactions and they have also been shown to be a useful tool for investigating virus and cell evolutions (Ahmed *et al.* 1981; Colbere-Garapin *et al.* 1989; de la Torre *et al.* 1988a; Kaplan *et al.* 1989). Persistent infection in cell culture could possibly serve as a model system for elucidating mechanisms of viral persistence *in vivo*.

In this introductory chapter, the molecular biology of EAV is addressed in section II with EAV infection and persistently infected stallions discussed in section III. Persistent infections of cell cultures with RNA viruses will be reviewed in section IV followed by the research objectives of this study in section V.

II. EQUINE ARTERITIS VIRUS

1. Classification and genome organization

Based on similarities in genomic organization and protein expression strategies, the *Arteriviridae* (genus *Arterivirus*), *Coronaviridae* (genera *Coronavirus* and *Torovirus*),

and *Roniviridae* (genus *Okavirus*) were grouped together in the order *Nidovirales* (Table 1.1; Cavanagh 1997; de Vries *et al.* 1997). Arteriviruses comprise four members: equine arteritis virus (EAV), porcine respiratory and reproductive syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (Snijder and Meulenberg 1998). Coronaviruses can be grouped into three clusters on the basis of serological and genetic properties (Lai and Cavanagh 1997; Stephensen *et al.* 1999). Grouping of newly discovered SARS-CoV has been of controversy. Phylogenetic analysis of different SARS-CoV proteins using unrooted trees showed that SARS-CoV does not segregate into any of the three established coronavirus groups and it was suggested that SARS-CoV may be classified as the prototype of a novel, fourth group of coronaviruses (Marra *et al.* 2003; Rota *et al.* 2003). However, phylogenetic analysis of coronavirus replicase genes using a rooted tree (the equine torovirus was used as an outgroup for coronaviruses) indicated that the SARS-CoV lineage was an early split-off from the coronavirus group 2 branch and it was suggested that SARS-CoV could be classified as the prototype of subgroup 2b if the established group 2 coronaviruses would be referred to as subgroup 2a (Snijder *et al.* 2003). Four torovirus species have been recognized: equine torovirus (Berne virus), bovine torovirus (Breda virus), human torovirus, and porcine torovirus (Koopmans and Horzinek 1994; Kroneman *et al.* 1998). The recently discovered okaviruses that infect prawns comprise two members thus far: gill-associated virus and yellow head virus (Cowley *et al.* 2002a; Cowley and Walker 2002b; Sittidilokratna *et al.* 2002).

Nidoviruses have in common a characteristic genome organization: the most 5' end open reading frames (ORF), 1a and 1b, encode the viral replicase polyproteins that are subsequently proteolytically processed; the 3'-terminals of the genome encode structural proteins (Snijder and Meulenberg 1998). Nidoviruses have a unique mechanism to regulate structural protein gene expression. The structural protein genes are not directly expressed from the genome, but from a nested set of 3'-coterminal subgenomic mRNAs (sg mRNAs). In the case of arteriviruses and coronaviruses, subgenomic mRNAs also contain a common 5' leader sequence derived from the 5' end of the genome (Lai and Cavanagh 1997; Snijder and Meulenberg 1998). On the other hand, subgenomic mRNAs

of okaviruses do not have a common 5' leader sequence (Cowley *et al.* 2002c), neither do torovirus sg mRNAs with exception of sg mRNA2 (Snijder *et al.* 1990).

The EAV genome is a single-stranded, positive-sense RNA molecule of 12,704 nucleotides [sizes excluding the 3' poly(A) tail] in length. The 3' end of the genome is polyadenylated (van Berlo *et al.* 1982) and the 5' end is believed to be capped (Snijder and Meulenberg 1998). Its genomic organization is depicted in Fig 1.1 and Table 1.2. The coding regions of the EAV genome are flanked by 5' and 3' non-translated regions (NTRs) of 224 nucleotides (nt) and 59 nt, respectively (Table 1.2). Nucleotides (1-211) are often referred to as the 5' leader sequence which is present in genomic RNA and in all sg mRNAs. An intraleader ORF (nt 14-127, encoding a hypothetical 37 aa peptide) was suggested by Kheyar *et al.* (1996). However, the expression of this ORF has never been observed and the role of this putative intraleader ORF has yet to be determined. The EAV genome includes nine functional ORFs. The replicase gene comprises the 5'-terminal three-quarters of the genome and includes two large ORFs (1a and 1b), which are both expressed directly from the genomic RNA. The translation of ORF1a generates a multi-domain precursor polyprotein: the 1727-amino-acid ORF1a polyprotein (187 kD). Following ORF1a translation, a -1 ribosome frameshift into ORF1b occurs with an estimated efficiency of 15 to 20% (den Boon *et al.* 1991). This results in the generation of a 3175-amino-acid ORF1ab polyprotein (345 kD). Two large replicase polyproteins (polyprotein 1a and polyprotein 1ab) are proteolytically processed by three ORF1a-encoded proteases (Snijder *et al.* 1992; 1995; 1996) and produce 12 nonstructural proteins (nsp1-12; Table 1.2) and multiple processing intermediates (den Boon *et al.* 1995a; Snijder *et al.* 1993, 1994; van Dinten *et al.* 1996; 1999; Wassenaar *et al.* 1997). The 3' one-quarter of the genome is composed of seven ORFs (2a, 2b, 3-7), which encode structural proteins E, GP2b (Gs), GP3, GP4, GP5 (G_L), M, and N, respectively. Most of these ORFs that encode structural proteins have both 5'- and 3'- terminal sequences that overlap with neighboring genes (Fig 1.1A and Table 1.2; de Vries *et al.* 1992; Snijder and Meulenberg 1998; Snijder *et al.* 1999). These structural proteins are expressed from a 3'-coterminal nested set of six subgenomic mRNAs that also contain a common 5' leader sequence, which is derived from the 5' end of the viral genome (Fig 1.1A; de Vries *et al.* 1990; van Berlo *et al.* 1982). The following 13, 3.2, 2.7, 2.2, 1.9, 1.2,

and 0.8 kb [including a poly(A) tail] were estimated for EAV genomic RNA and sg mRNAs 2-7, respectively (den Boon *et al.* 1991). In sg mRNAs, the leader sequence is linked to different sequences derived from the 3' region of the genome, which are referred to as "mRNA bodies". The leader sequences are connected to the mRNA bodies by a short, conserved transcription-regulating sequence (TRS) which is present both at the 3' end of the leader sequence and at the 5' end of the mRNA bodies (Fig 1.1A).

2. Virion architecture and properties

Equine arteritis virus is a small, spherical, enveloped, positive-strand RNA virus. The virus particles are 40-60 nm in diameter (Horzinek *et al.* 1971) and have a buoyant density of 1.17 to 1.20 g/ml in sucrose, 1.18 to 1.22 g/ml in cesium chloride (Hyllseth 1970). The sedimentation coefficient of EAV particles has been reported to be between 216S and 232S (van der Zeijst and Horzinek 1975). The stability of EAV is influenced by temperature and pH and EAV can be stored at -70ºC for years without significant loss of the infectivity (Burki 1966; Harry and McCollum 1981; McCollum *et al.* 1961). Equine arteritis virus can be inactivated by lipid solvents (ether and chloroform) and by disinfectants and detergents (Burki 1965; Zeegers *et al.* 1976).

A schematic representation of the EAV virion architecture is shown in Fig 1.1B. Virions consist of an icosahedral nucleocapsid (25-35 nm in diameter) which is surrounded by a lipid-containing envelope with tiny surface projections (Horzinek *et al.* 1971). The viral nucleocapsid is composed of the linear viral genomic RNA (12.7 kb) and a 14-kDa phosphorylated nucleocapsid (N) protein (de Vries *et al.* 1992; Hyllseth 1973; Zeegers *et al.* 1976). The virion envelope contains six envelope proteins. The two major envelope proteins are 16-kDa nonglycosylated membrane protein (M) and the relatively large envelope glycoprotein GP5 (previously named G_L) of 30 to 42 kDa (de Vries *et al.* 1992). The M and GP5 proteins occur in the virion as disulfide-linked heterodimers (de Vries *et al.* 1995b). The unglycosylated envelope protein E of 8 kDa is of intermediate abundance in the virion (Snijder *et al.* 1999). The three minor envelope proteins of EAV are 25-kDa glycoprotein GP2b (previously named Gs), the heterogeneously N-glycosylated GP3 glycoprotein of 36 to 42 kDa, and the 28-kDa GP4 glycoprotein, respectively (de Vries *et al.* 1992; 1995a; Hedges *et al.* 1999b; Wieringa *et al.* 2002). The GP2b, GP3, and GP4 proteins occur in the virion as heterotrimers (Wieringa *et al.* 2003a).

The EAV nucleocapsid protein, encoded by ORF7, is a small protein with a high content of basic amino acids and a hydrophilic nature (de Vries *et al.* 1992). The N protein is expressed abundantly in infected cells and constitutes about 35 to 40% of the protein moiety in the virion (de Vries *et al.* 1992). The N protein was shown to be phosphorylated and present in the virion as a monomer (Snijder and Meulenberg 1998). The N protein is necessary for virus assembly and the production of infectious virus particles (Molenkamp *et al.* 2000; Wieringa *et al.* 2004). It is predicted that the N protein plays an important role in encapsidating viral genomic RNA and in the interactions with envelope proteins during virus assembly. However, the regions of the N protein responsible for RNA encapsidation and interactions with envelope proteins have not yet been determined. The EAV virion biogenesis is assumed to be a strictly cytoplasmic event, however, besides existing in the cytoplasm, small quantities of the N protein have also been observed in the nucleus (Molenkamp *et al.* 2000; Tijms *et al.* 2002). The mechanism and significance of the nuclear translocation of EAV N protein remain unknown.

The GP5 and M proteins are the two major envelope proteins and are encoded by ORF5 and 6, respectively (de Vries *et al.* 1992). The M and GP5 proteins are present in virions in equimolar amounts and appear as disulfide-linked heterodimers which constitute the basic protein matrix of the envelope (de Vries *et al.* 1992; 1995b). In cells infected with EAV, disulfide-linked M protein homodimers were also observed but these were not incorporated into virions (Snijder and Meulenberg 1998). The M protein, like the N protein, lacks potential N-glycosylation sites and lacks a potential amino-terminal signal sequence (de Vries *et al.* 1992). The M protein is assumed to span the viral envelope three times with its internal transmembrane segments, leaving a short stretch of 10-18-amino-acid N-terminal domain (ectodomain) exposed at the outside of the virion and an approximately 72-residue C-terminal domain buried at the inside (de Vries *et al.* 1992; 1995b; Snijder and Meulenberg 1998). The single Cys residue (Cys-8) in the M ectodomain is crucial for the formation of both the GP5-M heterodimer and the M-M homodimer (Snijder *et al.* 2003). The GP5 protein (255 aa) is a heterogeneously glycosylated protein that contains an N-terminal signal sequence (aa 1-18) which is cleaved off during transport through the endoplasmic reticulum (ER). The hydropathy

profile of the GP5 protein would suggest that amino acid residues 19-116 constitute the hydrophilic ectodomain of the protein, while the C-terminal half consists of three membrane-spanning domains followed by an endodomain of about 64 amino acids (Balasuriya and MacLachlan 2004c; de Vries *et al.* 1992; Snijder *et al.* 2003). The ectodomain of the GP5 protein possesses one or two N-glycosylation sites (Asn-56 is a N-glycosylation site which is conserved amongst all strains of EAV and Asn-81 is another potential N-glycosylation site occurring in some field and laboratory strains of EAV) to which variable numbers of lactosamine side chains were added (Balasuriya *et al.* 1995a; de Vries *et al.* 1992). The ectodomain of the GP5 protein also contains five conserved Cys residues: Cys-34, Cys-57, Cys-63, Cys-66, and Cys-80. The Cys-34 in the GP5 ectodomain and the Cys-8 in the M ectodomain form a disulfide bond which mediates GP5-M heterodimerization (Snijder *et al.* 2003). The GP5 and M proteins are indispensable for both virus assembly (Wieringa *et al.* 2004) and the production of infectious virus particles (Molenkamp *et al.* 2000; Snijder *et al.* 2003; Wieringa *et al.* 2004). The GP5 protein expresses neutralization determinants of the virus. All of the neutralization determinants of EAV identified to date are located on the ectodomain of the GP5 protein (Balasuriya *et al.* 1993; 1995b; 1997; 2004b; Chirnside *et al.* 1995; Deregt *et al.* 1994; Glaser *et al.* 1995).

Besides the three major structural proteins, the EAV virion contains four other envelope proteins: small hydrophobic envelope protein E, and minor envelope proteins GP2b, GP3, and GP4 which occur as heterotrimers (Snijder *et al.* 2003; Wieringa *et al.* 2002). Also, it has been recently demonstrated that incorporation of the E protein is somehow linked to that of GP2b/GP4/GP3, suggesting the existence of a GP2b/GP4/GP3/(E) complex (Wieringa *et al.* 2004). The small envelope protein E (67 aa), encoded by EAV ORF2a, is an integral membrane protein with a central hydrophobic domain of 40 residues and a cluster of basic amino acid residues in the hydrophilic C-terminal domain (Snijder *et al.* 1999). The EAV E protein does not have a N-terminal signal sequence and lacks potential N-glycosylation sites (Snijder *et al.* 1999). The E protein proved to be stable and did not form covalently linked multimers (Snijder *et al.* 1999). The E protein was found to associate with intracellular membranes (both the ER and Golgi complex) in EAV-infected cells or upon independent expression(Snijder *et al.*

1999). Minor envelope proteins GP2b, GP3, and GP4, encoded by EAV ORF2b, 3, 4, respectively, are abundantly expressed in EAV-infected cells, but only a small fraction of them is assembled into the virion. In the virion, the molar ratio of GP2b to GP5 and M is approximately 1:25 (de Vries *et al.* 1992; 1995a; Wieringa *et al.* 2003a). Both GP2b and GP4 proteins are type I integral membrane proteins, containing one and three functional N-glycosylation sites, respectively (de Vries *et al.* 1995a; Wieringa *et al.* 2002). Both GP2b and GP4 proteins contain a 22-24 amino acid residue of an N-terminal signal sequence which is cleaved off during transport through the ER (de Vries *et al.* 1995a; Wieringa *et al.* 2002). The GP3 protein is a heavily glycosylated integral membrane protein with six N-glycosylation sites. It contains an uncleaved hydrophobic amino-terminal signal sequence and a hydrophobic C-terminus (Hedges *et al.* 1999b; Wieringa *et al.* 2002). The GP3 protein is anchored to the membrane by either one or both of its hydrophobic terminal domains; no part of its structure is detectably exposed cytoplasmically (Hedges *et al.* 1999b; Wieringa *et al.* 2002). It has been shown that the EAV E, GP2b, GP3, and GP4 proteins are dispensable for the formation of virus-like particles (Wieringa *et al.* 2004) while they are essential for the production of infectious virus particles (Molenkamp *et al.* 2000). It implies that the GP2b/GP4/GP3/(E) complex may be involved in the virus attachment and cell entry process.

3. The replication cycle of equine arteritis virus

Equine arteritis virus can be propagated in a variety of primary cultures such as equine macrophages (Moore *et al.* 2003a), equine endothelial cells (Moore *et al.* 2003b), equine kidney cells (McCollum *et al.* 1961), hamster kidney cells (Wilson *et al.* 1962), and so on. The virus can also replicate efficiently in several continuous cell lines such as rabbit kidney (RK-13; McCollum *et al.* 1962), baby hamster kidney (BHK-21; Hyllseth 1969), African green monkey kidney (Vero; Konishi *et al.* 1975) and so on. EAV infection of primary cells and continuous cell lines is highly cytocidal. The appearance of cytopathic effect (CPE) and the titer of infectious virus produced in different cell lines vary. The cytopathic effect exhibited in EAV-infected cells is characterized by rounding of cells and cell detachment from the culture plate surface (Hyllseth 1969; McCollum *et al.* 1962). Like other viruses, EAV infection of cells involves virus attachment and entry,

biosynthesis (viral genome replication, mRNA transcription, and viral protein synthesis), virus assembly, budding, and release.

(1) Virus attachment and entry

The first step of virus infection is viral attachment to a specific receptor on a susceptible cell. In the case of EAV, neither a virus attachment molecule nor a specific cell receptor has yet been identified. By analogy with many other animal RNA viruses and in view of its recognition by neutralizing antibodies, the EAV GP5 protein had been postulated to serve as the virus attachment protein and to mediate receptor recognition. However, exchange of the ectodomain of the EAV GP5 protein with that of PRRSV or LDV in the context of an infectious EAV cDNA clone did not alter the cell tropism of the virus (Dobbe *et al.* 2001). In that study, the EAV GP5 ectodomain (aa 1-114) in a full-length EAV cDNA clone was replaced by the GP5 ectodomain (aa 1-64) of PRRSV or LDV to yield chimeric EAV. It was found that these chimeric viruses were still able to infect BHK-21 and RK-13 cells. It is well known that these cells can be infected by EAV, but not by either PRRSV or LDV (Dobbe *et al.* 2001). The authors concluded that the ectodomain of GP5 is not the main determinant of EAV tropism in cell culture. Similarly, PRRSV mutants in which the ectodomain of the M protein was replaced by that of EAV or LDV still retained their ability to infect porcine alveolar macrophages and did not acquire tropism to cells susceptible to the respective viruses (e.g. BHK-21 cells for EAV and mouse macrophages fro LDV) from which the foreign ectodomains were derived (Verheije *et al.* 2002). This would suggest that, in the case of arteriviruses, the M protein is not responsible for receptor binding either. Recently, Wieringa *et al.* (2003a) have shown that minor structural proteins are present in EAV virions as disulfide-linked GP2b/GP4/GP3 heterotrimeric complexes. Also, there are indications that the E protein is noncovalently associated with the GP2b/GP4/GP3 trimers (Wieringa *et al.* 2004). It has also been demonstrated that the E, GP2b, GP3, and GP4 proteins are not required for the formation of EAV particles, although these proteins are believed essential for ensuring that the virus particles are infectious (Molenkamp *et al.* 2000; Wieringa *et al.* 2004). These findings would suggest that the GP2b/GP4/GP3/(E) complex might be involved in the EAV attachment/entry process. However, it must be realized that it is possible that a virus utilizes more than one attachment molecule for binding to cells. For example,

Delputte *et al.* (2002) showed that the PRRSV M protein alone or in a complex with GP5 were involved in virus attachment to the receptor heparan sulfate on porcine alveolar macrophages; it has also been found that sialic acids on the PRRS virion can mediate virus attachment to another receptor sialoadhesin on porcine alveolar macrophages (Delputte and Nauwynck 2004). Sialic acids may be present on one or more viral glycoproteins. Moreover, viruses do not necessarily utilize the same attachment molecule to attach to different cells, e.g. PRRSV uses some unidentified molecules other than sialic acids to attach to Marc-145 cells (Delputte and Nauwynck 2004).

The cell receptor for EAV has not been identified either. Asagoe *et al.* (1997) showed that heparin can reduce EAV infection of RK-13 cells and that this inhibition was due to the direct interaction between heparin and EAV rather than the interaction between heparin and RK-13 cells. Furthermore, treatment of RK-13 cells with heparinase before virus inoculation decreased EAV infection of the cells (Asagoe *et al.* 1997). The data suggested that a heparin-like molecule on the surface of RK-13 cells might serve as a cell receptor for EAV. Similar studies have shown that a heparin-like molecule on Marc-145 cells and porcine alveolar macrophages serves as the cell receptor for PRRSV (Delputte *et al.* 2002; Jusa *et al.* 1997). However, heparinase treatment of RK-13 cells could not completely block EAV infection (Asagoe *et al.* 1997). Furthermore, EAV infection could not be reduced below 13% even in the presence of a very high concentration of heparin (Asagoe *et al.* 1997). This implied that other molecules on the cell surface might serve as EAV receptors as well. In fact, for PRRSV, another arterivirus, two receptors have been identified on porcine macrophages: heparan sulfate and porcine sialoadhesin (Delputte *et al.* 2002; Vanderheijden *et al.* 2003). Both heparan sulfate and sialoadhesin mediate PRRSV attachment to porcine macrophages, and early attachment is mediated mainly via an interaction with heparan sulfate, followed by gradual increase of attachment via the interaction with sialoadhesin (Delputte *et al.* 2005). It was observed that PRRSV can attach to but not be internalized into wild-type CHO K1 cells, which express heparan sulfate but not sialoadhesin, suggesting that heparan sulfate alone is sufficient to mediate virus attachment but not entry (Delputte *et al.* 2005). Upon transfection and expression of recombinant sialoadhesin, CHO A745 and D667 cells which are naturally deficient in both heparan sulfate and sialoadhesin expression are capable of binding and internalizing

PRRSV, indicating that sialoadhesin expression is sufficient for both PRRSV attachment and internalization and heparan sulfate is not essential for internalization (Delputte *et al.* 2005). However, heparan sulfate may enhance PRRSV binding to porcine sialoadhesin (Delputte *et al.* 2005).

EAV is assumed to enter cells through a process of receptor-mediated endocytosis, since the same family member PRRSV has been shown to enter cells through a mechanism of clathrin-dependent, receptor-mediated endocytosis in which low pH is necessary for the fusion between the endosomal membrane and the viral envelope and subsequent virus uncoating (Kreutz and Ackermann 1996; Nauwynck *et al.* 1999). It was observed that PK-15 cells expressing recombinant sialoadhesin can bind and internalize PRRSV but they are not productively infected (Vanderheijden *et al.* 2003). It was also reported that Vero cells, which are non-permissive to PRRSV infection but can support PRRSV replication after transfection with viral RNA, were able to bind and internalize the virus almost as efficiently as the permissive cell line Marc-145 (Kreutz 1998). This may possibly be due to the fact that the fusion of viral and endosomal membranes does not occur after virus internalization, and virus remains in the endosome and the viral genome is not released into the cytoplasm. If this should prove to be true, it would indicate that some cellular factor(s), which are essential for viral and endosomal membrane fusion and virus uncoating, are lacking in these cells.

EAV attachment and the entry process still need further study. Firstly, the virus attachment molecule(s) need to be determined. Secondly, EAV specific cell receptor(s) wait to be defined. Thirdly, mechanism of fusion between EAV envelope and endosomal membrane need to be studied. It is still unknown how many receptors or coreceptors are needed for EAV attachment and entry. Also, it is not clear whether EAV utilizes the same receptor(s) in different cells and whether different EAV strains use the same receptor(s).

(2) Biosynthesis: viral genome replication, mRNA transcription, and viral protein synthesis

Once the viral RNA is released into the cytoplasm, arteriviruses start their replication cycle in the cell. The EAV replication cycle commences with translation of the replicase polyproteins from the genome, followed by genome replication, subgenomic

RNA transcription, and translation of structural proteins from sg mRNAs (Fig 1.2; Snijder and Meulenberg 1998).

A. Genome translation and post-translational processing of replicase polyproteins

There are two general mechanisms for translation initiation: classical cap-dependent initiation followed by linear ribosome scanning and initiation via internal ribosome entry site (IRES) element. The EAV genome is assumed to be 5' capped based on two findings: another arterivirus SHFV has been shown to contain a type I cap structure (Sagripanti *et al.* 1986) and a cap analogue is known to be essential to make *in vitro* transcribed full-length infectious RNA (Meulenberg *et al.* 1998; van Dinten *et al.* 1997). Therefore, it was suggested that EAV genome translation is initiated via a cap-dependent mechanism. van den Born *et al.* (2005) has further demonstrated that EAV genome translation is not IRES mediated. The EAV replicase polyprotein 1a is translated directly from ORF1a. However, ORF1b translation requires a -1 ribosomal frameshift just before ORF1a translation is terminated (den Boon *et al.* 1991). The ribosomal frameshift is assumed to be directed by two signals in the ORF1a/ORF1b overlap region: a 'shifty' heptanucleotide sequence 5' GUUAAAC 3', and a downstream RNA pseudoknot structure (Fig 1.3; Snijder and Meulenberg 1998).

Translation of the EAV replicase gene yields two polyproteins: the polyprotein 1a and the polyprotein 1ab. The EAV polyprotein 1a is cleaved seven times and the polyprotein 1ab is cleaved ten times by three different viral proteases, which in total, generates 12 end-products, named nonstructural protein (nsp) 1-12, and multiple processing intermediates (Fig 1.4A; Table 1.2). Three EAV proteases are located in nsp1, nsp2, and nsp4. The nsp1 protease has been characterized as a papain-like cysteine protease (PCP) (Snijder *et al.* 1992). The nsp1 of PRRSV and LDV contains two active proteases: PCP1a and PCP1ß (Ziebuhr *et al.* 2000). However, in the case of EAV, PCP1a is inactive and only PCP1ß is active (Ziebuhr *et al.* 2000). The catalytic dyad of the EAV PCP has been identified as Cys-164 and His-230 (den Boon *et al.* 1991; Snijder *et al.* 1992). The EAV PCP has been found to direct an autoproteolytic cleavage at its C terminus (Gly-260 | Gly-261 site) and to result in the rapid release of nsp1 (aa 1-260) from the polyprotein (Fig 1.4A and Table 1.2; Snijder *et al.* 1992). It was observed that

the EAV PCP mediates probably exclusively *in cis* cleavage, and attempts to achieve cleavage *in trans* were unsuccessful (den Boon *et al.* 1995a; Snijder *et al.* 1992). The second protease of EAV is the cysteine protease of nsp2 (CP2), which mediates the single cleavage at Gly-831 | Gly-832 to release nsp2 (Snijder *et al.* 1995). The putative catalytic residues of the EAV CP2 are Cys-270 and His-332, though the entire nsp2 protein appears to be essential for the nsp2|3 cleavage (Snijder *et al.* 1995). The nsp2|3 cleavage can be performed by the EAV CP2 *in cis* or *in trans*, though *trans*-cleavage activity is relatively low (Snijder *et al.* 1995; Ziebuhr *et al.* 2000). The EAV nsp4 protease has some unique characteristics. On one hand, the nsp4 protease utilizes the His-Asp-Ser (His-1103, Asp-1129, and Ser-1184 in EAV) catalytic triad, a typical characteristic of classical chymotrypsin-like proteases (Snijder *et al.* 1996). On the other hand, the putative substrate-binding region of the EAV nsp4 protease contains Thr and His residues (Thr-1179 and His-1198 in EAV), which are conserved in viral 3C-like cysteine proteases and determine their specificity for cleavage sites containing a (Glu/Gln) | (Gly/Ser) dipeptide (Snijder *et al.* 1996). Based on these characteristics, the EAV nsp4 protease was classified as the prototype of a novel group of chymotrypsin-like enzymes, the 3C-like serine proteases (3CLSP) (Snijder *et al.* 1996). The EAV 3CLSP was shown to mediate eight cleavages in the EAV replicase proteins: five in the polyprotein 1a and three in the ORF1b-encoded polypeptide (Snijder *et al.* 1996; van Dinten *et al.* 1999; Wassenaar *et al.* 1997). Four of the known EAV 3CLSP cleavages occur at Glu | Gly sites (nsp3|4, nsp5|6, nsp7|8/9, and nsp11|12), three at Glu | Ser sites (nsp4|5, nsp6|7, and nsp9|10), and one at Gln | Ser site (nsp10|11) (Fig 1.4A and Table 1.2; Snijder *et al.* 1996; van Dinten *et al.* 1999; Wassenaar *et al.* 1997; Ziebuhr *et al.* 2000). Whether EAV 3CLSP cleaves each site *in cis* or *in trans* has not yet been studied in detail. However, it is clear that EAV 3CLSP has *in trans* cleavage activity, since a nsp4 expression product has been shown to efficiently cleave the nsp9|10 and nsp10|11 sites in a separately expressed, ORF1b-encoded polypeptide (van Dinten *et al.* 1999).

As mentioned above, the nsp1|2 and nsp2|3 cleavages are mediated by EAV PCP and EAV CP2, respectively; the other sites in the EAV polyproteins 1a and 1ab are cleaved by EAV 3CLSP. But these sites are not equally sensitive to processing by EAV 3CLSP. In fact, two alternative pathways have been reported to process the carboxyl-

terminal part of the EAV polyprotein 1a (Fig 1.4B; Wassenaar *et al.* 1997). In the major processing pathway, the EAV 3CLSP first cleaves at the nsp4|5 site, followed by cleavage of the nsp3|4 and nsp7|8 junctions, and the nsp5-7 remained uncleaved (Fig 1.4B; Snijder *et al.* 1994; 1996). But it was found that cleavage at the nsp4|5 site requires the association of cleaved nsp2 with the nsp3-8 precursor (Wassenaar *et al.* 1997). If the nsp2 is absent, the nsp4|5 site cannot be processed and an alternative minor processing pathway is utilized (Wassenaar *et al.* 1997). In the minor processing pathway, the nsp4|5 site is not cleaved; instead, the nsp5|6 and nsp6|7 sites are cleaved. Subsequently, cleavages at the nsp3|4 and nsp7|8 sites occur (Fig 1.4B; Wassenaar *et al.* 1997). It appears that the two pathways are mutually exclusive (Wassenaar *et al.* 1997). In either processing pathway, multiple processing intermediates are generated. In the major processing pathway, the nsp2 is required for the cleavage at the nsp4|5 site. But nsp2 protease activity is not involved, since it has been demonstrated that the nsp2 mutants in which the nsp2 cysteine protease is inactivated can still induce the nsp4|5 cleavage (Wassenaar *et al.* 1997). Consequently, it was suggested that the association of nsp2 with nsp3-8 may affect conformation of the nsp2/nsp3-8 complex which allows the cleavage of the nsp4|5 site by EAV 3CLSP (Wassenaar *et al.* 1997).

B. Genome replication

The RNA synthesis (including genome replication and mRNA transcription) of RNA viruses (except retroviruses) relies on viral-encoded RNA-dependent RNA polymerase (RdRp). Equine arteritis virus replicase proteins are processed into 12 end products (nsp1-12) and multiple processing intermediates. The nsp1, nsp2, and nsp4 contain viral proteases (Snijder *et al.* 1992; 1995; 1996). The nsp9 contains the putative RNA-dependent RNA polymerase activity, the nsp10 comprises a zinc-binding domain and the nucleoside triphosphate-binding/helicase activity, and the nsp11 harbors a conserved nidovirus-specific domain with unknown function (Fig 1.4A and Table 1.2; van Dinten *et al.* 1996). Immunofluorescence studies have revealed that the nsp9, nsp10 and the majority of replicase polyprotein 1a cleavage products (nsp2, nsp4, nsp7-8, and nsp8) colocalize in the perinuclear region of EAV-infected cells and the distribution pattern of these proteins suggest that they are associated with intracellular membranes (van der Meer *et al.* 1998; van Dinten *et al.* 1996). Colocalization of these proteins

suggests that they might assemble into a large complex which is likely involved in viral RNA synthesis. *In situ* labeling of nascent viral RNA with bromouridine triphosphate (BrUTP) further revealed that viral RNA synthesis colocalizes with the membrane-bound complex in which the replicase subunits accumulate, demonstrating that the membrane-bound complex is indeed the site of viral RNA synthesis (van der Meer *et al.* 1998). A typical feature of EAV and other arterivirus infections is the formation of paired membranes and double-membrane vesicles (DMVs) observed under electron microscope (Breese and McCollum 1970; Pol *et al.* 1997; Stueckemann *et al.* 1982; Wood *et al.* 1970). These double-membrane vesicles appear to be derived from paired endoplasmic reticulum membranes and they are most likely formed by protrusion and detachment of vesicular structures with a double membrane (Pedersen *et al.* 1999). Using replicase-specific antibodies, BrUTP-specific monoclonal antibody, and cryoimmunoelectron microscopy, Pedersen *et al.* (1999) have shown that the EAV replicase subunits and RNA synthesis localize to double-membrane vesicles. This indicates that the EAV replicase subunits, including nsp9 which contains the putative RNA polymerase function, assemble into a viral replication complex, which is membrane-associated with DMVs where the viral RNA synthesis occurs. How are the ORF1b-encoded replicase subunits (nsp9, nsp10, etc), which do not contain any transmembrane hydrophobic domains, membrane-associated with DMVs? Hydrophobic domains located in nsp2, nsp3, and nsp5 were postulated to mediate the membrane association of the EAV replication complex (van der Meer *et al.* 1998). This also suggests the presence of multiple protein-protein interactions between replicase subunits in the replication complex. Interestingly, it was observed that double-membrane vesicles could be induced, in the absence of EAV infection, upon expression of ORF1a-encoded replicase subunits nsp2 to nsp7 from an alphavirus-based expression system (Pedersen *et al.* 1999). Snijder *et al.* (2001) further demonstrated that the co-expression of nsp2 and nsp3 is both necessary and sufficient to induce the formation of double-membrane vesicles. Taken together, these data indicate that EAV ORF1a replicase proteins play an important role in the formation of a membrane-bound scaffold for the viral replication/transcription complex (Snijder *et al.* 2001).

As described above, the EAV replication complex, assembled on the membrane of virus-induced and host cell-derived DMVs, directs the viral RNA synthesis. The RNA

polymerase copies (+) genomic strands into full-length (-) genomic strands and then utilizes (-) genomic strands as templates to synthesize (+) genomic strands. But the *cis*-acting elements essentially involved in EAV replication and transcription have not been defined. Also, the exact components of the EAV replication complex need to be elucidated. It has been shown that the EAV replicase is the only viral protein required for genome replication and none of the structural proteins (E, GP2b, GP3, GP4, GP5, M, and N) are essential for genomic replication (Molenkamp *et al.* 2000). Among EAV replicase proteins, nsp1 was also shown to be dispensable for genome replication (Tijms *et al.* 2001). However, it is still uncertain whether any host cell proteins are involved in genomic replication. Recently, four MA-104 cell proteins have been shown to bind to *in vitro*-generated transcripts representing the 3' noncoding region of the genomic negative strand of several arteriviruses, including EAV (Hwang and Brinton 1998). This region, which is complementary to the (+) strand genomic leader sequence, is assumed to be important for the initiation of (+) strand RNA synthesis. Archambault *et al.* (2005) have also shown that some proteins in Vero cell cytoplasmic extracts can bind to the (+) and (-) strands of the EAV leader sequence. These suggest that host cell proteins may be utilized as components of the EAV replication complex. However, this topic definitely needs further study. Construction of an EAV replicon may be a solution to resolve these issues concerning EAV replication.

C. Subgenomic RNA transcription

One striking characteristic of the nidovirus replication cycle is the generation of a nested set of 3'-coterminal sg mRNAs from which the structural proteins are expressed (Snijder and Meulenberg 1998). For coronaviruses and arteriviruses, these sg mRNAs also contain a common 5' leader sequence, which is derived from the 5' end of the viral genome (Fig 1.1A and Fig 1.2; Snijder and Meulenberg 1998). These sg mRNAs comprise leader and body sequences that are not contiguous on the viral genome. It was observed that coronaviruses and arteriviruses use transcription-regulating sequences to join the leader and the body of sg mRNAs (Chen *et al.* 1993; de Vries *et al.* 1990; den Boon *et al.* 1996; Godeny *et al.* 1998; Makino *et al.* 1991; Meulenberg *et al.* 1993; Spaan *et al.* 1983; Zeng *et al.* 1995).

How is the 5' leader sequence joined to the sg mRNA bodies? Studies for both coronaviruses and arteriviruses have ruled out conventional *cis*-splicing as the major mechanism for the production of sg mRNAs (den Boon *et al.* 1995b; Jacobs *et al.* 1981; Yokomori *et al.* 1992). Moreover, nidoviruses replicate in the cytoplasm, while conventional splicing mainly occurs in the nucleus. Thus, it is generally accepted that nidovirus sg mRNAs are generated via a discontinuous transcription process. The first model proposed to explain this discontinuous process was the so-called 'leader-primed transcription' model (Fig 1.5A; Baric *et al.* 1983; Spaan *et al.* 1983). In this model, discontinuous transcription was assumed to occur during positive-strand RNA synthesis using the full-length negative genomic strand as a template. The sense TRS at the 3' end of the leader transcript base pairs with the antisense body TRS in the negative-strand template, after which the leader transcript is extended to generate a sg mRNA. In this model, the genome-length negative strand serves as the template for the synthesis of genomic RNA as well as sg mRNAs. However, the discovery of sg negative-strand RNAs and the fact that these sg negative-strand RNAs appear to be transcriptionally active argued against the above model (Baric and Schaad 1995; Brian *et al.* 1994; den Boon *et al.* 1996; Sawicki and Sawicki 1990; Schaad and Baric 1994; Sethna *et al.* 1989, 1990; 1991). Subsequently, several models were put forward to explain the presence of sg negative strands. Sethna *et al.* (1989; 1990) hypothesized that sg mRNAs could be amplified as independent replicons. According to their hypothesis, leader-primed transcription could produce the first generation of sg mRNAs and these sg mRNAs could subsequently function as templates for the synthesis of sg negative strands, which could in turn be used to generate the second generation of sg mRNAs. In Sethna's model, sg mRNAs are generated from two templates: genome-length negative strands and sg negative strands. However, direct evidence for replication of sg mRNAs has never been obtained. When sg-length RNA was transfected into coronavirus-infected cells, amplification of sg RNA was not observed (Chang *et al.* 1994; Makino *et al.* 1991). Sawicki and Sawicki (1990; 1995) proposed another model called 'discontinuous negative strand extension' model (Fig1.5B,C). In their model, discontinuous transcription was proposed to occur during negative-strand RNA synthesis using the full-length positive genomic strand as a template. The antisense body TRS at the 3' end of the

nascent (-) strand base pairs with the sense leader TRS in the positive genomic template, thereby allowing the addition of the anti-leader sequence. The sg negative-strand RNAs further function as templates for sg mRNAs synthesis. In this model, sg negative strands are produced directly from genome-length positive strand and are the only templates for sg mRNAs synthesis. More and more studies of arterivirus and coronavirus sg mRNAs synthesis have provided convincing evidence which is consistent with the discontinuous negative strand extension model (Baric and Yount 2000; Pasternak *et al.* 2001; Sawicki *et al.* 2001; van Marle *et al.* 1999a).

The availability of full-length infectious cDNA clones of arteriviruses has provided a powerful tool to investigate sg mRNAs synthesis. Through genetic manipulation of the body and leader TRS of an infectious EAV clone, van Marle *et al.* (1999a) has experimentally demonstrated that EAV discontinuous mRNA transcription is guided by a direct base pairing interaction between the sense leader TRS and the antisense body TRS. Using a number of TRS mutants with partial transcriptional activity, they also showed that the TRS sequence at the leader-body junction of the sg mRNA is derived exclusively from the body TRS rather than from the leader TRS, supporting the theory that sg mRNAs are generated by a 'discontinuous negative strand extension' mechanism rather than by a 'leader-primed transcription' mechanism (van Marle *et al.* 1999a). For discontinuous transcription, viral RNA polymerase has to stop transcription at one site and reinitiate transcription at another site. Therefore, it has been proposed that nidovirus discontinuous negative strand extension resembles similarity-assisted, copy-choice RNA recombination (Fig 1.5C; Brian and Spaan 1997; Nagy and Simon 1997; van Marle *et al.* 1999a). During this process, the RdRp complex and nascent (-) strand transcript are released from the primary site (a body TRS) in the positive-strand genomic template and translocated to the secondary site (the leader region) of the template where transcription is reinitiated. Base pairing between the sense leader TRS and the antisense body TRS at the 3' end of the nascent (-) strand plays a crucial role in guiding this RNA strand transfer (Pasternak *et al.* 2003; van Marle *et al.* 1999a). Recent studies have shown that, besides base pairing between sense leader TRS and antisense body TRS, the secondary structure of the leader is also essential for efficient sg RNAs synthesis (van den Born *et al.* 2004; 2005). According to the predicted secondary structure of the leader sequence of

arteriviruses (in the case of EAV, leader sequence is nt 1-211), the leader TRS is located in the loop of a hairpin structure and this hairpin is named as leader TRS hairpin (LTH) (Fig 1.5C; van den Born *et al.* 2004; van Marle *et al.* 1999a). By mutagenesis studies of the leader sequence using an infectious EAV clone, it has been demonstrated that the leader TRS hairpin and its immediate flanking sequences are essential for efficient sg RNA synthesis (van den Born *et al.* 2004; 2005). It is predicated that proably the antisense body TRS at the 3' end of the nascent (-) strand can base pair with the sense leader TRS only when the latter is present in the loop of a hairpin structure (van den Born *et al.* 2004; 2005; van Marle *et al.* 1999a).

Another open question is about the functionality of body TRSs and the heterogeneity of sg mRNAs. In addition to the leader TRS (5'UCAAC3'), the EAV genome contains 17 other 5'UCAAC3' motifs, of which only 6 function as body TRSs participating in leader-body junction to generate major sg mRNAs2-7 (den Boon *et al.* 1991) from which structural proteins E/GP2b, GP3, GP4, GP5, M, and N are expressed, respectively. The proteins E and GP2b are expressed from the same sg mRNA2. Using sg mRNA-specific RT-PCR approach, studies have shown that there exist subspecies for sg mRNAs 3, 4, and 5: namely sg mRNA3.1, -3.2, -3.3, sg mRNA4.1, -4.2, sg mRNA5.1, and -5.2 (den Boon *et al.* 1996; Pasternak *et al.* 2000). This heterogeneity of sg mRNAs originates from the use of alternative body TRSs for the joining of leader and body (den Boon *et al.* 1996; Pasternak *et al.* 2000). The reason that sg mRNA3.3, -4.1, and -5.2 subspecies went unnoticed in previous studies may be due to two facts: a) these subspecies are minor mRNA subspecies and are in low abundance in infected cells. For example, sg mRNA5.2 is 125- to 625-fold less abundant than the major sg mRNA5.1 subspecies; body TRS4.1 is 40 times less active than body TRS4.2. b) The size differences of subspecies for respective mRNA are very small. For example, sg mRNA3.1 is 32 nt larger than sg mRNA3.2 which is 42 nt larger than sg mRNA3.3; sg mRNA4.1 is 17 nt larger than sg mRNA4.2. These small size differences can hardly be detected by northern hybridization assay used in previous studies. Taken together, it must be realized that the same sequence motif 5'UCAAC3' can be actively, less actively, or not used for leader-body junction to generate sg mRNAs. Thus, the primary sequence 5'UCAAC3' itself is clearly not sufficient to determine whether it is used for sg mRNA

transcription or not. Other factors, such as flanking sequences and/or RNA secondary structure, are likely to contribute to determining the functionality of the sequence motif 5'UCAAC3'. Probably the sequences 5'UCAAC3' at certain positions are more active and at other positions are less active or not active.

Just as in viral genomic replication, synthesis of sg mRNAs is also directed by the RdRp complex. So, it can be assumed that sg mRNA transcription of EAV also occurs on the membrane of virus-induced and host cell-derived double membrane vesicles. Similar to viral replication, none of the structural proteins is required for EAV sg mRNA transcription (Molenkamp *et al.* 2000). However, the EAV nsp1, which is dispensable for viral replication, has been found to be essential for sg mRNA transcription (Tijms *et al.* 2001). How the nsp1 is involved in sg RNA synthesis is still unclear. Interestingly, it has been found that the EAV nsp1 is able to interact with the cellular transcription co-factor p100 (Tijms and Snijder 2003). It remains to be determined whether the interaction between the nsp1 and p100 plays any role in sg mRNA transcription.

EAV structural proteins are expressed from respective sg mRNAs. Since all of the EAV sg mRNAs are 5' and 3' co-terminal as the viral genome, it is reasonable to assume that translational machinery for genomic RNA and sg mRNAs are the same. With exception of the sg mRNA7, the EAV sg mRNAs are structurally polycistronic (Fig 1.2). However, in each of these sg mRNAs, only the most 5'-proximal gene positioned immediately downstream of the leader sequence is translated, with exception of sg mRNA2, from which both E and GP2b proteins are translated (Snijder *et al.* 1999). As mentioned afore, there exist subspecies for sg mRNAs 3, -4, and -5. But, since all TRSs used to generate sg mRNA subspecies are located upstream of the translation initiation codons in respective ORFs (Fig 1.6), sg mRNA subspecies can be used to express the same corresponding structural protein.

D. The link among translation, replication, and transcription

The EAV replicase is expressed directly from the viral genome as two polyproteins which subsequently are extensively processed by three viral proteases to generate 12 end-products and multiple processing intermediates which direct viral replication and sg mRNA transcription. Equine arteritis virus genomic replication and sg mRNA transcription are both based on viral replicase proteins and occur at the same intracellular

location, following the same principles of RNA synthesis. The only difference is that genomic replication is a continous process and sg mRNA transcription involves a discontinuous mechanism. The continuity or discontinuity may be directed by some viral or cellular proteins which are associated with the transcription complex but not the replication complex.

(3) Virus assembly, budding, and release

Equine arteritis virus acquires its envelope from internal membranes of the infected cell rather than from the plasma membrane. The assembly of EAV takes place at the cytoplasmic faces of the endplasmic reticulum (ER), and/or the Golgi complex (Magnusson *et al.* 1970).

It has been shown that all seven EAV structural proteins (E, GP2b, GP3, GP4, GP5, M, and N proteins) are indispensable for the production of infectious progeny virus (Molenkamp *et al.* 2000; Snijder *et al.* 1999; Snijder *et al.* 2003). However, only the structural proteins GP5, M, and N are essential for the formation of virus-like particles (VLPs) (Wieringa *et al.* 2004). None of the other structural proteins, E, GP2b, GP3, and GP4, is required to generate virus-like particles (Wieringa *et al.* 2004). When any one of GP5, M, and N genes was disrupted in the infectious cDNA clone, the virus particle formation was abrogated (Wieringa *et al.* 2004). In contrast, following inactivation of any of E, GP2b, GP3, and GP4 genes, virus particles were still generated although these particles were noninfectious (Wieringa *et al.* 2004). Strangely, all attempts to produce virus-like particles by the cotransfection of cells with expression plasmids encoding the EAV GP5, M, and N proteins were unsuccessful (Snijder *et al.* 1999; Wieringa *et al.* 2004). This suggests that, besides the GP5, M, and N proteins, there must be additional factors involved in EAV particle formation. For instance, EAV particle formation may depend on pre-formation of the nucleocapsid which constitutes of the N protein and the viral genomic RNA; if the icosahedral nucleocapsid containing viral genomic RNA is not formed, the subsequent steps of EAV assembly will not take place.

How the EAV N protein interacts with viral genomic RNA to form the icosahedral nucleocapsid remains unknown. Is the EAV N protein the sole protein required to form the icosahedral nuclocapsid or does the formation of icosahedral nucleocapsids need the assistance of other viral or cellular proteins? How many copies of the EAV N protein are

needed to form the nucleocapsid? Is the EAV nucleocapsid assembly a concerted assembly or a sequential assembly? In a concerted assembly, the structural units of the protein shell assemble productively only in association with the viral genome. In the alternative sequential assembly, the viral genome is inserted into a preformed protein shell. In EAV-infected cells, there are many forms of RNAs, e.g. full-length (+) and (-) viral genomic RNAs, (+) and (-) viral sg mRNAs, and cellular RNAs. But only full-length (+) viral genomic RNA is selectively packaged into the EAV virion (Molenkamp *et al.* 2000). How does the EAV N protein distinguish (+) viral genomic RNA from other forms of RNAs? The RNA sequences required for encapsidation of the EAV genome have not yet been determined. What parts of the EAV N protein recognize the RNA-packaging sequences? All of these questions remain to be answered.

How does the EAV nucleocapsid acquire an envelope? As mentioned above, the EAV N, GP5, and M proteins are the only structural proteins required for the production of viral particles (Wieringa *et al.* 2004). It is generally thought that the disulfide-linked GP5/M heterodimers constitute the basic protein matrix of the envelope (de Vries *et al.* 1995b) and are a prerequisite for virus assembly (Snijder *et al.* 2003). When expressed individually, EAV GP5 and M proteins were observed to be retained only in the endoplasmic reticulum (ER); in contrast, when co-expressed, the M protein localized both in ER and the Golgi complex and the GP5 protein consistently colocalized with the M protein in the Golgi complex (Balasuriya *et al.* 2000). The authors concluded that transport of EAV GP5 and M proteins from ER to the Golgi complex is dependent upon the formation of a GP5-M heterodimer (Balasuriya *et al.* 2000). Snijder *et al.* (2003) further confirmed this conclusion with their observation that mutations of Cys residues in GP5 or M, which would block GP5-M heterodimer formation, abrogated the transport of both GP5 and M to the Golgi complex and resulted in the accumulation of both proteins in the ER with no infectious virus produced. The authors assumed that the GP5/M heterodimers are a prerequisite for virus assembly, which obviously needs to be confirmed. Actually this can be easily affirmed by investigating whether mutations blocking GP5-M heterodimerization will also abrogate the virus particle formation. The above data also suggested that the heterodimerization of GP5 and M proteins occur in the ER. The luminal (ecto-) domains of GP5 and M proteins form a disulfide linkage

between GP5 Cys-34 and M Cys-8 (Snijder *et al.* 2003). The cytoplasmically exposed domains of the disulfide-bonded GP5/M heterodimers may interact with the synthesized nucleocapsid. Thus, the EAV nucleocapsids bud into the lumen of ER or Golgi network and acquire lipid membrane carrying viral envelope proteins. Since the EAV E, GP2b, GP3, and GP4 proteins are also integral membrane proteins and are anchored to the lipid membrane of ER or Golgi complex, the EAV nucleocapsids also acquire these envelope proteins when budding into the lumen. However, the formation of GP2b/GP4/GP3 heterotrimers is not simple. In EAV-infected cells, the GP2b protein occurs in four monomeric conformations due to the formation of different intrachain disulfide bonds between the cysteine residues (Cys 48, 102, 137), and a disulfide-linked dimer (de Vries *et al.* 1995a; Wieringa *et al.* 2003a; 2003b). The disulfide-linked dimer, previously identified as homodimer of the GP2b protein (de Vries *et al.* 1995a), has been redefined as a covalently linked heterodimer of GP2b and GP4 (Wieringa *et al.* 2003a). In EAV-infected cells, no disulfide-bonded GP2b/GP3/GP4 heterotrimers could be detected, despite the fact that such heterotrimers occur in the EAV virion (Wieringa *et al.* 2003a). It is speculated that the formation of disulfide-bonded GP2b/GP3/GP4 heterotrimers involves two disulfide-bond linkages: the disulfide bond between GP2b and GP4 and the disulfide bond between GP4 and GP3. It is known that GP2b Cys-102 is responsible for the disulfide bond linkage with GP4 (Wieringa *et al.* 2003b). However, it is still unknown which cysteine residue in GP4 interacts with Cys-102 of GP2b. Likewise, the cysteine residues that are involved in the disulfide bond formation between GP4 and GP3 remain undefined. So, what is the process of the formation of disulfide-bonded GP2b/GP4/GP3 heterotrimers in the virion? It has been postulated that the GP2b protein and the GP4 protein first form a covalently linked heterodimer, then GP3 protein interacts noncovalently with the disulfide-bonded GP2b/GP4 heterodimer and the complex is finally assembled into the virion, but disulfide bond linkage between GP2b/GP4 and GP3 takes place only after virus particles have been released (Wieringa *et al.* 2003a; 2003b). Also, it has been recently found that, when one of the GP2b, GP3, or GP4 proteins was missing, incorporation of the remaining two minor envelope proteins was completely blocked and incorporation of the E protein was greatly reduced (Wieringa *et al.* 2004). The absence of E protein entirely prevented incorporation of the GP2b, GP3, and GP4

proteins into the virion (Wieringa *et al.* 2004). The detailed molecular interactions between EAV structural proteins are unclear. Wieringa *et al.* (2004) proposed a model to describe the interactions between EAV structural proteins. In this model, the E protein is thought to be the component which, on the one hand interacts with the GP2b/GP4/GP3 heterotrimer, and on the other hand interacts with the GP5/M heterodimer and/or the nucleocaspid. It is through these interactions that the E protein draws the GP2b/GP4/GP3 complex into nascent particles.

After the EAV nucleocapsids bud into the lumen of ER or Golgi complex and acquire a lipid membrane carrying viral envelope proteins, virus particles are formed. Then the virus particles are transported from the intracellular compartments to the plasma membrane where the virus particles are released via exocytosis. After the virus particles are released, the noncovalent linkage between the GP3 protein and the GP2b/GP4 heterodimer become covalently linked; thus, the disulfide-bonded GP2b/GP4/GP3 heterotrimers are formed.

4. Neutralization determinants of equine arteritis virus

Long-lived EAV-neutralizing antibodies are induced following natural infection, experimental infection or vaccination (with the modified live virus vaccine) of horses (Timoney and McCollum 1993), and neutralizing antibodies can protect horses from subsequent challenge with EAV (Doll *et al.* 1968; McCollum 1970, 1976, 1986; McCollum *et al.* 1988). Identification and characterization of immunogenic epitopes has important implications for disease diagnosis and development of improved vaccines. Neutralizing monoclonal antibodies to EAV have been developed by several laboratories, and all of them have been shown to recognize the GP5 protein using Western blot and/or immunoprecipitation assays (Balasuriya *et al.* 1993; 1995b; 1997; Deregt *et al.* 1994; Glaser *et al.* 1995). The hydropathy profile of the GP5 protein predicts that amino acid residues 1-18 constitute the putative signal sequence, 19-116 constitute the hydrophilic ectodomain of the protein, while the C-terminal half consists of three membrane-spanning domains followed by an endodomain of about 64 amino acids (de Vries *et al.* 1992; Snijder *et al.* 2003). Chirnside *et al.* (1995) have shown that horses developed EAV-neutralizing antibodies after they were inoculated with either a fusion protein covering GP5 aa 55-98 or a synthetic peptide corresponding to GP5 aa 75-97. It was

further demonstrated that an *E. coli*-expressed recombinant protein comprising the entire ectodomain (aa 18-122) of EAV GP5 protein induced high levels of neutralizing antibodies in ponies (Castillo-Olivares *et al.* 2001). These support the hypothesis that the ectodomain (aa 19-116) of EAV GP5 protein is immunodominant. By comparing the sequences of neutralization-sensitive EAV and neutralization-resistant viruses (escape mutants, EM), Glaser *et al.* (1995) identified that amino acid residues at 99 and 100 are critical for virus neutralization. After extensive comparison of GP5 amino acid sequences and neutralization phenotypes of a large number of field and laboratory EAV strains using both neutralizing MAbs and EAV strain-specific polyclonal equine antisera, Balasuriya *et al.* (1993; 1995b; 1997) have identified four distinct neutralization sites. These sites include amino acids 49 (site A), 61 (site B), 67-90 (site C), and 99-106 (site D) in the GP5 protein. In a recent study, the ORF5 of an infectious EAV clone was replaced with that of different laboratory, field, and vaccine strains of EAV, thus generating various chimeric viruses (Balasuriya *et al.* 2004b). The comparative characterization of neutralization phenotypes of chimeric viruses and parental viruses has further confirmed the importance of the aforementioned four neutralization sites in the GP5 protein. Taken together, these studies demonstrated that the ectodomain of the GP5 protein contains EAV neutralization determinants. Site D includes several overlapping linear epitopes. The four neutralization sites (A-D) are conformationally interactive (Balasuriya *et al.* 1997; 2004b). However, it was also observed that a monoclonal antibody (1H7) failed to neutralize some chimeric viruses but was able to neutralize their respective parental viruses, though chimeric viruses and parental viruses have the same ORF5 sequences (Balasuriya *et al.* 2004b). This suggests that some of the neutralization epitopes are conformationally dependent and may interact with other structural proteins. Moreover, it remains to be determined whether there exist additional neutralization determinants in the EAV GP5 protein. The ectodomain of the EAV GP5 protein possesses one or two N-glycosylation sites: Asn-56 is the conserved N-glycosylation site amongst all strains of EAV and Asn-81 is another potential N-glycosylation site occurring in some field and laboratory strains of EAV. There is no evidence that the presence of a second glycosylation site on EAV GP5 protein interferes with virus neutralization. In the case of EAV, all of the neutralization determinants identified thus far are located in the GP5

protein; however, neutralization determinants have been found in both the GP4 and GP5 proteins of PRRSV. Monoclonal antibodies against the GP4 protein of the Lelystad strain of PRRSV have been reported to be neutralizing (Meulenberg *et al.* 1997), although MAbs against the GP5 protein of PRRSV are more effective in virus neutralization than MAbs against the GP4 protein (Weiland *et al.* 1999). The primary neutralization determinants of PRRSV are located in the middle of the GP5 ectodomain (Pirzadeh and Dea 1997; Plagemann *et al.* 2002; Zhang *et al.* 1998), but a neutralization epitope (aa 24) has also been observed in the predicted signal sequence of the GP5 protein of PRRSV (Wissink *et al.* 2003).

5. Genetic and phenotypic diversity of equine arteritis virus

The virus-encoded RNA-dependent RNA polymerases (RdRp) are low fidelity enzymes with mutation rates as high as the order of 10^{-3} to 10^{-5} errors per nucleotide per replication cycle (Domingo and Holland 1997; Drake 1993). In addition, RNA viruses have very short replication times and generate very large populations during replication. Therefore, RNA viruses exist not as a single genotype but rather as a heterogeneous mixture of related genomes known as a viral quasi-species (Holland *et al.* 1992). The quasi-species nature of RNA viruses allows viruses to adapt to environmental changes more rapidly and facilitates the emergence of viral variants (Castro *et al.* 2005). Genetic and phenotypic variation among equine arteritis virus isolates have been demonstrated, although only one neutralization serotype of EAV has been identified so far (Fukunaga and McCollum 1977; Golnik *et al.* 1986). Murphy *et al.* (1988; 1992) first demonstrated that genomic variation exists among EAV isolates using RNase T1 oligonucleotide fingerprinting. Nucelotide sequence comparison of ten EAV isolates revealed that the leader sequence identity between different isolates and the Bucyrus reference strain ranged from 94.2 to 98.5% (Kheyar *et al.* 1998). In the case of the replicase gene, the nsp2 is highly variable among field isolates of EAV (Balasuriya *et al.* 2004a). Most studies of genetic variation of EAV have focused on structural proteins. Nucleotide and amino acid comparison of various EAV isolates in ORFs 2-7 have been performed (Archambault *et al.* 1997; Balasuriya *et al.* 1995a; 2004a; Chirnside *et al.* 1994; Hedges *et al.* 1996; 2001; Larsen *et al.* 2001; Lepage *et al.* 1996; St-Laurent *et al.* 1997; Stadejek *et al.* 1999). These studies revealed that the EAV M and N proteins are more conserved

than other structural proteins, the ORF4 and ORF2 protein sequences are moderately conserved, and the ORF3 and ORF5 protein sequences are most variable. Phylogenetic analysis of EAV ORF5 sequences have suggested classification of various EAV isolates into two large groups: North American group (group I) and European group (group II) (Balasuriya *et al.* 1995a; Stadejek *et al.* 1999). The geographic origin of the viruses (where the viruses were isolated) may be different from phylogenetically predicted relationships. For example, some of the EAV strains were isolated from horses in North America, but are grouped with European isolates according to phylogenetic analysis, suggesting dispersal of the virus between continents by movement of horeses. Therefore, phylogenetic analyses of ORF5 sequences between strains are a useful molecular epidemiological tool for tracing the origin of an EAV isolate.

As mentioned above, there is only one serotype of EAV (Fukunaga and McCollum 1977; Golnik *et al.* 1986) and all strains evaluated thus far are neutralized by polyclonal equine sera raised against the virulent Bucyrus strain of EAV (Balasuriya and MacLachlan 2004c). However, EAV strains frequently exhibit neutralization phenotypic differences; e.g., different EAV strains are neutralized to various degrees with different polyclonal antisera and monoclonal antibodies (Balasuriya *et al.* 1997; 2004a; 2004b; Hedges *et al.* 1999a). In addition, geographically and temporally distinct EAV isolates vary markedly in the severity of the clinical disease they induce and in their abortigenic potentials (Balasuriya *et al.* 1998; 1999b; Balasuriya and MacLachlan 2004c; McCollum and Timoney 1999; Timoney and McCollum 1993). For example, the virulence of some EAV strains for horses have been defined: severely virulent (VBS53 and ATCC EAV), moderately virulent (KY84, CAN86, AZ87, and IL93), mildly virulent (SWZ64, AUT68, IL94, and CA97), and putatively avirulent (KY63, PA76, KY77, CA95G, and ARVAC vaccine) strains of EAV (MacLachlan *et al.* 1996; McCollum 1981; McCollum and Timoney 1984; McCollum *et al.* 1995; McCollum and Timoney 1999; Moore *et al.* 2003b; Patton *et al.* 1999; Timoney and McCollum 1993). However, the genetic determinants of viral virulence have not yet been defined.

III. EAV INFECTION AND PERSISTENTLY INFECTED STALLIONS

Natural EAV infection occurs in both horses and donkeys, and the virus is distributed in many equid populations throughout the world (McCollum *et al.* 1995; Timoney and McCollum 1993). Most EAV infections are subclinical in nature; occasionally, infection results in a respiratory type syndrome, abortion in pregnant mares, and interstitial pneumonia or pneumonoenteritis in young foals (Timoney and McCollum 1993; 1996). Up to 30% to 70% of stallions infected with EAV can subsequently become persistently infected and constantly shed the virus in semen. On the other hand, neither mares, geldings, nor foals have been shown to become carriers of the virus (Timoney and McCollum 1993).

1. Modes of transmission

The two major routes of EAV transmission between horses are the respiratory and venereal routes (Timoney and McCollum 1993). During the acute phase of the infection, virus is shed significant amounts in the respiratory tract secretions and transmission to other susceptible horses occurs through aerosolized respiratory tract secretions (Timoney and McCollum 1993). Acutely infected horses also shed virus in their urine, feces, vaginal and other body secretions, although in smaller amounts than via the respiratory tract (McCollum *et al.* 1971; Timoney and McCollum 1993). EAV can also be found in aborted fetuses, placenta, and their membranes and fluids from a mare that has aborted as a result of EAV infection (Timoney and McCollum 1993). These sources of virus may also contribute to aerosol transmission of EAV to other horses. Aerosol transmission of infectious particles is the most important and primary route of EAV dissemination during outbreak of the disease. However, it should be emphasized that direct and close contact appears to be necessary for aerosol transmission of EAV (Collins *et al.* 1987; Timoney 1988). Venereal transmission is another important route of dissemination of EAV. The virus can be transmitted venereally not only by the acutely infected mare and stallion, but also by persistently infected stallions (Timoney *et al.* 1986; 1987). Approximately 85 to 100% of seronegative mares become infected when they are bred to persistenlty infected stallions or artificially inseminated with infective semen from these carrier animals (Timoney and McCollum 1993). The infected mares can in turn transmit the virus to susceptible horses via the respiratory route. Reciprocal venereal transmission from an

acutely infected mare to a seronegative stallion, though plausible, has not been documented. Carrier stallions are believed to be the natural reservoir of EAV and are responsible for perpetuation and dissemination of EAV in equine populations (Timoney *et al.* 1987; Timoney and McCollum 1993).

Besides the two aforementioned major routes of transmission, EAV can also be transmitted less commonly by other means. For example, the foal can congenitally acquire EAV infection through transplacental transmission when a pregnant mare is exposed to the virus in late gestation (Vaala *et al.* 1992). In this case, infected foals may develop rapidly progressive fulminating interstitial pneumonia and in some cases, also a fibronecrotic enteritis (Carman *et al.* 1988; Del Piero *et al.* 1997; Vaala *et al.* 1992; Wilkins *et al.* 1995). Transmission of EAV through indirect contact with virus-contaminated fomites or by an infected teaser stallion or nurse mare may also occur infrequently (Timoney and McCollum 1996).

2. Carrier state

A. General information

As early as the latter half of the nineteeth century, it had been observed that healthy stallions could transmit so called pink-eye or influenza disease, which very likely was equine viral arteritis, to mares at breeding (Clark 1892; Pottie 1888). At that time, it was postulated that the causative agent is shed in the semen of those healthy stallions (Clark 1892; Pottie 1888). These are the earliest reports of occurrence of an 'EAV' carrier state. However, not until the 1984 epizootic of equine viral arteritis in Kentucky was the EAV carrier state extensively studied by Timoney and McCollum (Timoney *et al.* 1986; 1987). After natural infection with EAV, up to 30% to 70% of stallions can become persistently infected with the virus (Timoney and McCollum 1993). Persistently infected stallions shed EAV constantly in their semen; virus has not been detected in their urine, blood, or nasopharyngeal secretions (Timoney and McCollum 1993). Equine arteritis virus appears to be restricted to the reproductive tract of carrier stallions, and the ampulla of the vas deferens seems to be the primary site of EAV persistence (Neu *et al.* 1987; Timoney and McCollum 1993). Virus is present in the sperm-rich fraction of the ejaculate and is not detected in the preejaculatory fluids (Timoney *et al.* 1987). There appears to be little variation of virus titer among semen samples sequentially collected from the same

stallion over time. Duration of the carrier state varies and it has been arbitrarily divided into three categories: short-term or convalescent carrier state lasting several weeks; intermediate carrier state lasting 3 to 7 months; and the long-term or chronic carrier state lasting for years and even the lifetime of particular stallions (Timoney *et al.* 1986; Timoney and McCollum 1993). It has also been observed that some carrier stallions spontaneously cease to shed virus after years of persistent infection, with no indication of reversion to a shedding state later on (Timoney and McCollum 1993). A longitudinal field study provided no evidence that carrier stallions are or can become intermittent shedders of the virus (Timoney *et al.* 1991) .

B. The mechanisms of EAV persistence in carrier stallions

The detailed mechanisms of virus persistence in the host have not been clearly understood. It has been proposed that viral persistence maybe involves two essential ingredients: an ineffectual immune response, and a unique component or strategy of viral replication (de la Torre *et al.* 1991; Oldstone 1989, 1991). EAV persistence in carrier stallions may also involve the factors in these two aspects.

In carrier stallions, EAV persists exclusively in the reproductive tract and not in other sites. It seems unlikely that the EAV has obtained a restricted tropism to the reproductive tract of stallions, since EAV in the reproductive tract of carrier stallions can be venereally transmitted to susceptible mares in which the virus has an apparent wide tissue distribution (McCollum *et al.* 1988; Timoney *et al.* 1987). It seems more likely that the immune effectors have eliminated EAV from other sites but have failed to eliminate the virus from reproductive tract of carrier stallions. The observation that carrier stallions usually have moderate to high titers of serum neutralizing antibody to EAV (Timoney and McCollum 1993) further support the conclusion that humoral immunity does not prevent establishment or maintenance of persistent EAV infection in the reproductive tract of stallions. How the virus in the reproductive tract of carrier stallions evades immune clearance by neutralizing antibodies is unknown. It seems unlikely that immune escape mutants of virus play a significant role, because the emerging virus variants in carrier stallions can still be neutralized by polyclonal neutralizing antibodies (Balasuriya and MacLachlan 2004c). Virus persistence may be related to the fact that the reproductive tract of mature male is an immunologically priviledged site (Johnson 1973).

The physiologic mechanisms responsible for this privileged site are multifactorial, develop at puberty, and in some species depend on androgens (Pelletier 1986). Maybe the virus in the reproductive tract of stallions is inaccessible to circulating neutralizing antibodies or that neutralizing antibodies can only partially downregulate virus replication but are inadequate to completely prevent ongoing virus replication. Also, the role of cellular immune responses in persistent EAV infection is not known.

There is convincing evidence that establishment and maintenance of the carrier state in the stallion is testosterone-dependent (Little *et al.* 1991; McCollum *et al.* 1994). When persistently infected stallions were castrated and administered testosterone, they continued to shed virus in semen, whereas castrated stallions given a placebo ceased shedding virus (Little *et al.* 1991). Investigation of the persistence of EAV in prepubertal and peripubertal colts showed that EAV can replicate in the reproductive tract of a significant proportion of colts for a variable period of time after clinical recovery in the absence of circulating concentrations of testosterone equivalent to those found in sexually mature stallions. However, long-term persistent EAV infection does not appear to occur in colts exposed to the virus before the onset of peripubertal development (Holyoak *et al.* 1993). The mechanism by which testosterone contributes to the establishment and maintenance of the persistent EAV infection in stallions remains undetermined. It is speculated that testosterone may be involved in (i) the development of mature reproductive tract and (ii) the replication of androgen-dependent cells in the reproductive tract of stallions to maintain EAV persistence.

It remains to be determined whether other host factor(s) or viral factor(s) contribute to the establishment and maintenance of persistent EAV infection in stallions. Frequency of the carrier state varies between different groups of stallions; however, extensive epidemiological study involving Standardbred and Thoroughbred horses failed to demonstrate any breed predilection for establishment of persistent EAV infection (Timoney *et al.* 1986; 1987). Analysis of two carrier stallions and a number of their male offspring did not demonstrate a significant association between inherited MHC haplotype and the carrier state (Albright-Fraser 1998). Moreover, the mechanism accounting for the spontaneous clearance of EAV from some carrier stallions is not clear. So far, no fully validated therapy (except castration) has been available to eliminate the EAV carrier state

in stallions. One group of researchers have investigated the effects of a GnRH antagonist on testosterone secretion, spermatogenesis, and viral excretion in EAV carrier stallions, but the results were not conclusive (Fortier *et al.* 2002).

C. The significance of EAV carrier stallions

Carrier stallions occupy a significant niche in the epidemiology of EAV infection. Carrier stallions are not only a natural reservoir of EAV responsible for maintenance and dissemination of EAV in equine populations (Timoney *et al.* 1987; Timoney and McCollum 1993), but also a natural source of genetic and phenotypic diversity of EAV (Balasuriya *et al.* 2004a; Balasuriya and MacLachlan 2004c; Hedges *et al.* 1999a). Equine arteritis virus exists as a quasi-species. It has been observed that EAV quasispecies was relatively limited and the virus remained relatively genetically stable during horizontal and vertical transmission in the course of an outbreak of EVA (Balasuriya *et al.* 1999a); in contrast, virus quasi-species expanded and genetic and phenotypic variants of EAV emerged during persistent infection of stallions (Balasuriya *et al.* 2004a; Hedges *et al.* 1999a). Therefore, carrier stallions are probably the major source of EAV evolution. Some new virus variants generated during the course of persistent infection may have significant pathogenic potential and give rise to occurrences of EVA.

IV. PERSISTENT INFECTION OF CELL CULTURES WITH RNA VIRUSES

Viral infection of various hosts result in an acute infection, which is characterized by rapid production of infectious virus followed by rapid resolution and clearance of the infection by the host, or persistent infection, in which viral particles or viral products continue to be produced for long periods of time. The mechanisms employed by different viruses for their persistence *in vivo* vary. One common feature is that the host defense systems fail to clear the virus from the host. It is generally thought that both host and viral factors may contribute to the establishment and maintenance of persistent infection. Persistent viral infections in cell culture are valuable in studying virus-host cell interactions and in providing insights into how virus persistence is established and maintained. The following is an attempt to summarize persistent infection in cell culture by RNA viruses.

1. Properties of persistent infection

Many RNA viruses have been found capable of establishing persistent infection in tissue culture cells (Rima and Martin 1976). One virus may establish persistent infections in a number of cell lines, e.g. poliovirus is able to establish persistent infections in human neuroblastoma cells (Colbere-Garapin *et al.* 1989), human erythroblastoid K562 cells (Lloyd and Bovee 1993), Hep-2 cells (Calvez *et al.* 1993), and Hela cells. Persistent infection in Hela cells was established by cotransfecting the cells with poliovirion type 2 RNA and R1, an in vitro-synthesized poliovirus subgenomic replicon which contains a deletion of nearly all the capsid region (Kaplan *et al.* 1989). Sindbis virus is able to initiate persistent infections in BHK-21 cells (Schwobel and Ahl 1972; Weiss *et al.* 1980) and mouse L cells (Inglot *et al.* 1973). Persistently infected cell cultures may have common properties in some respects, as well as distinct properties in other respects.

A. Cytopathic effect, virus-positive cells, and production of virus

Persistently infected cells may or may not exhibit virus-specific cytopathic effect despite the fact that they continuously produce infectious virus. For example, CV-1 cells persistently infected with human parainfluenza virus-3 (Moscona and Galinski 1990) and rhabdomyosarcoma cells persistently infected with coxsackievirus B4 (Frisk *et al.* 1999) continuously produced infectious virus without cellular destruction; however, Hela cells persistently infected with human rhinovirus-2 and Hela cells persistently infected with poliovirus continually produced low levels of infectious virus and cell cultures underwent multiple episodes of partial destruction and subsequent recovery (Gercel *et al.* 1985; Kaplan *et al.* 1989). The percentage of virus-positive cells varies from one persistent system to another. In some persistent systems, the percentage of virus-positive cells even undergoes cyclic variation. For instance, in rabies virus/BHK21 cells, the percentage of cells showing positive immunofluorescence varied from 5% to 100% in cycles of 6-8 subcultures (Wiktor and Clark 1972); in human rhinovirus-2/Hela cells, the percentage of cells producing infectious centers varied from 0.03% to 23% (Gercel *et al.* 1985). Most of the persistently infected cells continuously produce small amounts of infectious virus. In measles virus/Hela cells, the yield of cell-associated virus was high and similar to that in lytic infections, whereas the yield of released (extracellular) virus was very small (Rustigian 1966a). In mumps virus/conjunctiva cells, the yield of cell-associated virus

was consistently about one log unit lower than that of released virus (Walker and Hinze 1962). The amount of released virus may show cyclic variation coinciding with variation in the appearance of CPE and cell destruction in some persistent systems such as Sindbis virus/BHK-21 cells (Schwobel and Ahl 1972), Sindbis virus/mouse L cells (Inglot *et al.* 1973), lymphocytic choriomeningitis virus (LCMV)/BHK-21 cells (Staneck *et al.* 1972), rabies virus/BHK-21 cells (Kawai *et al.* 1975; Wiktor and Clark 1972) and so on. Occasionally persistently infected cells may stop producing infectious virus after long-term subculturing. For example, during routine subcultivation of Hela cells persistently infected with human rhinovirus-2, some spontaneous cures were observed (Gercel *et al.* 1985). In spontaneously cured cells, no extracellular virus or infectious centers could be detected and no viral antigen could be detected by immunofluorescence (Gercel *et al.* 1985). Whether these cells were free of viral RNA was not reported.

B. Effect of antibodies and anti-viral compounds

Addition of virus-specific antibodies or anti-viral compounds to the media of persistently infected cells may result in distinct outcomes: non-cured or cured. It has been shown that cloning or subculture of measles virus/Hela cells in the presence of anti-measles serum in the culture medium led to a state in which no infectious virus was released (non-yielder state), whereas the large majority of these cells were still immunofluorescence positive (Rustigian 1966b), indicating the existence of viral antigen. When human influenza C virus/MDCK cells (Goshima and Maeno 1989) or poliovirus/human erythroblastoid K562 cells (Lloyd and Bovee 1993) were subcultured with growth medium containing virus-neutralizing antibodies for several continuous passages, extracellular virus became undetectable. However, further passage in the absence of antiserum resulted in the return of infectious virus yield. Similarly, addition of the antiviral compound disoxaril did not cure rhabdomyosarcoma cells persistently infected with coxsackievirus B4 (Frisk *et al.* 1999). In contrast, it was observed that three serial subcultivations of human rhinovirus-2/Hela cells in the presence of antiserum resulted in a cure (no extracellular virus or infectious centers or viral antigens could be detected) (Gercel *et al.* 1985). In addition, after removal of antiserum, no renewed virus production or viral antigen expression could be detected upon 12 further passages (Gercel *et al.* 1985), indicating that the persistently infected cells were cured. Similarly, it has

been reported that mouse L cells persistently infected with reovirus can be cured by antireovirus serum (Ahmed *et al.* 1981). It was also found that anti-viral compound ribavirin was able to eliminate foot-and-mouth disease virus (FMDV) from persistently infected BHK-21 cells (de la Torre *et al.* 1987). In ribavirin-cured cultures, no FMDV RNA or antigens were detected by dot-blot hybridization to cDNA probes or by indirect immunofluorescence, respectively. No renewed FMDV production occurred after the cells underwent at least 20 serial passages in the absence of ribavirin (de la Torre *et al.* 1987). Curing of FMDV from persistently infected cells by ribavirin involves ribavirin's ability to enhance mutagenesis (Airaksinen *et al.* 2003). RNA viruses replicate with a high spontaneous mutation frequency which is near the error threshold for maintenance of genetic information. When the mutation rate of the viral genome increases and exceeds this error threshold, the viral fitness decreases significantly and the virus may fall into error catastrophe or lethal mutagenesis leading to virus extinction (Castro *et al.* 2005; Domingo *et al.* 2005; Manrubia *et al.* 2005). Ribavirin is an RNA virus mutagen which can increase mutation frequency during virus replication and increase in mutation frequency may drive RNA viruses to error catastrophe leading to extinction of viruses (Crotty *et al.* 2000; 2001; 2002; Pariente *et al.* 2003).

C. Resistance to superinfection

Another interesting property of persistently infected cells is that they are immune to superinfection with homologous virus but are still susceptible to infection with heterologous viruses. In an investigation of human rhinovirus-2 (HRV-2)/Hela cells to superinfection, normal Hela cells infected with homologous viruses (HRV-2, HRV-2 TS-1 mutant, and HRV-4) or heterologous viruses (poliovirus type 2 and vesicular stomatitis virus (VSV)), HRV-2/Hela cells superinfected with the same homologous or heterologous viruses, and HRV-2/Hela cells not superinfected with any virus were compared (Gercel *et al.* 1985). It was found that the cytopathic effects and virus yields in HRV-2/Hela cells superinfected with HRV-2, TS-1 mutant, and HRV-4 were similar to those produced in HRV-2/Hela cells alone (not superinfected) but were less pronounced than those produced in normal Hela cells infected with the respective virus. In contrast, virus yields in HRV-2/Hela cells superinfected with poliovirus type 2 or VSV were comparable to those obtained in normal Hela cells infected with respective virus (Gercel

et al. 1985). Both yielder (with production of infectious virus) and non-yielder (with no production of infectious virus but with existence of viral antigen) measles virus/Hela cells were shown to be resistant to superinfection with measles virus, but these cells supported normal growth of vaccinia virus, herpes virus and poliovirus (Rustigian 1966a). Similarly, Sindbis virus/mouse L cells were found to be resistant to superinfection with Sindbis virus but not with VSV or encephalomyocarditis virus (Inglot *et al.* 1973). LCMV/BHK-21 cells were resistant to superinfection with homologous arenaviruses but not with dengue virus, rubella virus, Sindbis virus, and VSV (Staneck *et al.* 1972). Rabies virus/BHK-21 cells were resistant to superinfection with rabies virus but not with VSV (Kawai *et al.* 1975). FMDV/BHK-21 cells were resistant to superinfection with FMDV but not with encephalomyocarditis virus, VSV, and Semliki Forest virus (de la Torre *et al.* 1985). Poliovirus/human neuroblastoma IMR-32 cells were resistant to superinfection with Sabin 1, 2, and 3 poliovirus but sensitive to another enterovirus coxsackievirus B3 (Colbere-Garapin *et al.* 1989). Above examples uniformly demonstrated that all persistently infected cells are resistant to superinfection with the same virus that was used to initiate the persistent infection. The mechanism by which persistently infected cells are resistant to superinfection with homologous viruses but are sensitive to heterologous virus infection is not clear. It is speculated that it may be associated with virus-specific cell receptors or the production level of interferon: i) interference at one of the early steps of virus growth cycle (e.g. attachment, penetration, or uncoating) is probably involved in the resistance of persistently infected cells to superinfection by the homologous virus; ii) in contrast, cell receptors of 'heterologous' viruses are distinctly different from that of homologous virus, thus there are no such interferences with heterologous virus infection; lack or low levels of interferon produced in these persistently infected cells may also contribute to the growth of heterologous virus in carrier cultures.

D. Evolution of viruses and cells

A number of studies have shown that the viruses recovered from persistently infected cells are genetically and phenotypically different from the parental viruses which were used to establish the persistent infections. Temperature-sensitive (ts) virus mutants were isolated from mouse L cells persistently infected with Newcastle disease virus (Preble and Youngner 1972, 1973a, 1973b) and mouse L cells persistently infected with

VSV (Youngner *et al.* 1976). The production of defective interfering (DI) viral particles was reported in mouse L cells persistently infected with LCMV (Lehmann-Grube *et al.* 1969; Welsh *et al.* 1972) and BHK-21 cells persistently infected with Parana virus (Staneck and Peau 1974). Defective interfering viral RNA has also been detected in Vero cells persistently infected with Murray Valley encephalitis (MVE) virus (Lancaster *et al.* 1998). Small plaque virus mutants were observed in BHK-21 cells persistently infected with rabies virus (Kawai *et al.* 1975) and in mouse neuroblastoma cells persistently infected with yellow fever 17D virus (Vlaycheva and Chambers 2002). Fusion-defective mutants of mouse hepatitis virus A59 were produced in persistently infected primary mouse glial cells and the phenotype was probably due to a mutation at the viral spike (fusion) protein cleavage signal (Gombold *et al.* 1993). Genetic and antigenic changes have been demonstrated in hepatitis A virus (HAV) variants arising during persistent infection of BS-C-1 cells (Lemon *et al.* 1991). FMDV is able to initiate persistent infection in BHK-21 cells (de la Torre *et al.* 1985) and the virus isolated from persistently infected culture (carrier culture) was more cytolytic than parental FMDV for BHK-21 cells (de la Torre *et al.* 1988a). Further studies showed that nine amino acid substitutions were fixed on the viral capsid protein during persistence and these amino acid substitutions resulted in altered antigenicity, as revealed by reactivity with monoclonal antibodies (Diez *et al.* 1990a). Moreover, FMDV became progressively less virulent for mice and cattle during the course of persistence in BHK-21 cells (Diez *et al.* 1990b). Virus evolution was also observed in carrier cultures of poliovirus. The poliovirus mutants selected from human neuroblastoma IMR-32 cells persistently infected with the Sabin strains could reach 1-3 $\log_{10}$ units higher titer in IMR-32 cells than that in non-neural Hep-2 cells, while parental viruses had similar titers in both cell lines (Colbere-Garapin *et al.* 1989). Correlated with this modified cell tropism, the poliovirus mutants recovered from carrier culture could establish secondary persistent infection in non-neural Hep-2 cells (Pelletier *et al.* 1991). Interestingly, these poliovirus mutants harbor major mutations at capsid proteins VP1 and VP2 which are known to be involved in interactions between poliovirus and its receptor (Borzakian *et al.* 1993; Calvez *et al.* 1993; Colbere-Garapin *et al.* 1989; Duncan *et al.* 1998; Pavio *et al.* 1996; Pelletier *et al.* 1991; 1998a). Poliovirus type 1 mutants selected from human neuroblastoma cells persistently

infected with the wild-type Mahoney strain exhibited neurovirulent phenotype in mice, while the wild-type Mahoney strain is avirulent in mice (Couderc *et al.* 1994). And this conversion of mouse-neurovirulent phenotype is caused by several amino acid substitutions on the viral capsid protein (Couderc *et al.* 1993; 1994).

Coevolution of viruses and the host cells have been observed in mouse L cells persistently infected with reovirus (Ahmed *et al.* 1981), BHK-21 cells persistently infected with FMDV (de la Torre *et al.* 1988a), and Hela cells and human neuroblastoma cells persistently infected with poliovirus (Kaplan *et al.* 1989; Pavio *et al.* 2000). In the study performed by Ahmed *et al* (1981), persistent infection was established in mouse L cells with a reovirus stock, more than 90% of which was made up of defective interfering particles. Mutant mouse L cells and reovirus were isolated from carrier cultures. Reovirus isolated from carrier culture grew more efficiently than cloned wild-type reovirus in mutant L cells, indicating the evolution of viruses during persistent infection. On the other hand, infection of mutant L cells with either wild-type reovirus or reovirus isolated from carrier cultures led to establishment of persistent infections, while infection of the original L cells with either wild-type reovirus or reovirus isolated from carrier cultures resulted in a lytic infection with no surviving cells, demonstrating the evolution of the host cells during persistent infection. Virus evolution during persistent FMDV infection of BHK-21 cells has been discussed above. It has also been observed that BHK-21 cell mutants selected during serial passage of the carrier culture were constitutively more resistant to the parental FMDV infection than the parental BHK-21 cells (de la Torre *et al.* 1988a), indicating the evolution of the host cells during persistent infection. The increased resistance of BHK-21 cell mutants to parental FMDV was not due to an impairment of attachment, internalization, or uncoating of the viral particles but was because of some intracellular block that downregulated FMDV RNA synthesis (de la Torre *et al.* 1988a). Further studies showed that BHK-21 cells evolved and exhibited extensive heterogeneity during persistent infection with FMDV (de la Torre *et al.* 1989). Analysis of 248 stable cell clones isolated from FMDV carrier culture revealed that at least six distinct cell phenotypes could be distinguished with regard to cell morphology, susceptibility to parental FMDV infection, and cell growth characteristics (de la Torre *et al.* 1989). These altered phenotypes were maintained as stable genetic traits. Genetic and

phenotypic variation of the host cells enables the selection of cells with increased resistance to FMDV and thus contributes to the maintenance of viral persistence (Martin Hernandez *et al.* 1994). During persistent poliovirus infection in Hep-2 cells, the evolution and heterogeneity of the host cells have also been reported (Borzakian *et al.* 1992). Cell evolution was also observed in other persistent poliovirus infections. After passage of poliovirus/Hela cells for 6 months, a stable cell line termed SOFIA emerged that no longer produced infectious virus and did not contain viral proteins or viral RNA (Kaplan *et al.* 1989). In SOFIA cells, the poliovirus receptor (PVR) gene did not contain gross alteration at DNA level; however, the transcription of PVR-specific RNA was reduced. Therefore, it was suggested that resistance of SOFIA cells to poliovirus infection appears to result from down-regulation of PVR RNA which leads to lack of PVR protein expression at the cell surface (Kaplan and Racaniello 1991). During persistent poliovirus infection in human neuroblastoma IMR-32 cells, cells expressing mutated poliovirus receptor emerged and the mutations at the N-terminal domain of PVR increased the resistance of cells to poliovirus-induced lysis (Pavio *et al.* 2000). Moreover, when the wild-type PVR and mutated PVR were expressed independently in murine LM cells which lack the poliovirus receptor gene, it was found that the level of poliovirus-induced apoptosis was lower in cells expressing mutated PVR which was selected during persistent poliovirus infection in IMR-32 cells than in cells expressing the wild-type receptor (Gosselin *et al.* 2003).

2. Virus-specific factors involved in persistent infection

Various viral factors could be involved in the establishment and maintenance of persistent infection, e.g. temperature-sensitive (ts) viral mutants, defective interfering (DI) viral particles, alteration of viral RNA or protein synthesis, and so on.

A few studies have shown that viruses released from persistently infected cells contain ts mutants (Preble and Youngner 1972, 1973a, 1973b; Youngner *et al.* 1976). However, there has been no convincing evidence to demonstrate the role of ts mutants in the establishment or maintenance of persistent infection.

The possible involvement of defective interfering viral particles in some persistent infections is based upon: i) the presence of DI particles in the viral preparation used to initiate persistent infection; or ii) the presence of DI particles in the medium of

persistently infected cells. A number of viruses are usually highly cytolytic in permissive cells and within a few days after infection all of the cells die. However, when infected with the viral preparation containing DI particles, cells can survive the infection and eventually became persistently infected. In 1970s, it was reported that noncytocidal persistent infection of BHK-21 cells (Holland and Villarreal 1974) or mouse L cells (Youngner *et al.* 1976) with wild-type VSV particles could be established only in the presence of large number of DI particles. During persistent infection, wild-type and DI VSV particles were continuously produced accompanied with viral protein changes and RNA sequence alterations (Holland and Villarreal 1974; Holland *et al.* 1976; 1979; Rowlands *et al.* 1980; Semler and Holland 1979; Villarreal and Holland 1976). Similarly, in the presence of their respective DI particles, wild-type reovirus, Sindbis virus, and Ebola virus establish persistent infections in mouse L cells, BHK-21 cells, and Vero cells, respectively (Ahmed *et al.* 1981; Calain *et al.* 1999; Weiss *et al.* 1980). Interestingly, when Hela cells were cotransfected with poliovirus type 2 RNA plus R1, an *in vitro*-synthesized poliovirus subgenomic replicon which contains a deletion of nearly all the capsid region, persistent infection was established, whereas Hela cells transfected only with poliovirion RNA all died (Kaplan *et al.* 1989). The detailed mechanism by which DI particles change a lytic infection to a persistent infection is unclear. DI genomes are truncated and their replication depends on the replicative enzymes encoded by the helper virus. Since DI genomes are usually much smaller than the helper virus genome, they are replicated more efficiently. Because of competition of DI RNAs, synthesis of the helper virus genome is decreased. It is speculated that the decrease of the helper virus genome may result in downregulation of determinants of cell killing which have yet to be identified.

The foregoing discussion of virus evolution during persistent infection would suggest that viral factors play an important role in establishment and maintenance of persistent infection. The following are several additional examples supporting the hypothesis that alteration of viral gene products is involved in persistent infections. It was observed that persistence of Japanese encephalitis virus (JEV) in several cell lines (murine neuroblastoma N18 cells, Vero cells, and murine astrocytoma DBT cells) was associated with expression of truncated viral nonstructural protein 1 (NS1) in host cells

(Chen *et al.* 1996). Sindbis virus is highly cytopathic in BHK-21 cells and establishment of persistent infection with wild-type Sindbis virus needed the presence of high concentration of DI particles (Weiss *et al.* 1980). However, Sindbis virus mutants (free of DI particles) isolated from the carrier culture were able to establish secondary persistent infection in BHK-21 cells. Through reverse genetics studies, it was found that a single coding mutation in the nsP2 gene (a predicted change of Pro-726? Ser) was responsible for the ability of mutated virus to establish persistent infection in BHK-21 cells (Dryga *et al.* 1997). It has been suggested that persistence of VSV mutants on fibroblast cells is associated with shutoff levels of cellular gene expression which may be determined by the VSV matrix protein (Desforges *et al.* 2001). Poliovirus mutants selected in the persistently infected human neuroblastoma cells initiated by either the Sabin 1 strain or the type 3 wild-type Leon strain can establish secondary persistent infection in non-neural Hep-2 cells, while both the Sabin 1 strain and the type 3 wild-type Leon strain cause a lytic infection in Hep-2 cells (Duncan *et al.* 1998; Pelletier *et al.* 1991). Further studies have shown that several amino acid residues in capsid proteins VP1 and VP2 are the viral determinants which control whether the outcome of an infection in Hep-2 cells is a lytic infection or a persistent infection (Borzakian *et al.* 1993; Calvez *et al.* 1993; Duncan *et al.* 1998; Pelletier *et al.* 1998a). It was proposed (Duncan *et al.* 1998) that these viral determinants may affect the early steps of the virus life cycle such as virus attachment and the receptor-mediated conformational changes which are believed to be necessary for viral penetration and uncoating. Modification of these steps could be the mechanism by which poliovirus mutants are able to establish persistent infection in Hep-2 cells. It has also been reported that a single amino acid change (Phe-260? Leu) in viral glycoprotein markedly increased the capacity of lymphocytic choriomeningitis virus to persist in adult mice (Matloubian *et al.* 1990).

3. Host-specific factors involved in persistent infection

Various host factors such as host cell type, host cell mutants, host cell differentiation stage, production level of interferon and so on may be involved in the establishment and maintenance of persistent viral infection.

Many viruses show marked differences in interaction with various types of host cells. For example, Newcastle disease virus (NDV) infection in BHK-21 cells led to large

amounts of fusion of the cells with production of infectious virus; in NDV-infected Hep-2 cells, less cell fusion was observed; while in NDV-inoculated mouse L cells, no apparent cell fusion was observed and a persistent infection was established (Hecht and Summers 1974; Poste *et al.* 1972). Mouse cells are not susceptible to echovirus 1 (EV-1) infection since they lack the viral receptor, human VLA-2. By transforming mouse L cells and 3T3 cells with cDNAs of human VLA-2, stable mouse L cell and 3T3 cell transformants that express human VLA-2 were obtained. Upon infection with EV-1, cytopathic effect developed on mouse L cell transformants but not on 3T3 cell transformants and a persistent infection was established on 3T3 cell transformants (Zhang and Racaniello 1997). The study also revealed that receptor down-regulation is not involved in the establishment of persistent infection (Zhang and Racaniello 1997).

The contribution of host cell mutants to the maintenance of persistent infection has been addressed in discussion of cell evolution during persistent infection.

Effects of the state of cell differentiation on viral infection have been documented. Human erythroleukemia K562 cells can differentiate into several cell lineages. Four cell strains were analyzed for their responses to poliovirus infection (Benton *et al.* 1995). Poliovirus readily establishes persistent infections in K562-Mu cells and K562-ATCC cells, while the majority of K562-KI cells and K562-We cells were killed after poliovirus infection (Benton *et al.* 1995). Surface poliovirus receptor levels are similar in all K562 cell strains and there are no significant differences in viral RNA and protein synthesis between these cell strains. It was proposed that some host factors related to virus-mediated cytopathology may be responsible for the observed phenomenon (Benton *et al.* 1995). Further studies showed that after hemin-induced differentiation, poliovirus infection of K562-Mu cells became cytolytic rather than leading to persistent infection (Benton *et al.* 1996), demonstrating the influence of cell differentiation on the outcome of virus infection. It is speculated that hemin treatment alters the expression of specific host proteins in K562-Mu cells, which changes their response to poliovirus infection from persistent to lytic (Benton *et al.* 1996). In addition, it has been observed that upon poliovirus infection, well differentiated human blood cell lines develop CPE more rapidly than less differentiated cell lines (Okada *et al.* 1987).

In some persistent infections, such as Newcastle disease virus/mouse L cells (Thacore and Youngner 1969) and human influenza C virus/MDCK cells (Goshima and Maeno 1989), low levels of interferon were detected. However it is unclear whether interferon plays a role in the maintenance of persistent infection in these cells. In most persistent viral infections, such as measles virus/Hela cells (Rustigian 1966a), mumps virus/conjunctiva cells (Walker and Hinze 1962), human rhinovirus-2/Hela cells (Gercel *et al.* 1985), and poliovirus/human neuroblastoma IMR-32 cells (Colbere-Garapin *et al.* 1989), interferon was below detectable levels. It would appear therefore, that interferon does not play a significant role in establishment and maintenance of persistent infection.

V. RESEARCH OBJECTIVES OF THIS DISSERTATION

As discussed previously, neither a virus attachment molecule nor a specific cell receptor has been identified for EAV. The early steps (e.g. attachment, penetration, and uncoating) of the EAV infection cycle have not been elucidated. **A major goal** of this study is to identify and characterize the cell receptor(s) involved in EAV attachment to and entry into cells. However, identification of the cellular receptor(s) used by EAV for attachment and entry has been hampered by the unavailability of a cell line that lacks the appropriate EAV receptor(s). Identification of EAV non-permissive cell lines in which EAV infection is restricted at different steps in its replication and comparison of these non-permissive cell lines with permissive cell lines should help to identify the cellular factor(s) that are involved in EAV infection. **The specific objective** of this research is to evaluate a variety of cell lines for their permissiveness to EAV infection and then identify the mechanism that restricts EAV infection. To achieve this objective, two hypotheses and aims are proposed.

Hypothesis 1: Within selected cell lines (RK-13, BHK-21, C2C12, Hela, Hep-2, and L-M), some cell lines are permissive to VBS53 EAV infection and some cell lines are non-permissive to VBS53 EAV infection.

Aim 1: to test the permissiveness of various cell lines to EAV infection by one-step growth of the virus in these cell lines as well as by indirect immunofluorescence assay using EAV-specific monoclonal antibodies.

Hypothesis 2: In non-permissive cell lines, EAV infection is restricted at certain step(s) of the virus life cycle.

Aim 2: to determine whether restriction of EAV infection in non-permissive cell lines is due to a block at the virus attachment step, a block at the cell entry step, or an intracellular block.

After EAV infection, up to 30% to 70% of stallions can subsequently become persistently infected. The detailed mechanism by which EAV establishes persistent infection in stallions is unknown. Carrier stallions are not only a natural reservoir of EAV responsible for maintenance and dissemination of EAV in equine populations, but also a natural source of genetic and phenotypic diversity of EAV. Persistent viral infections in cell culture have been demonstrated as a valuable tool to study virus-host cell interactions, virus and host cell evolutions, and to elucidate the mechanisms of viral persistence. In fact, genetic and phenotypic characterization of virus mutants and host cell mutants during persistent infection has already greatly helped study virus-receptor interactions, define virus virulence gene, and identify viral and host cell determinants involved in persistent viral infection. **The second major goal** of this study is to establish persistent EAV infection in cell cultures and then use the persistently infected cultures as a tool to study virus-host cell interactions, virus and host cell evolutions, and to identify viral and host cell determinants involved in EAV persistence. **Specific hypotheses and objectives** relating to this goal are as follows.

Hypothesis 1: Persistent EAV infection can be established in certain cell lines.

Aim 1: to test a variety of cell lines and determine in which cell lines persistent EAV infection can be established.

Hypothesis 2: During persistent infection of cell cultures, EAV undergoes genetic and phenotypic evolution; genetic variations result in phenotypic changes.

Aim 2: to investigate genetic and phenotypic (such as growth characteristics, neutralization phenotypes, and pathogenicity) variations of the viruses recovered from different passages of the persistently infected culture; to define genetic variations that are responsible for corresponding phenotypic changes.

Table 1.1. The order of *Nidovirales*

Order	*Nidovirales*				
Family	*Arteriviridae*	*Coronaviridae*			*Roniviridae*
Genus	*Arterivirus*	*Coronavirus*		*Torovirus*	*Okavirus*
Species	Equine arteritis virus (EAV)	Canine coronavirus (CCV)		Equine torovirus (EqTV)	Gill-associated virus (GAV)
	Porcine respiratory and reproductive syndrome virus (PRRSV)	Feline coronavirus (FCoV)		Bovine torovirus (BoTV)	Yellow head virus (YHV)
	Lactate dehydrogenase-elevating virus (LDV)	Human coronavirus 229E (HCoV-229E)	Group 1 species	Human torovirus (HuTV)	
	Simian hemorrhagic fever virus (SHFV)	Porcine epidemic diarrhea virus (PEDV)		Porcine torovirus (PoTV)	
		Transmissible gastroenteritis virus (TGEV)			
		Bovine coronavirus (BCoV)			
		Human coronavirus OC43 (HCoV-OC43)			
		Mouse hepatitis virus (MHV)	Group 2 species		
		Porcine hemagglutinating encephalomyelitis virus (HEV)			
		Rat coronavirus (RCV)			
		Infectious bronchitis virus (IBV)	Group 3 species		
		Turkey coronavirus (TCoV)			
		Severe acute respiratory syndrome coronavirus (SARS-CoV)	Not grouped		

Table 1.2. The ORFs of the EAV genome

Leader or ORFs (nt location)[*]	Protein (aa length[†] and molecular weight[§] kDa)	Putative functional domain[‡]
5' NTR (1-224)	N/A	
5' Leader (1-211)	N/A	
	Nonstructural proteins (nsp)	
ORF1a (225-5408)	**1a polyprotein (1727 aa; 187 kDa)**	
ORF1ab (225-5405, 5405-9751)	**1ab polyprotein (3175 aa; 345 kDa)**	
	nsp1: Met1-Gly260 (260 aa; 29 kDa)	Zinc finger, PCP1ß
	nsp2: Gly261-Gly831 (571 aa; 61 kDa)	CP2
	nsp3: Gly832-Glu1064 (233 aa)	HD
	nsp4: Gly1065-Glu1268 (204 aa)	3CLSP
	nsp5: Ser1269-Glu1430 (162 aa)	HD
	nsp6: Gly1431-Glu1452 (22 aa)	?
	nsp7: Ser1453-Glu1677 (225 aa)	?
	nsp8: Gly1678-Asn1727 (50 aa)	?
	nsp 9: Gly1678-Glu2370 (693 aa; 80 kDa)	RdRp
	nsp10: Ser2371-Gln2837 (467 aa; 50 kDa)	MB, NTPase, HEL
	nsp11: Ser2838-Glu3056 (219 aa; 26 kDa)	?
	nsp12: Gly3057-Val3175 (119 aa; 12 kDa)	?
	Structural proteins	
ORF2a (9751-9954)	E (67 aa; 8 kDa)	intermediate envelope protein
ORF2b (9824-10507)	GP2b (227 aa; 25 kDa)	minor envelope protein; 1 N-glycosylation
ORF3 (10306-10797)	GP3 (163 aa; 36-42 kDa)	minor envelope protein; 6 N-glycosylation
ORF4 (10700-11158)	GP4 (152 aa; 28 kDa)	minor envelope protein; 3 N-glycosylation
ORF5 (11146-11913)	GP5 (255 aa; 30-42 kDa)	major envelope protein; 1 N glycosylation; expressing neutralization determinants
ORF6 (11901-12389)	M (162 aa; 16 kDa)	major envelope protein
ORF7 (12313-12645)	N (110 aa; 14 kDa)	nucleocapsid protein; 3 phosphorylation
3' NTR (12646-12704)	N/A	

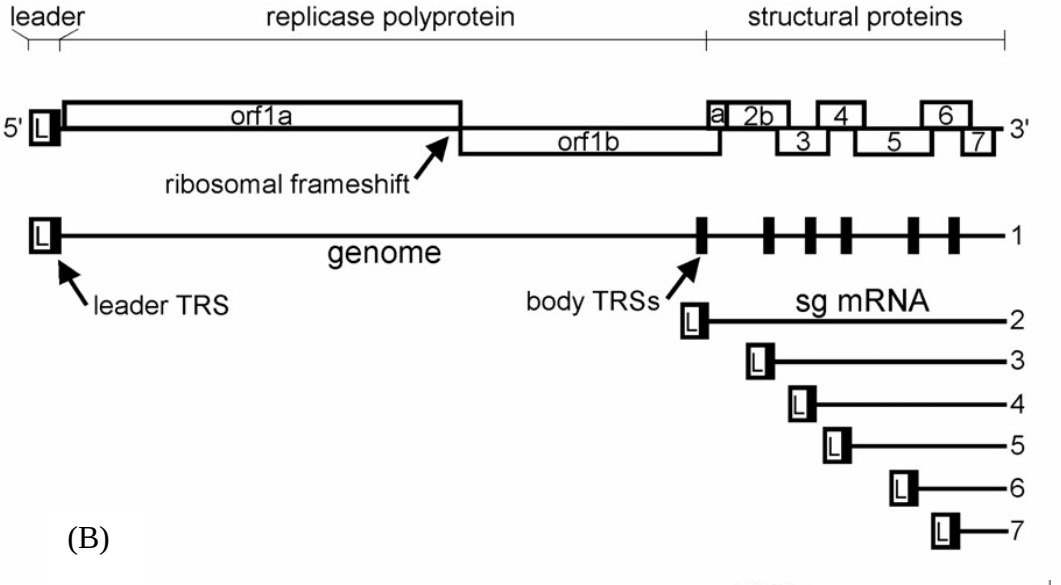
[*] Nucleotides are numbered according to the published sequence of EAV030 virus (van Dinten *et al.*, 1997).

[†] Amino acids of non-structural proteins are numbered according to their locations in polyprotein 1ab. Amino acids of structural proteins are numbered according to their locations in individual structural protein. N/A: Not Applicable.

[§] Molecular weight (kDa) of structural proteins shown here was the size after modification, e.g. glycosylation.

[‡] PCP1ß: papain-like cysteine proteinase 1ß; CP2: cysteine proteinase of nsp2; HD: hydrophobic domain; 3CLSP: 3C-like serine proteinase; RdRp: RNA-dependent RNA polymerase; MB: metal ion-binding domain; NTPase: NTPase domain; HEL: helicase domain; ?: function unknown.

(A)



(B)

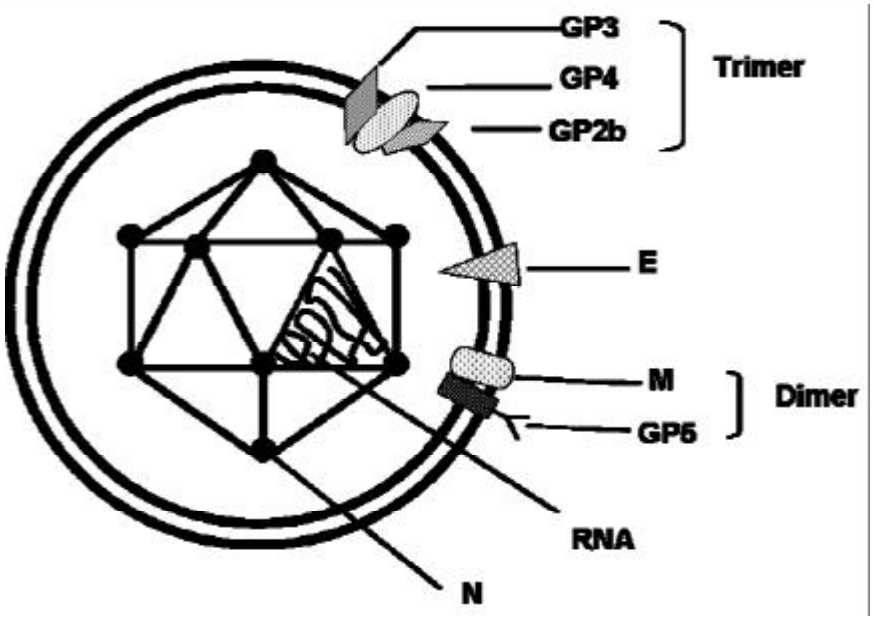


Fig. 1.1. (A) Schematic diagram of the genome organization and expression of EAV. The regions of the genome specifying the leader (L) sequence, the replicase gene (ORFs 1a and 1b), and the structural protein genes are indicated. The nested set of EAV mRNAs (genome and sg mRNAs 2 to 7) is depicted below. The black boxes in the genomic RNA indicate the position of leader and major body TRSs. The figure A is reprinted from van den Born *et al.* (2005). (B) Schematic representation of EAV virion, showing an icosahedral nucleocapsid and an envelope containing six viral membrane proteins which include a GP5-M heterodimer, an E protein, and a GP2b/GP3/GP4 heterotrimer. The E protein is proposed to be the component which, on the one hand interacts with the GP2b/GP4/GP3 heterotrimer, and on the other hand interacts with the GP5/M heterodimer and/or the nucleocaspid. It is through these interactions that the E protein draws the GP2b/GP4/GP3 complex into nascent virus particles (Wieringa *et al.* 2004).

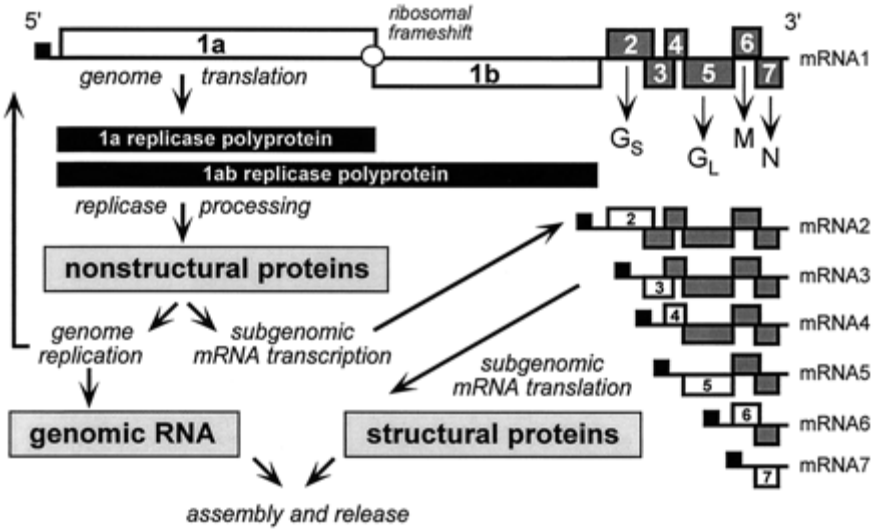


Fig 1.2. Overview of the replication cycle of EAV. The viral genome and the nested set of subgenomic mRNAs are indicated, with the small black boxes representing the common 5' leader sequence. The white boxes in sg mRNAs represent the translationally active ORF(s) for each mRNA. The figure is reprinted from Snijder *et al.* (1999).

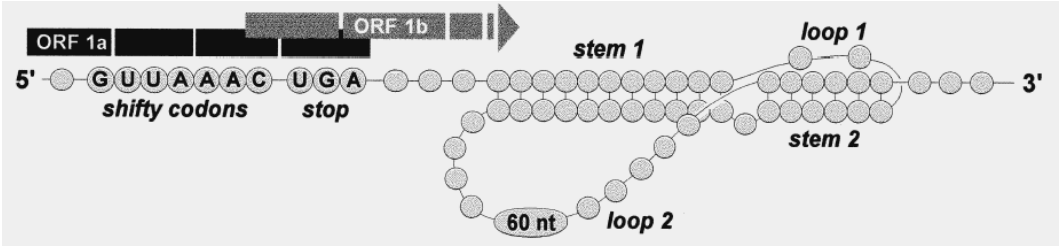


Fig 1.3. Schematic diagram of the EAV ORF1a/1b frameshift-directing signals: the 'shifty' codons (5' GUUAAAC 3') and RNA pseudoknot structure. The codons of the reading frames 1a including its termination codon and 1b are indicated. The figure is reprinted from Snijder and Meulenberg (1998) with permission.

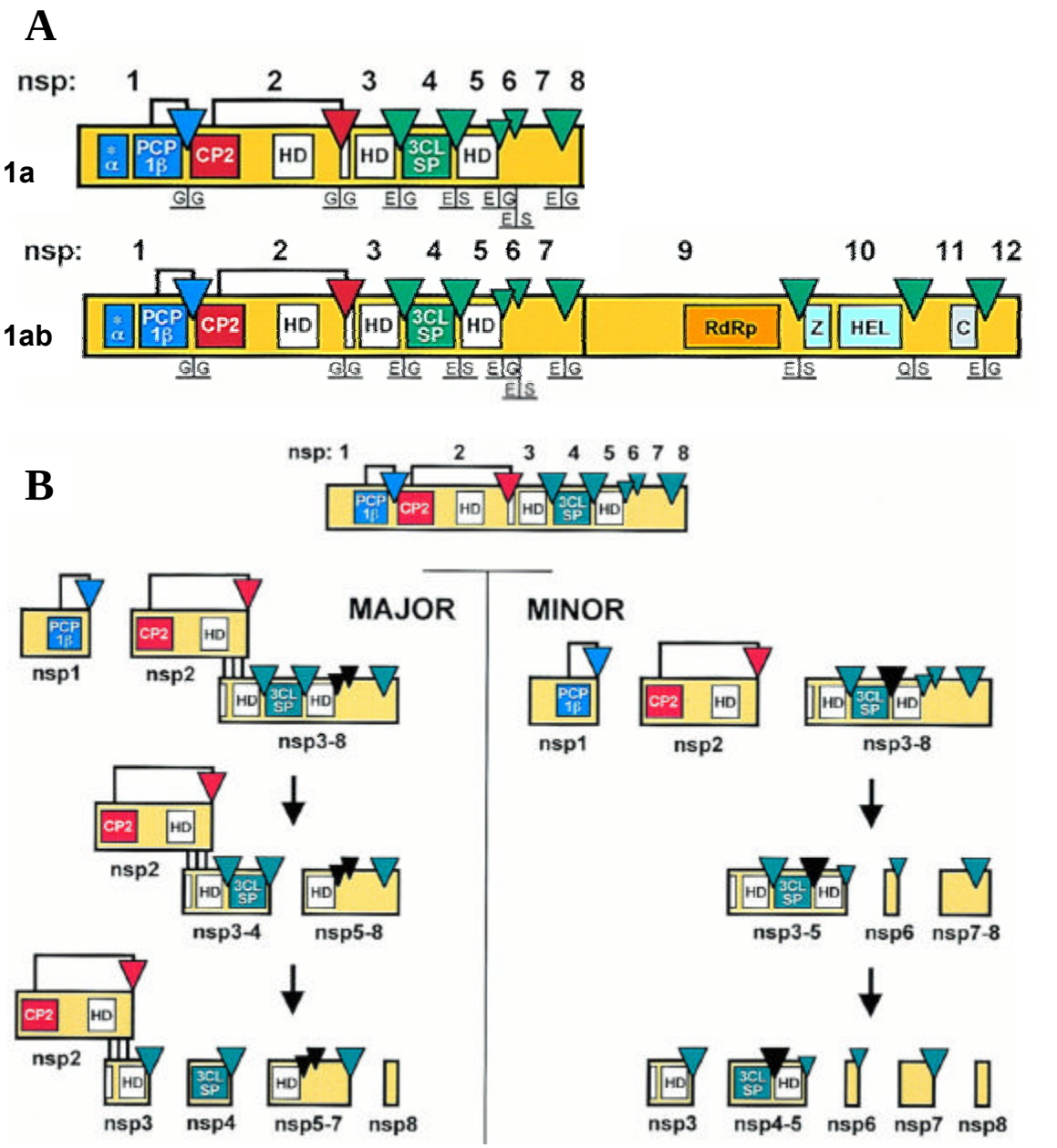


Fig 1.4. Overview of the proteolytic processing of the EAV replicase polyproteins. (A) The processing schemes for the EAV polyproteins 1a and 1ab are depicted. The three EAV proteases and their corresponding cleavage sites are shown: PCP1ß, nsp1 papain-like cysteine protease 1ß; CP2, nsp2 cysteine protease; 3CLSP, nsp4 3C-like serine protease; the a* domain represents the inactive PCP1a domain. Hydrophobic domains (HD) in nsp2, nsp3, and nsp5 are indicated. In the ORF1b-encoded polypeptide, the four major conserved domains are depicted: RdRp, RNA-dependent RNA polymerase; Z, putative zinc-binding domain; HEL, helicase domain; C, conserved, nidovirus-specific C-terminal domain. (B) Two alternative processing pathways of the EAV ORF1a protein. The association of cleaved nsp2 with nsp3-8 was shown to direct cleavage at the nsp4|5 site by the nsp4 3CLSP (major pathway). Alternatively, in the absence of nsp2, the nsp5|6 and 6|7 sites were cleaved and the nsp4|5 junction remained uncleaved. The diagrams are reprinted from Ziebuhr *et al.* (2000) with permission.

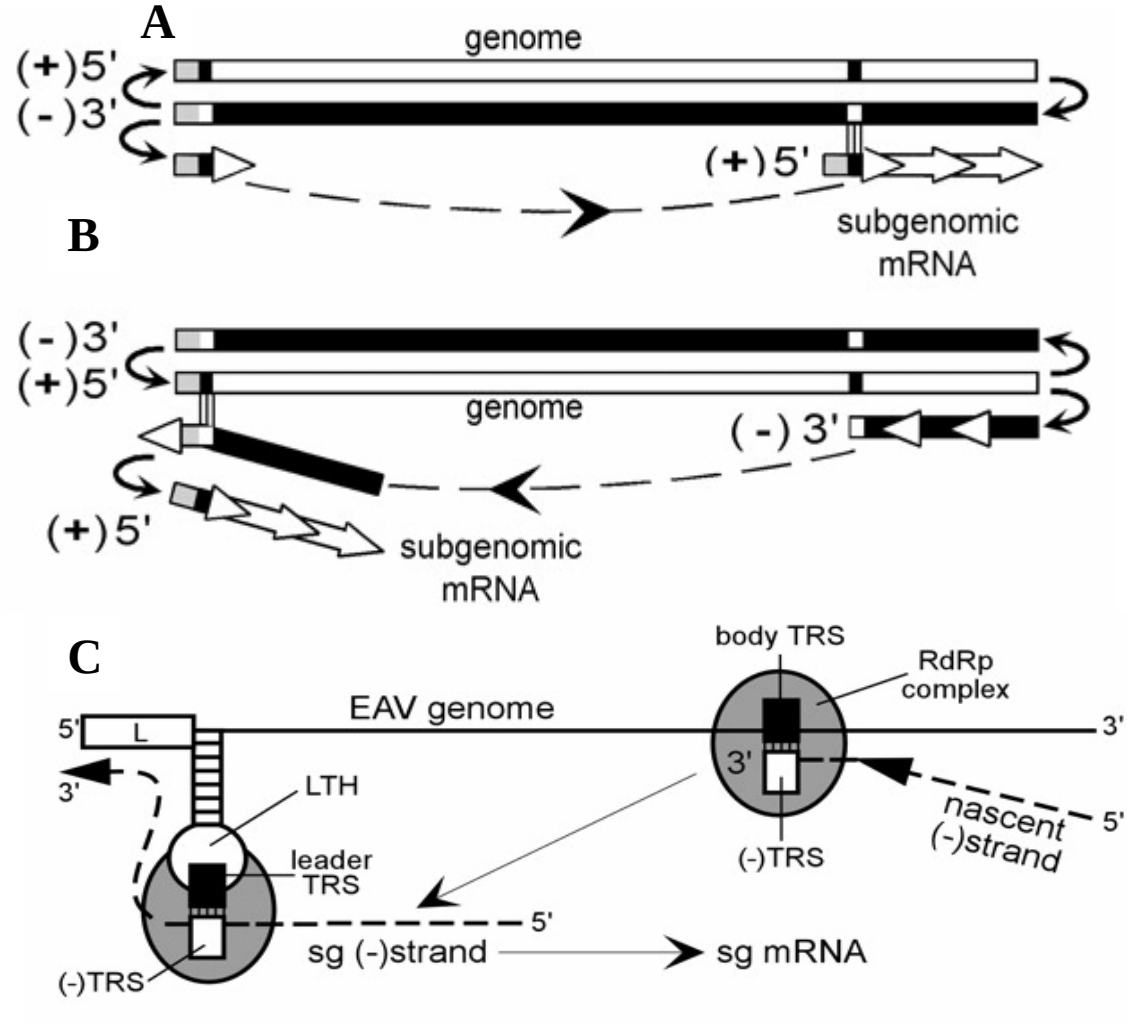


Fig 1.5. Transcription models illustrating the discontinuous step during nidoviral subgenomic mRNA synthesis. (A) 'Leader-primed transcription' model. Discontinuous transcription was proposed to occur during positive-strand RNA synthesis using the full-length negative genomic strand as a template. The sense TRS at the 3' end of the leader transcript base pairs with the antisense body TRS in the negative-strand template, after which the leader transcript is extended to produce a sg mRNA. (B, C) 'Discontinuous negative strand extension' model. In C, EAV is used as an example. Discontinuous transcription was proposed to occur during negative-strand RNA synthesis using the full-length positive genomic strand as a template. After attenuation of the RdRp complex at a body TRS in the positive-strand genomic template, the nascent (-) strand is translocated to the leader TRS region. Following base pairing between the antisense body TRS and sense leader TRS, negative strand RNA synthesis resumes to add the anti-leader sequence. Subsequently, the sg (-) strand RNA serves as a template for sg mRNA synthesis. The positive- (white) and negative- (black) strand RNAs are shown. The sense leader and body TRS (black boxes) in positive strand RNA and antisense leader and body TRS (white boxes) in negative strand RNA are indicated. The diagrams A and B are modified from Snijder and Meulenberg (1998) and the diagram C is reprinted from van den Born *et al.* (2005) with permission.

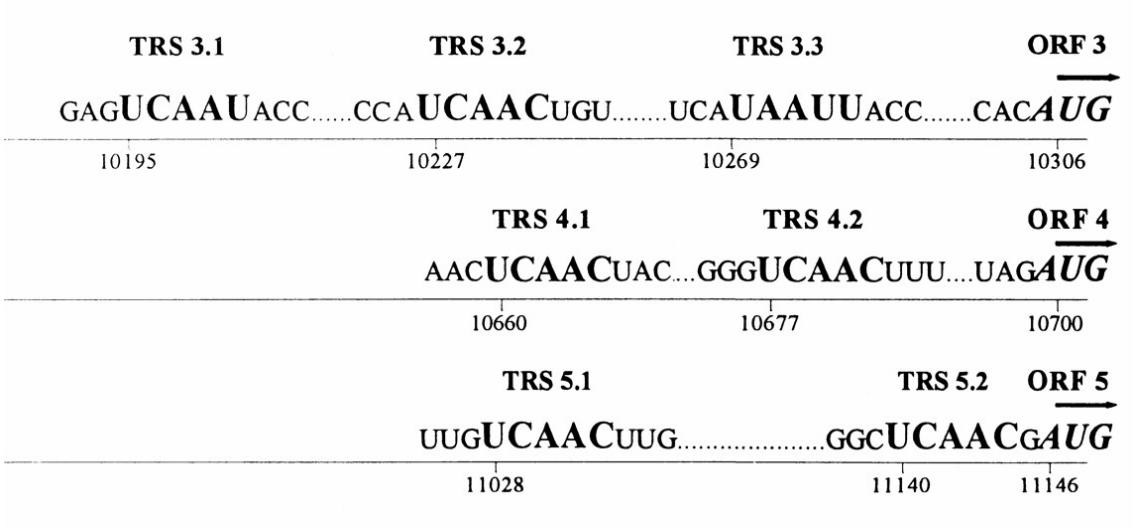


Fig 1.6. EAV sg mRNA3, -4, and -5 body TRS regions. The sequences are aligned with respect to the translation initiation for the corresponding structural protein. The body TRSs and translation initiation codon are shown in bold. The figure is reprinted from Pasternak *et al.* (2000).

Chapter Two

Determination of the Permissiveness of Various Cell Lines to Equine Arteritis Virus Infection and Demonstration That Equine Arteritis Virus Infection Is Restricted at the Entry Step in Non-Permissive Cell Lines

ABSTRACT

Neither the virus attachment molecule nor the specific cell receptor has been identified for EAV. The early cell-EAV interactions (e.g. attachment, penetration, and uncoating) have not been elucidated. Although several permissive cell lines have been identified which support productive EAV infection, it is unknown which cell lines are non-permissive to EAV infection and at which steps EAV infection is blocked in these cell lines. In this study, a variety of cell lines of different species and tissue origin were tested for their permissiveness to infection with EAV strain VBS53, and the mechanisms that restrict EAV infection in non-permissive cell lines were investigated. The cell lines baby hamster kidney (BHK-21; ATCC CCL-10), rabbit kidney (RK-13; KY & ATCC CCL-37) and mouse muscle (C2C12; ATCC CRL-1772) were found to support productive infection with EAV strain VBS53 as demonstrated by infectious virus yield and indirect immunofluorescence assay using EAV-specific monoclonal antibodies. On the other hand, human cervix (Hela; ATCC CCL-2), human epidermoid larynx (Hep-2; ATCC CCL-23), and mouse connective tissue (L-M; ATCC CCL-1.2) cell lines exhibited limited susceptibility to infection with EAV strain VBS53. Interestingly, Hela cells became more susceptible to the virus infection after extended serial passage. The respective cell lines were referred to as Hela 'High' (passage 170-221) and Hela 'Low' (passage 95-115). While Hela 'High' cell line was more susceptible than Hela 'Low' cell line, it was still considerably less susceptible than BHK-21 cell line to EAV infection. Karyotypic analysis of Hela 'High' and Hela 'Low' cell lines using G-banding technique revealed chromosomal differences. Transfection of Hela 'High', Hela 'Low', Hep-2, and L-M cell lines with viral RNA *in vitro*-transcribed from a full-length EAV cDNA clone induced virus replication, assembly, and release. Virus levels were comparable to those in transfected BHK-21 cells 24 h post transfection, indicating that these cells fully support EAV replication when the attachment and entry steps are bypassed. Thus, interference

with the attachment or entry steps might account for resistance of these cells to infection with EAV. Attachment of EAV strain VBS53 to the cells was determined by directly titrating the virus bound to the cells. It was found that EAV was able to bind to Hela 'High', Hela 'Low', Hep-2, and L-M cell lines almost as efficiently as it did to the fully permissive cell line BHK-21. Attachment of EAV strain VBS53 to each cell line was further confirmed using a fluorescence-activated cell sorter (FACS) assay in which binding of biotinylated EAV was detected with streptavidin-FITC. All of the cell lines under study bound EAV in a dose-dependent manner and nonbiotinylated EAV competitively inhibited binding, indicating that the binding was specific. Virus entry studies showed that the efficiency of EAV strain VBS53 entry into Hela 'High', Hela 'Low', Hep-2, and L-M cell lines was significantly lower than that of entry into BHK-21 cells. In addition, it was observed that the entry of EAV into Hela 'High' cell line was more efficient than virus entry into Hela 'Low', Hep-2, and L-M cell lines. In conclusion, we have identified several cell lines that exhibit varying susceptibility to infection with EAV strain VBS53 and have found that permissiveness of a cell line to EAV is determined at the entry step. Results of this study would suggest that interaction of EAV with its attachment receptor on the cell surface may be necessary, but of itself not sufficient to initiate a productive infection.

INTRODUCTION

Equine arteritis virus (EAV) is the prototype member of the family *Arteriviridae* which also contains porcine respiratory and reproductive syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (Snijder and Meulenberg 1998). The EAV genome is a single-stranded, positive-sense RNA molecule of 12.7 kb, which includes nine functional open reading frames (ORFs) (Snijder and Meulenberg 1998; Snijder *et al.* 1999). The replicase gene comprises the 5'-terminal three-quarters of the genome and includes two ORFs (1a and 1b). ORFs 1a and 1b are expressed directly from the genomic RNA and encode replicase proteins involved in viral replication and transcription (den Boon *et al.* 1991; 1995a; Snijder *et al.* 1992, 1994; 1995; 1996; van Dinten *et al.* 1996; 1999; Wassenaar *et al.* 1997). The 3' terminal one-quarter length of the genome is composed of seven ORFs (2a, 2b, and 3-7), which encode structural proteins E, GP2b (Gs), GP3, GP4, GP5 (G_L), M, and N, respectively (den Boon *et al.* 1991; Snijder and Meulenberg 1998; Snijder *et al.* 1999). These structural proteins are expressed from 5'- and 3'-coterminal nested set of six subgenomic mRNAs.

EAV virions consist of an icosahedral nucleocapsid which is surrounded by a lipid-containing envelope with tiny surface projections (Horzinek *et al.* 1971). The viral nucleocapsid is composed of the linear viral genomic RNA and a phosphorylated nucleocapsid (N) protein (de Vries *et al.* 1992; Hyllseth 1973; Zeegers *et al.* 1976). The virion envelope contains six envelope proteins. The two major envelope proteins include the nonglycosylated membrane protein (M) and the relatively large envelope glycoprotein GP5 (de Vries *et al.* 1992). The M and GP5 proteins occur as disulfide-linked heterodimers (de Vries *et al.* 1995b). The unglycosylated envelope protein E is of intermediate abundance in the virion (Snijder *et al.* 1999). The three minor envelope proteins of EAV are glycoproteins GP2b, GP3, and GP4, which occur as heterotrimers in the virion (de Vries *et al.* 1992; 1995a; Hedges *et al.* 1999b; Wieringa *et al.* 2002; 2003a).

The first step in virus infection is viral attachment to a specific receptor on a susceptible cell. In the case of EAV, neither the virus attachment molecule nor a specific cell receptor has yet been clearly identified. By analogy with many other animal RNA viruses and in view of its recognition by neutralizing antibodies, the EAV GP5 protein

had been postulated to serve as the virus attachment protein and to mediate receptor recognition. However, exchange of the ectodomain of the EAV GP5 protein with that of PRRSV or LDV in the context of an infectious EAV cDNA clone did not alter the cell tropism of the virus, suggesting that the ectodomain of GP5 is not the main determinant of EAV tropism in cell culture (Dobbe *et al.*, 2001). Similarly, PRRSV mutants in which the ectodomain of the M protein was replaced by that of EAV or LDV still retained their ability to infect porcine alveolar macrophages and did not acquire tropism for cells susceptible to the respective viruses (e.g. BHK-21 cells for EAV and mouse macrophages for LDV) from which the foreign ectodomains were derived (Verheije *et al.* 2002). This would suggest that, in the case of arteriviruses, the M protein is not responsible for receptor binding either. Recently, Wieringa *et al.* (2003a) have shown that minor structural proteins are present in EAV virions as disulfide-bonded GP2b/GP4/GP3 heterotrimeric complexes. Also, there are indications that the E protein is noncovalently associated with the GP2b/GP4/GP3 trimers (Wieringa *et al.* 2004). It has also been demonstrated that the E, GP2b, GP3, and GP4 proteins are not required for the formation of EAV particles, although these proteins are believed essential for ensuring that the virus particles are infectious (Molenkamp *et al.* 2000; Wieringa *et al.* 2004). These findings would suggest that the GP2b/GP4/GP3/(E) complex might be involved in the EAV attachment/entry process.

The cell receptor for EAV has not been identified either. Asagoe *et al.* (1997) showed that heparin can reduce EAV infection of RK-13 cells and that this inhibition was due to the direct interaction between heparin and EAV rather than the interaction between heparin and RK-13 cells. Furthermore, treatment of RK-13 cells with heparinase before virus inoculation decreased EAV infection of the cells (Asagoe *et al.* 1997). The data suggested that a heparin-like molecule on the surface of RK-13 cells might serve as cell receptor for EAV. However, heparinase treatment of cells could not completely block EAV infection and EAV infection could not be reduced below 13% even in the presence of a very high concentration of heparin (Asagoe *et al.* 1997), implying that other molecules on the cell surface might serve as EAV receptors as well.

Identification of the receptor used by EAV for cell attachment and entry has been hampered by the unavailability of a known cell line that lacks the appropriate EAV

receptor(s). Identification of EAV non-permissive cell lines in which EAV infection is restricted at different steps and comparison of these non-permissive cell lines with permissive cell lines should help to identify the cellular factor(s) that are involved in each step of EAV infection. In this study, a variety of cell lines of different species and tissue origin were assessed for their permissiveness to infection with the strain VBS53 of EAV and the mechanism that restricts EAV infection in the non-permissive cell lines was investigated.

MATERIALS AND METHODS

Cells and viruses. The origin and morphological description of the cell lines used in this study are listed in Table 2.1. The RK-13 (ATCC CCL37) cell line is routinely used in our laboratory between passage level 192 and 204. An additional RK-13 cell line, RK-13 (KY), is also available in the laboratory and it is routinely used between passage level 397 and 409. The RK-13 (ATCC CCL-37) and RK-13 (KY) cell lines are morphologically and karyotypically distinct. Hela cells were obtained from the American Type Culture Collection (ATCC CCL-2) at passage level 95. Since it was found that Hela cells had different susceptibilities to EAV strain VBS53 infection after extensive serial passage, it was decided to categorize Hela cells from passage 95 to 115 as Hela 'Low' cells and these are not susceptible to EAV strain VBS53 infection. On the other hand, Hela cells from passage 170 to 221 were referred to as Hela 'High' cells and these were found to be partly susceptible to EAV strain VBS53 infection. All the cell lines were grown in Eagle's minimum essential medium (EMEM) with 10% ferritin supplemented calf serum (FSCS), penicillin, streptomycin, fungizone, and sodium bicarbonate. The RK-13, BHK-21, Hela, Hep-2, and L-M cell lines were grown at 37ºC in an aerobic incubator. The C2C12 cell line was grown at 37ºC in the presence of 5% CO_2. The RK-13 cell lines were subcultured once weekly (1:5 split), whereas BHK-21, L-M, and Hep-2 cell lines were subcultured twice weekly (1:6 split), and the C2C12 and Hela cell lines were subcultured once every 4 days (1:4 split) in accordance with routine procedures. The origin, passage history and horse-pathogenicity of the virulent Bucyrus strain (VBS53) of EAV are summarized in Fig 2.1. The original isolate of EAV in 1953 was passaged 15 times in horses and the pleural fluid collected from the 15^{th} inoculated horse

was named as the VBS53 strain of EAV, which was highly virulent for horses. The EAV strain VBS53 was propagated twice in BHK-21 cells to produce virus stocks which were used in this study. Viral infectivity was determined by plaque assay on RK-13 (KY) cells as previously described (McCollum *et al.* 1962).

Antibodies. The development and characterization of monoclonal antibodies to the nucleocapsid protein (N; MAb 3E2) and the non-structural protein 1 (nsp1; MAb 12A4) of EAV have been previously described (MacLachlan *et al.* 1998; Wagner *et al.* 2003). Both MAbs 3E2 and 12A4 were kindly provided by Drs. Udeni Balasuriya and James MacLachlan at University of California, Davis.

Growth of EAV in various cell lines. Growth of EAV strain VBS53 in RK-13, BHK-21, C2C12, Hela, Hep-2, and L-M cell lines was compared. Subconfluent monolayers of each cell line grown in T-25 flasks (approximately 2.2×10^6 cells/T-25 flask) were inoculated with EAV strain VBS53 at a multiplicity of infection (m.o.i.) of 3. Flasks were incubated at 37°C for 1 h. The inoculum was aspirated, and cell sheets washed three times with PBS to remove unbound virus and the sheets overlaid with 10 ml of EMEM medium. This was designated time zero with respect to infection. Inoculated cultures were incubated at 37°C. At 0, 12, 24, 36, 48, 60, and 72 h post infection (p.i.), both supernatants and cells were harvested and sonicated three times (20V, 15 sec/time) to fully disrupt the cells to release virus particles. Viral infectivity was determined by plaque assay on RK-13 (KY) cells as previously described (McCollum *et al.* 1962). Mock-infected cultures of each cell line were included in the experiment. Cell lines were also grown in 8-chamber slides and infected with EAV strain VBS53. At 24 h and 72 h post infection, cell sheets were examined by indirect immunofluorescence assay for expression of the viral nucleocapsid (N) protein and non-structural protein 1 (NSP1). To determine the effect of m.o.i. on the outcome of infection, RK-13 (KY), Hela, Hep-2, and L-M cell lines were infected with EAV strain VBS53 at m.o.i. of 1, 3, 10, and 50. At 0 h and 40 h after infection, extracellular virus (represented by culture supernatants) and cell-associated virus (represented by cell-lysates) were titrated separately by plaque assays.

Serial amplification of EAV in Hela 'High' and Hela 'Low' cell lines. Subconfluent monolayers of Hela cells (low passage P109 and high passage P200) were inoculated with EAV strain VBS53 at an m.o.i of 3 (using an inoculum of 0.3 ml). Mock-

inoculated Hela cells (P109 and P200) were included. The same volume of EAV infective inoculum was added to 10% EMEM medium without cells and included as an additional control. After 1 h of incubation at 37ºC, without washing, 10 ml medium was added to each culture. After 4 days of incubation at 37ºC, the supernatant (P1) (10 ml) was harvested and centrifuged at 1900 rpm for 10 min to remove cell debris. The supernatant was then filtered through a 0.45 µm membrane to ensure removal of cell debris. Filtered supernatant (0.3 ml) from mock-inoculated Hela cells (P1 mock), EAV-inoculated Hela cells (P1 EAV-Hela), and EAV-inoculated medium (P1 EAV-medium) were added to uninfected Hela cell cultures (P110 or P201) or medium without cells, respectively, and incubated at 37ºC for 1 h. A 10 ml volume of medium was added and the cultures incubated for 4 days at 37ºC to provide 'P2 mock', 'P2 EAV-Hela', and 'P2 EAV-medium', respectively. The same procedure was followed in attempting to amplify the virus up to P7.

Chromosomal analysis. Karyotypic analysis of low (P98) and high (P202) passage Hela cells was performed using the conventional G-banding technique (ISCN 1985; Yunis 1976) at Department of Cytogenetics, University of Kentucky. Twenty giemsa-banded metaphase cells from passage 98 and passage 202 Hela cells were analyzed, respectively.

***In vitro* transcription and transfection of cells with *in vitro* transcribed EAV RNA.** Viral RNA transcripts were *in vitro*-transcribed from the full-length infectious EAV cDNA clone pEAV2421/211EB which was derived from EAV strain VBS53 (Balasuriya *et al.*, unpublished). The *in vitro* transcription was carried out as previously described (van Dinten *et al.* 1997) with minor modifications. Briefly, the plasmid DNA was linearized with *Xho*I, digested with proteinase K, purified by phenol-chloroform extraction, precipitated with ethanol, and dissolved in water. The *in vitro* transcription was conducted in a reaction mixture of 50.0 µl: linear DNA 15.0 µl, rNTPs mix (10mM each, Amersham) 5.0 µl, acetylated BSA (1 mg/ml, Promega) 5.0 µl, DTT (100 mM, Promega) 2.5 µl, 5' cap analog M^7G(ppp)G (10 mM, New England Biolabs) 5.0 µl, RNA guard RNase inhibitor (Pharmacia) 2.5 µl, T7 RNA polymerase (Promega) 2.5 µl, 5x T7 transcription buffer 10.0 µl, nuclease free water 2.5 µl. After 2 h of incubation at 37ºC, the RNA quality and yield were assessed by agarose gel electrophoresis and

spectrophotometry. RNA transcripts were introduced into BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cell lines by means of electroporation following previously described protocols (van Dinten *et al.* 1997) with minor modifications. Briefly, cells were grown to subconfluence, trypsinized, washed with PBS, and resuspended in PBS at a concentration of 1 x 10^7 cells per ml. A 15 µl aliquot of the transcripts mixture was added to 500 µl of the cell suspension, which was then transferred into electroporation cuvettes (0.4 cm electrode gap; Bio-Rad). Using a Gene Pulser II system (Bio-Rad), the cells were pulsed twice at 850V and 25 µF, with the pulse controller set at 'infinite' ohms. The cells were incubated at room temperature for a 10 min 'recovery period' after electroporation and then resuspended in 15 ml of culture medium which was previously warmed to room temperature. The cells were seeded into 8-chamber slides for immunofluorescence assays or into a T-25 flask to monitor virus yield at 12, 24, 48, 72, and 96 h post transfection.

Indirect immunofluorescence assay (IFA). Mock-, EAV-infected, or EAV RNA-transfected cells grown in 8-chamber slides were fixed with cold acetone for 10 min, and washed three times with PBS containing 10mM glycine. Slides were then incubated with MAb 3E2 (ascitic fluid) specific for EAV nucleocapsid protein, or MAb 12A4 (ascitic fluid) specific for EAV NSP1, followed by fluorescein-conjugated goat antimouse immunoglobulin (Pierce). The cell sheets were counterstained with Evans blue. The images were recorded using a LEICA confocal microscope.

Measurement of virus binding to and entry into cells. EAV binding to and etnry into various cell lines were determined by modifying previously described asssays (de la Torre *et al.* 1988a; Vlaycheva and Chambers 2002). Subconfluent monolayers of BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cell lines were trypsinized and resuspended in culture medium at a concentration of 2 x 10^6 cells per ml, and cooled down at 4ºC for at least 30 min. In the case of each cell line, 2 x 10^6 PFU of EAV strain VBS53 (in 200 µl) was added to four replicates of 1 x 10^6 cells (500 µl) and held at 4ºC for 1 h. The cells were then washed three times with cold PBS to remove unbound virus. Two of the cell replicates were resuspended in 1 ml of cold culture medium. Cell suspensions were subjected to three cycles of freezing and thawing to release attached viruses, and then centrifuged for 10 min at 1900 rpm at 4ºC. Clarified supernatants were titrated by plaque assay on RK-13 (KY) cells as previously described (McCollum *et al.*

1962). The amount of virus attached to Hela 'High', Hela 'Low', Hep-2, and L-M cells was calculated as a percentage of the virus attached to BHK-21 cells. The other duplicate of the cells were resuspended in 1 ml of pre-warmed culture medium and incubated at 37ºC for 1 h with agitation. After incubation for 1 h at 37ºC, the cells were treated with 1.0 ml acid glycine (pH 3.0) to inactivate non-internalized virus. The cells were then washed four times with PBS and resuspended in 1 ml of culture medium. The last wash was saved for infectivity titration to confirm that no residual extracellular virus was detectable in the cell suspensions. Serial decimal dilutions of the cell suspensions were made in culture medium and then inoculated onto monolayers of RK-13 (KY) cells in 6-well plates. Inoculated cultures were incubated at 37ºC for 1 h. Cell monolayers were overlaid with culture medium containing 0.75% carboxymethyl cellulose and incubated at 37ºC for 4 days. Infectious centers were counted after staining of plates with 10% formalin buffered crystal violet. Efficiency of virus entry into Hela 'High', Hela 'Low', Hep-2, and L-M cell lines was calculated as a ratio of infectious center counts in each cell line being evaluated to infectious center counts in BHK-21 cell line.

Virus purification and biotinylation. Purification and biotinylation of EAV were carried out according to previously described assay with minor modifications (Nauwynck *et al.* 1999). The stocks of EAV strain VBS53 were pelleted by ultracentrifugation through a 20% (wt/wt) sucrose cushion in TNE buffer (20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA) for 5 h at 25,000 rpm (SW28 rotor) at 4°C. Virus pellets were resuspended in TNE buffer and further purified by ultracentrifugation using a discontinuous 20%-50% sucrose gradient for 16 h at 32,000 rpm (SW41 rotor) at 4ºC. Visible virus bands were harvested and rinsed with bicarbonate buffer (pH 8.6) by centrifugation for 5 h at 34,000 rpm (SW41 rotor) at 4ºC. In the final step, the purified virus particles were resuspended in sodium bicarbonate buffer. The infectivity titer of the resulting virus particles was approximately 5×10^9 pfu/ml, as determined by plaque assay in RK13 (KY) cells. The protein concentration was 2.0 mg/ml, as determined by the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as a standard. Purified EAV particles in suspension were biotin labeled using a protein biotinylation kit (Amersham). Briefly, virus particles were diluted to a concentration of 1 mg/ml in sodium bicarbonate buffer and 40 µl of biotinylation reagents were added per mg of virus protein. The

mixture was shaken for 1 h at 4°C. Biotinylated virus was collected after purification on a Sephadex G25 column, aliquoted (0.5 ml/tube, 0.4 mg/ml), and stored at -70°C. The infectivity titer of the biotinylated virus was 4×10^9 pfu/ml.

Analysis of biotinylated EAV attachment to cells by flow cytometry. Attachment of biotinylated EAV to various cell lines were carried out by modifying previously described procedures (Borrow and Oldstone 1992; Nauwynck *et al.* 1999). Subconfluent monolayers of RK-13 (KY), RK-13 (ATCC), BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cell lines were washed three times with cold PBS and then treated with non-enzymatic cell dissociation solution (Sigma) for cell detachment. Various amounts of biotinylated EAV [0, 2.5 µg (2.5×10^7 pfu), 5.0 µg (5.0×10^7 pfu), 7.5 µg (7.5×10^7 pfu), 10.0 µg (1.0×10^8 pfu), 12.5 µg (1.25×10^8 pfu)] were added to aliquots of a suspension of each cell line containing 5×10^5 cells in a total volume of 500 µl of PBS containing 0.2% bovine serum albumin (PBSA) and incubated at 4ºC for 1 h. The cells were washed three times with cold PBSA and incubated with 200 µl of a 1:100-dilution of streptavidin-fluorescein conjugate in PBSA for 1 h at 4ºC. The cells were then washed three times in PBS containing 2% fetal bovine serum (FBS), followed by resuspension in 1.0 ml of PBS containing 2% FBS. Propidium iodide was added to the cell suspensions (1 µg propidium iodide per ml of cell suspension) just before analysis by flow cytometry. The relative fluorescence intensity of each sample was determined by flow cytometry and 30,000 cells were counted in each sample. Dead cells labeled with propidium iodide were excluded.

The specificity of the attachment of biotinylated EAV to cells was demonstrated by two methods: (i) a competitive inhibition test with nonbiotinylated EAV; and (ii) binding experiments with biotinylated BHK-21 cellular proteins. In the first case, 5×10^5 cells were first incubated at 4ºC for 1 h with different amounts of nonbiotinylated EAV (0, 1×10^8 pfu, 2.5×10^8 pfu, 5×10^8 pfu, 7.5×10^8 pfu) in a final volume of 300 µl of PBSA. Then, 1×10^8 pfu of biotinylated EAV was added and the total volume was increased to 500 µl with PBSA. The cells were incubated at 4ºC for a further hour, and fluorescence intensities were determined in accordance with previously described protocols. Inhibition of attachment was calculated as follows: % inhibition = [Mean fluorescence intensity of cells without nonbiotinylated EAV - Mean fluorescence intensity of cells with different

amounts of nonbiotinylated EAV]/[Mean fluorescence intensity of cells without nonbiotinylated EAV] × 100%. In the case of the second method used to confirm the specificity of virus attachment, biotinylated BHK-21 cellular proteins (0, 2.5 µg, 5.0 µg, 7.5 µg, 10.0 µg, and 12.5 µg) were incubated with 5×10^5 cells of each cell line at 4ºC for 1 h, and the fluorescence intensities determined as already described.

RESULTS

Determination of the permissiveness of various cell lines to infection with the VBS53 strain of EAV. In order to identify a cell line that lacks the receptor(s) for EAV attachment and entry, a variety of cell lines of different species and tissue origin (Table 2.1) were tested for their permissiveness to infection with the VBS53 strain of EAV (Fig 2.1). The first approach used to achieve this objective was to determine whether each of the selected cell lines supported efficient growth of the virus. In EAV-inoculated BHK-21, RK-13 (ATCC), RK-13 (KY), and C2C12 cell lines, virus-specific cytopathic effects (CPE) were first visualized from 24 h p.i. with all the cell monolayers destroyed by 48 h p.i., confirming that EAV is very cytolytic in each of these cell lines. In contrast, virus-specific CPE were not observed in EAV-inoculated Hela, Hep-2, and L-M cell lines even at 96 h after inoculation. Replication of EAV in BHK-21, RK-13 (ATCC), RK-13 (KY), and C2C12 cells exhibted a typical growth kinetic curve, with 4-5 $\log_{10}$ increases in infectivity titers within 24 h, followed by a gradual decline in titers after 48 h (Fig 2.2). In contrast, virus infectivity titers in Hela (ATCC), Hep-2, and L-M cells did not increase markedly (less than 2 $\log_{10}$ increase) during the entire observation period (Fig 2.2), indicating that growth of this strain of EAV in these cell lines was restricted and not efficient.

To determine whether virus antigens were synthesized during infection, indirect immunofluorescence assays were performed. In EAV-infected BHK-21 (Fig 2.3A and B), RK-13 (ATCC), RK-13 (KY), and C2C12 cells (data not shown), almost 100% cells were positive for the structural protein N (Fig 2.3A) and for the non-structural protein nsp1 (Fig 2.3B) by 24 h post inoculation, confirming that EAV efficiently replicated in each of these cell lines. However, in EAV-inoculated Hela (ATCC) and L-M cells, only a small number of cells were fluorescent positive for N- (Fig 2.3C and K) or nsp1 protein (Fig

2.3D and I) by 24 h after inoculation. The number of EAV fluorescent-positive cells in Hela (ATCC) and L-M cell lines was less than 2% and 6%, respectively. Even by 72 h post inoculation, the percentage of EAV-positive cells in Hela (ATCC) and L-M cell lines had not increased (Fig 2.3E, F, M, and N), indicating that the spread of virus from the initially infected cells to uninfected cells had not occurred. No EAV-positive cells were observed in the EAV-inoculated Hep-2 cell line throughout the period of observation (Fig 2.3O, P, Q, and R). Failure to detect expression of selected non-structural and structural viral proteins in the majority of cells of the EAV-inoculated Hela (ATCC), Hep-2, and L-M cell lines would suggest that EAV infection of these cell lines might be restricted at steps that precede viral replication, e.g. viral attachment or entry steps.

In order to determine whether increase of m.o.i. can result in productive infection of EAV in Hela (ATCC), Hep-2, and L-M cells, various cell lines were inoculated with VBS53 EAV at m.o.i. of 1, 3, 10, and 50, respectively. As shown in Table 2.2, with an increase of m.o.i., there was a concomitant increase in residual virus in each cell line at 0 h post inoculation. However, with the increase in m.o.i., virus yields in RK-13 (KY) cells had declined by 40 h post inoculation. In Hela (ATCC), Hep-2, and L-M cell lines, on the other hand, an increase in m.o.i. resulted in increase in virus titer by 40 h post inoculation. This increase probably reflected the increase amount of virus in the inoculum. Even at a high m.o.i. (e.g. m.o.i. = 50), virus replication in Hela (ATCC), Hep-2, and L-M cell lines was not as efficient as in the fully permissive RK-13 cell line. It should be pointed out that in EAV-inoculated RK-13, Hep-2, and L-M cells, the percentage of extracellular virus to the total virus present (the sum of extracellular virus and cell-associated virus) was greater than 80%, while it was only around 50% in Hela (ATCC) cells (Table 2.2).

Taken collectively, these findings confirm that RK-13, BHK-21, and C2C12 cell lines are fully susceptible to infection with the VBS53 strain of EAV, whereas Hela (ATCC), Hep-2, and L-M cell lines only have limited susceptibility to infection with this virus.

Enhancement of susceptibility of Hela cells to EAV infection after extended serial passages. In the course of this study, it was found that the Hela cell line became more susceptible to infection with EAV strain VBS53 after extended serial passage. Hela

cells which were obtained from ATCC at passage level 95 were serially passed up to passage 221 as described in Materials and Methods. As indicated in Fig 2.2, the virus infectivity titer in EAV-inoculated Hela P176 cells was 1.5-2 $\log_{10}$s higher than that in Hela P98 cells. Using the IFA test, it was found that at 24 h post inoculation, EAV-positive cells were approximately 12% in EAV-inoculated Hela P176 cells but less than 2% in Hela P98 cells (Fig 2.3C, D, G, and H). Moreover, spread of the virus from the initially infected cells to uninfected cells was observed in Hela P176 cells as reflected by the increase in percentage of EAV fluorescent-positive cells from 12% at 24 h post inoculation to approximately 19% at 72 h post inoculation (Fig 2.3G, H, I, and J). This was not observed in Hela P98 cells (Fig 2.3C, D, E, and F). Furthermore, it was shown that Hela cells between passage level 170 and 221 have higher susceptibility to EAV infection than Hela cells between passage level 95 and 115, which were found to be non-susceptible to infection. Hela cells from passage 95 to 115 were referred to as Hela 'Low' cell line and Hela cells from passage 170 to 221 as Hela 'High' cell line. It should be pointed out that the morphology and growth rate of the Hela 'High' cell line were indistinguishable from those of the Hela 'Low' cell line. While Hela 'High' cell line was more susceptible than Hela 'Low' cell line to infection with EAV strain VBS53, susceptibility of Hela 'High' cell line to infection was remarkably less than that of RK-13, BHK-21, and C2C12 cell lines (Fig 2.2 and Fig 2.3). Also, no apparent EAV-induced CPE was observed during one-step growth curve experiment of EAV in the Hela 'High' cell line.

In order to further confirm that Hela 'High' cell line is more susceptible than Hela 'Low' cell line to EAV infection, serial passage of EAV strain VBS53 in low (P109) and high (P200) passage Hela cells were carried out. As shown in Table 2.3, the VBS53 strain of EAV was efficiently amplified in Hela 'High' cell line but not in Hela 'Low' cell line. Furthermore, this strain of EAV was able to initiate persistent infection in Hela 'High' cell line but not in Hela 'Low' cell line (see Chapter 3), confirming that Hela cells evolved and acquired some new characteristics during extended subculturing.

Chromosomal analysis of Hela 'High' and Hela 'Low' cell lines. To investigate the possible changes between Hela 'High' and Hela 'Low' cell lines and also to rule out the possibility that Hela 'High' cell line was contaminated by other cells during the serial

subculturing of Hela ‘Low’ cell line, karyotypic analyses of low (P98) and high (P202) passage Hela cells were performed using the conventional G-banding technique. Analyses of 20 Giemsa-banded metaphase cells from passage 98 and passage 202 Hela cells are presented in Table 2.4. It has been established that Hela cells have four typical marker chromosomes: one copy of marker M1, one copy of marker M2, four to five copies of marker M3, and two copies of marker M4. Marker M1 is the der(1;3)(q10;q10), marker M2 is the der(3;5)(p10;q10), marker M3 is the i(5)(p10), and marker M4 consists of the long arm of chromosome 11 and an arm of chromosome 19 (http://www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=CCL-2). Both Hela ‘High’ and Hela ‘Low’ cell lines were observed to contain one copy of marker M1, one copy of marker M2, and 3-5 copies of marker M3 (Table 2.4). Marker M4 may be present, because a derivative chromosome consisting of the long arm of chromosome 11 and an arm of an unidentified chromosome was observed in both Hela ‘High’ and Hela ‘Low’ cell lines. This unidentified chromosome resembles the long arm of chromosome 20 more closely than either arm of chromosome 19 [?der(11;20)(q10;q10)] (Table 2.4). The fact that Hela ‘High’ cells analyzed contained typical marker chromosomes of Hela cells confirms that they had not been contaminated with other cells. Karyotypic analyses also revealed some differences between Hela ‘High’ and Hela ‘Low’ cell lines at chromosomal level. Hela cells at passage level 202 are karyotypically more heterogenous than those at passage level 98. Cells at both passage levels have a hyperdiploid count with modal number of 81-82 chromosomes, but the hyperdiploid count ranges from 78 to 94 chromosomes in passage 202 cells and from 81 to 82 in passage 98 cells (Table 2.4). In comparing the cells at passage level 202 to those at passage level 98, both gain and loss of chromosomal material appear to have occurred. For example, cells at passage level 202 acquired the following materials: an isochromosome for the short arm of chromosome 2, i(2)(p10); an isochromosome for the long arm of chromosome 2, i(2)(q10); an additional copy of chromosome 6 (Table 2.4). In contrast, a derivative chromosome der(15;15)(q10;q10) which exists in passage 98 cells was absent in passage 202 cells (Table 2.4). In summary, karyotypical analyses revealed that cells at both passage level 98 and passage level 202 contain typical Hela

marker chromosomes. However, chromosomal changes were demonstrated in Hela cells between passage levels 98 and 202.

Transfection of cells with viral RNA *in vitro* transcribed from an infectious EAV cDNA clone. As has been shown, RK-13, BHK-21, and C2C12 cell lines are fully susceptible to infection with EAV strain VBS53, whereas Hela 'High', Hela 'Low', Hep-2, and L-M cell lines are of limited susceptibility to infection. In order to determine whether restriction of EAV infection is due to an intracellular block, viral RNA *in vitro* transcribed from an infectious EAV cDNA clone pEAV2421/211EB which was derived from EAV strain VBS53 (Balasuriya *et al.*, unpublished) was transfected into BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cell lines. Cytopathic effects appeared and progressed in BHK-21 cells 72 h after transfection. No CPE was observed in the transfected Hela 'High', Hela 'Low', Hep-2, and L-M cell lines even at 96 h post transfection. However, based on the results of virus titration and IFA testing, EAV in fact replicated in all of the transfected cell lines (Fig 2.4 and Fig 2.5). At 24 h post transfection, the percentage of EAV nsp1- and N-protein positive cells was approximately 20% in all transfected cell lines (Fig 2.5A, C, E, G, I and data not shown). Consistently, at 24 h post transfection, virus yields in transfected Hela 'High', Hela 'Low', Hep-2, and L-M cell lines were similar (approximately 1.4×10^5 pfu/ml); these were even higher than the corresponding titer in transfected BHK-21 cells (approximately 2.4×10^4 pfu/ml) (Fig 2.4). This indicated that Hela 'High', Hela 'Low', Hep-2, and L-M cell lines support EAV replication as efficiently as the permissive BHK-21 cell line once the viral RNA is released into the cytoplasm. After 24 h post transfection, virus yields in transfected BHK-21 cells increased to markedly higher levels than those in transfected Hela 'High', Hela 'Low', Hep-2, and L-M cells (Fig 2.4). This probably reflects the inefficiency of the released virus to initiate secondary rounds of infection in Hela, Hep-2, and L-M cell lines. This was corroborated by the IFA results. In transfected BHK-21 cells, efficient spread of the infection to nontransfected cells was observed by 48 h post transfection (Fig 2.5A and B), followed by the development of CPE and cell death by 72 h post transfection. In transfected Hela 'Low', Hep-2, and L-M cell lines, however, spread of the infection from transfected cells to nontransfected cells was not observed at 48 h (Fig 2.5C and D; G and H; I and J) or even up to 72 h (data not shown) post

transfection. In transfected Hela ‘High’ cells, spread of infection from transfected cells to nontransfected cells was not apparent (Fig 2.5E and F). Differences of virus yields in transfected Hela ‘High’ and Hela ‘Low’ cell lines are not as marked as those in infected Hela ‘High’ and Hela ‘Low’ cell lines (Fig 2.4 and Fig 2.2). Taken together, the transfection experiments demonstrated that Hela ‘High’, Hela ‘Low’, Hep-2, and L-M cell lines support EAV replication when the attachment and entry steps are bypassed, suggesting the absence of an intracellular block later in the virus replication cycle. Thus, EAV infection of these cell lines appears to be restricted at the attachment or entry steps.

Binding assays. In order to determine whether EAV infection of Hela ‘High’, Hela ‘Low’, Hep-2, and L-M cell lines is restricted at virus attachment step, virus binding to these cell lines was carried out. The first approach used to study virus attachment was to incubate cells with EAV strain VBS53 for 1 h at 4ºC and then the virus bound to cells was titrated after unattached viruses were removed through extensive washing. As shown in Fig 2.6, Hela ‘Low’, Hela ‘High’, and Hep-2 cell lines were able to bind EAV strain VBS53 almost as efficiently as the fully susceptible BHK-21 cell line. The amount of virus attached to L-M cell line was 25% greater than that attached to BHK-21 cell line (Fig 2.6).

In order to confirm the attachment of EAV to these cell lines, another approach was taken using flow cytometry to examine the binding of biotinylated EAV strain VBS53 to each cell line. RK-13 (KY), RK-13 (ATCC), BHK-21, Hela ‘Low’, Hela ‘High’, Hep-2, and L-M cell lines were incubated with various amounts (0, 2.5 µg, 5.0 µg, 7.5 µg, 10.0 µg, and 12.5 µg) of biotinylated EAV, respectively. The biotinylated EAV was found to bind to all of the cell lines under study and the binding level (fluorescence intensity) increased with the increase in the amount of biotinylated virus (Fig 2.7A, C, D, E, F, G and H), suggesting that EAV binds to these cells in a dose-dependent manner. However, using the same amount of virus, binding differences were observed between these cell lines. RK-13 (Fig 2.7C), BHK-21 (Fig 2.7D), Hela ‘Low’ (Fig 2.7E), Hela ‘High’ (Fig 2.7F), and Hep-2 (Fig 2.7G) cells had similar levels of virus binding (fluorescence intensities), while L-M cells had a significantly higher level of virus binding (Fig 2.7H). Since EAV strain VBS53 was amplified in BHK-21 cells, the purified virus particles used for biotinylation may still have contained some BHK-21 cellular proteins. In order to

exclude the possibility that the binding observed was caused by BHK-21 cellular proteins, mock-infected BHK-21 cell supernatants which underwent the same purification procedure as used for EAV particles was biotinylated and various amounts of biotinylated BHK-21 cellular proteins (0, 2.5 µg, 5.0 µg, 7.5 µg, 10.0 µg, and 12.5 µg) equivalent to those of biotinylated EAV were used to perform binding assays. As shown in Fig 2.7, in the case of each cell line, the fluorescence intensities resulting from biotinylated BHK-21 cellular proteins were significantly lower than those caused by biotinylated EAV. Moreover, unlike biotinylated EAV, an increase in the amount of biotinylated BHK-21 cellular proteins did not remarkably increase the binding levels (fluorescence intensities). This indicated that fluorescence intensities observed in binding experiments mainly resulted from the biotinylated EAV rather than from biotinylated BHK-21 cellular proteins. The specificity of biotinylated EAV attachment to cells was also confirmed by the fact that the binding of biotinylated EAV to the various cell lines was competitively inhibited by nonbiotinylated EAV (Fig 2.8).

These findings demonstrate that EAV strain VBS53 attaches to Hela 'Low', Hela 'High', Hep-2, and L-M cell lines as efficiently as it attaches to RK-13 and BHK-21 cell lines, suggesting that virus attachment is not the major step in restricting EAV infection in Hela, Hep-2, and L-M cell lines.

Entry assay. To determine whether infection of Hela 'Low', Hela 'High', Hep-2, and L-M cell lines with EAV strain VBS53 is restricted at the entry step, the virus entry into these cell lines was investigated. In attempting to use confocal microscopy to follow the attachment and entry of biotinylated EAV into BHK-21, Hela, Hep-2, and L-M cell lines, it was difficult to clearly distinguish attached virus from internalized virus (data not shown). An alternative approach was taken using a modified conventional infectious center assay to study EAV entry. A standardized amount of cells were incubated with a standardized amount of EAV strain VBS53 for 1 h at 4ºC for virus attachment. After extensive washing to remove unbound virus, cells were incubated at 37ºC for 1 h to allow for virus entry. Then, cells were treated with acid glycine (pH 3.0) to inactivate non-internalized viruses. Subsequently, serial decimal dilutions of the cells were made and respective dilutions were plated onto monolayers of RK-13 (KY) cells for quantitation of infectious foci by plaque formation. Since it has been shown that Hela 'High', Hela

'Low', Hep-2, and L-M cell lines support EAV replication as efficiently as BHK-21 cells (Fig 2.4 and Fig 2.5) once viral RNA is introduced into the cell cytoplasm, it is reasonable to propose that counts of infectious centers reflect the efficiency of EAV entry into each cell line. As shown in Fig 2.9, the efficiency of VBS53 EAV entry into Hela 'Low', Hela 'High', Hep-2, and L-M cell lines was significantly lower than that of virus entry into BHK-21 cells. In addition, it was observed that the efficiency of EAV entry into Hela 'High' cell line was approximately five-fold higher than those of virus etnry into Hela 'Low', Hep-2, and L-M cell lines (Fig 2.9). These findings indicate that infection with EAV strain VBS53 is restricted at the entry step in Hela, Hep-2, and L-M cell lines.

DISCUSSION

Neither viral molecules nor cellular factors involved in the early events (e.g. attachment, penetration, uncoating) of the replication cycle of EAV have been elucidated. One approach to studying host requirements for EAV infection is to identify cell lines that are defective in their ability to support viral infection. In this study, we first investigated the permissiveness of various cell lines to infection with EAV strain VBS53 and then attempted to establish the mechanism that restricts infection in non-permissive cell lines. It had been previously demonstrated that RK-13, BHK-21, and Vero cell lines are permissive to EAV infection and infection of these cell lines is highly cytocidal (Snijder and Meulenberg 1998). In this study the cell line C2C12, derived from the mouse muscle, was also found to be permissive to EAV infection with the development of typical EAV-induced cytopathic effects. Furthermore, it was found that EAV infection was restricted in Hela, Hep-2, and L-M cell lines in which no apparent CPE was observed during one-step growth of EAV nor was efficient virus replication demonstrated. The mechanism that restricts EAV infection in these cell lines was further investigated. When attachment and entry steps are bypassed by transfection of Hela, Hep-2, and L-M cell lines with viral RNA, these cell lines support EAV replication as efficiently as the permissive BHK-21 cell line, suggesting the absence of an intracellular block later in the virus replication cycle. Binding studies revealed that Hela, Hep-2, and L-M cell lines were able to bind the VBS53 strain of EAV efficiently. Although it has been reported to

use confocal microscopy to study the attachment and entry of biotinylated PRRSV into alveolar macrophages (Nauwynck *et al.* 1999), it was difficult to clearly distinguish attached virus from internalized virus when similar approach was used to study EAV entry into BHK-21, Hela, Hep-2, and L-M cell lines (data not shown). Thus, a modified infectious center assay was used in this study to investigate EAV entry into cells. After one hour incubation of virus with cells at 4ºC for virus attachment, the temperature was increased to 37ºC to allow for virus entry. Treatment of the cell suspensions with acid glycine (pH 3.0) inactivates non-internalized virus and thus only the virus that has successfully entered the cells can initiate subsequent replication. Since Hela, Hep-2, and L-M cell lines support EAV replication as efficiently as BHK-21 cells, the plaque number should reflect the efficiency of the virus entry into these cell lines. However, since no apparent CPE was observed in EAV-inoculated Hela, Hep-2, and L-M cell lines, a plaque assay cannot be directly performed in these cell lines. Therefore, after virus entry, the serial decimal dilutions of suspensions of Hela, Hep-2, L-M, and BHK-21 cells are plated onto monolayers of RK-13 (KY) cells which are overlaid with carboxymethyl cellulose. With this assay, EAV infection of Hela, Hep-2, and L-M cell lines was shown to be restricted at the entry step. However, there are shortcomings to this entry assay. It is predicated on the fact that virus entering cells must first complete a single round of infection in BHK-21, Hela, Hep-2, and L-M cells before infecting RK-13 cells and forming infectious foci. Essentially, this assay does not establish virus entry directly although it logically can reflect the entry efficiency. Furthermore, this assay cannot distinguish defects at penetration step from those at the fusion/uncoating step. A more direct virus entry assay will be performed to confirm these findings. For example, radio-labeled EAV particles can be used in a virus entry assay in which radioactivity of the cell suspensions is measured to reflect entry efficiency after non-internalized viruses are inactivated by acid glycine (pH 3.0) treatment.

In EAV-inoculated RK-13, Hep-2, and L-M cells, the percentage of extracellular virus to the total virus present (the sum of extracellular virus and cell-associated virus) was greater than 80%, while it was only around 50% in Hela cells. This would suggest that virus release from Hela cells might be less efficient than that from RK-13, Hep-2, and L-M cells.

Another interesting finding from this study is that Hela cells become more susceptible to EAV infection after extended serial passage. Hela 'Low' cells (P95 to P115) are not susceptible to infection with EAV strain VBS53 whereas Hela 'High' cells (P170 to P221) are partly susceptible to infection. Increased susceptibility of Hela 'High' cells to EAV infection appears to be a stable genetic trait since 51 subsequent serial passages (from 170 to 221) did not result in any apparent alteration in the degree of susceptibility of the cells to EAV (data not shown). This would suggest that in serially subculturing of Hela cells up to passage 170, Hela cells acquire certain characteristics which would appear stable. Furthermore, EAV strain VBS53 was able to initiate persistent infection in Hela 'High' cell line but not in Hela 'Low' cell line (see Chapter 3), confirming that the Hela cell line evolved during long-term serial passage. The results of karyotypic analysis did not support the possibility that Hela 'High' cell line was contaminated by other cells. The Hela 'High' cell line still contained the marker chromosomes characteristic of the Hela cell line. Karyotypic analysis also revealed changes between Hela 'Low' and Hela 'High' cell lines at chromosomal level. However, the molecular changes that are responsible for susceptibility differences of Hela 'Low' and Hela 'High' cell lines to EAV infection are not yet determined. Based on the following i) virus yields in EAV-inoculated Hela 'High' cell line are significantly lower than those in EAV-inoculated BHK-21, RK-13, and C2C12 cell lines, ii) less than 100% of Hela 'High' cells are infected with EAV, and iii) the efficiency of EAV entry into Hela 'High' cell line is only about 10% of that with respect to BHK-21 cell line, it is postulated that Hela 'High' cell line comprises a heterogenous population of cells of which only a certain percentage of cells are fully permissive to EAV infection.

The evolution of cells and emergence of cell diversity is not an unusual event. Normal tissues and cultured cells can exhibit a degree of heterogeneity (Ingram *et al.* 1985; Schafer *et al.* 1987). It has been reported that BHK(TK^-) cells on subculture spontaneously give rise to cells that are highly resistant to herpes simplex virus type-1 infection (Roller and Roizman 1994). Clonal selections of cell lines resistant to Theiler's murine encephalomyelitis virus or herpes simplex virus types 1 and 2 infections have been reported as well (Hertzler *et al.* 2000; Roller and Herold 1997). The extent of cellular heterogeneity is greatly increased in tumors and tumor cell heterogeneity appears

to result from an enhanced genetic instability of transformed cells (Cifone and Fidler 1981; Crouch *et al.* 1987; Fidler *et al.* 1981; Lichtner *et al.* 1987; Nicolson 1987a, 1987b). Hela cells were originally derived from a cervical adenocarcinoma and perhaps retain the potential to evolve if they undergo extended serial passage. Cell evolution has also been frequently observed in cell cultures persistently infected with a virus such as mouse L cell evolution during persistent infection with reovirus (Ahmed *et al.* 1981), BHK-21 cell evolution during persistent infection with foot-and-mouth disease virus (de la Torre *et al.* 1988a; 1989), and Hela cell and human neuroblastoma cell evolution during persistent infection with poliovirus (Kaplan *et al.* 1989; Pavio *et al.* 2000).

In recent years it has become evident that viral attachment to and entry into the cell are frequently more complex than previously thought. A number of viruses have been shown to enter cells via receptor complexes rather than by an individual cell surface molecule. For example, human immunodeficiency virus attaches to CD4 molecule (Maddon *et al.* 1986) but requires the presence of chemokine receptors as its coreceptor for virus entry (Choe *et al.* 1996; Deng *et al.* 1996; Doranz *et al.* 1996; Dragic *et al.* 1996; Feng *et al.* 1996). Adenovirus subgroups A, C, D, E, and F bind to molecule CAR (coxsackievirus and adenovirus receptor) (Roelvink *et al.* 1998) and adenovirus types 2 and 5 bind to heparan sulfate glycosaminoglycans (Dechecchi *et al.* 2001), however, these binding molecules are insufficient for adenovirus entry which needs the involvement of coreceptor α_v–integrins (Wickham *et al.* 1993). Coxsackievirus A21 (CAV21) has been shown to bind to both decay-accelerating factor (DAF) and intercellular adhesion molecule 1 (ICAM-1) (Shafren *et al.* 1997). However, DAF just functions as a low-affinity attachment receptor and does not initiate a productive infection; high-affinity binding molecule ICAM-1 is required for virus entry into cells (Shafren *et al.* 1997). Similarly, PRRSV, another arterivirus, has been shown to attach to heparan sulfate and porcine sialoadhesin on porcine macrophages (Delputte *et al.* 2002; Vanderheijden *et al.* 2003). Heparan sulfate alone is sufficient to mediate PRRSV attachment but not entry, whereas sialoadhesin alone is sufficient for both virus attachment and virus entry (Delputte *et al.* 2005).

Cell receptors mediating EAV attachment and entry have not been identified. One previous study suggested that a heparin-like molecule on the surface of RK-13 cells

might serve as cell receptor for EAV (Asagoe *et al.* 1997). However, heparinase treatment of RK-13 cells could not completely block EAV infection (Asagoe *et al.* 1997), implying that other molecules on the cell might serve as EAV receptors as well. Data from this study indicate that Hela, Hep-2, and L-M cell lines are able to bind EAV strain VBS53 efficiently but cannot provide mechanism for virus entry. This would suggest that cell receptor(s) for attachment of EAV strain VBS53 are present on the surface of these cell lines, but these attachment receptor(s) are not sufficient to enable efficient virus entry. Additional cellular factor(s) are therefore likely to be required for entry of EAV strain VBS53 and these cellular factor(s) are probably absent or expressed at very low levels on Hela 'Low', Hep-2, and L-M cell lines. Compared to Hela 'Low' cell line, Hela 'High' cell line is more susceptible to infection with EAV strain VBS53, and this increased susceptibility appears to be due to increased virus entry efficiency. It would appear that some Hela 'High' cells probably have acquired the ability to express the cellular factor(s) which are necessary for EAV entry. RK-13, BHK-21, and C2C12 cell lines being fully susceptible to EAV infection presumably constitutively express the cellular factor(s) essential for both EAV attachment and entry. The availability of cell lines with varying susceptibility to EAV infection should be useful for studying post-attachment events involved in EAV entry.

Table 2.1 Source and passage level of cell lines used in study

Cell line	Species	Tissue	Morphology	Source
BHK-21 (ATCC CCL-10)	Hamster	Kidney	Fibroblast	Dr. George Allen, University of Kentucky
RK-13 (ATCC CCL-37) (passage 192-204)	Rabbit	Kidney	Epithelial	Dr. William McCollum, University of Kentucky
RK-13 (KY) (passage 397-409)	Rabbit	Kidney	Epithelial	Dr. William McCollum, University of Kentucky
C2C12 (ATCC CRL-1772)	Mouse	Muscle, myoblast	Fibroblast	Dr. Carole Moncman, University of Kentucky
Hela 'Low' (ATCC CCL-2) (passage 95-115)	Human	Cervical Carcinoma	Epithelial	American Type Culture Collection
Hela 'High' (passage 170-221)	Human	Cervical Carcinoma	Epithelial	Derived from Hela 'Low' cell line by the author
Hep-2 (ATCC CCL-23)	Human	Epidermoid Laryngal Carcinoma	Epithelial	Dr. William McCollum, University of Kentucky
L-M (ATCC CCL-1.2)	Mouse	Connective tissue	Fibroblast	Dr. George Allen, University of Kentucky

Table 2.2 Effect of multiplicity of infection on outcome of infection of selected cell lines with EAV strain VBS53

cells and m.o.i[a]	Virus Titer (pfu/ml) (0 h p.i.)		Virus Titer (pfu/ml) (40 h p.i.)	
	cell-associated virus[b]	extracellular virus	cell-associated virus	extracellular virus
RK-13 (KY) P406				
m.o.i. = 1	1.3×10^{1}	1.1×10^{2}	1.5×10^{6}	6.1×10^{7} (97.6)[c]
m.o.i. = 3	5.0×10^{1}	3.0×10^{2}	2.1×10^{6}	4.5×10^{7} (95.5)
m.o.i. = 10	1.4×10^{2}	1.6×10^{3}	5.2×10^{5}	3.0×10^{7} (98.3)
m.o.i. = 50	1.8×10^{3}	5.0×10^{4}	4.4×10^{5}	1.7×10^{7} (97.5)
Hela P100				
m.o.i. = 1	3.2×10^{1}	1.6×10^{2}	1.4×10^{3}	1.7×10^{3} (54.8)
m.o.i. = 3	8.0×10^{1}	6.1×10^{2}	6.3×10^{3}	7.6×10^{3} (54.7)
m.o.i. = 10	5.2×10^{2}	3.1×10^{3}	2.4×10^{4}	2.3×10^{4} (48.9)
m.o.i. = 50	2.2×10^{3}	3.8×10^{4}	6.5×10^{4}	7.8×10^{4} (54.5)
Hep-2 P454				
m.o.i. = 1	6.7×10^{1}	1.8×10^{2}	1.6×10^{2}	6.6×10^{2} (80.5)
m.o.i. = 3	1.4×10^{2}	3.3×10^{2}	8.6×10^{2}	3.2×10^{3} (78.8)
m.o.i. = 10	8.7×10^{2}	1.7×10^{4}	2.3×10^{3}	1.1×10^{4} (82.7)
m.o.i. = 50	3.5×10^{3}	6.7×10^{4}	8.0×10^{3}	3.9×10^{4} (83.0)
L-M P305				
m.o.i. = 1	7.0×10^{0}	2.7×10^{2}	6.9×10^{1}	3.6×10^{3} (98.1)
m.o.i. = 3	2.7×10^{1}	1.9×10^{2}	3.8×10^{2}	1.7×10^{4} (97.8)
m.o.i. = 10	1.4×10^{2}	1.2×10^{3}	1.8×10^{3}	8.3×10^{4} (97.9)
m.o.i. = 50	2.2×10^{3}	4.3×10^{4}	3.6×10^{3}	3.6×10^{5} (99.0)

[a] Various cells were inoculated with EAV strain VBS53 at an m.o.i. of 1, 3, 10, 50 at 37ºC for 1 h. The inoculum was removed and the cells were washed three times with PBS. The cells were supplemented with 10 ml culture medium and incubated at 37°C. This was designated time zero of infection. At 0 h and 40 h after infection, the extracellular virus (the supernatants) and cell-associated virus (the cell lysate) were harvested and titrated on RK-13 (KY) cells.
[b] For harvesting cell-associated viruses, the cells were resuspended in 10 ml culture medium, sonicated three times (20V, 15 sec/time), and centrifuged at 1900 rpm for 10 min to remove cell debris.
[c] The numbers in parentheses represent percentage of extracellular virus titer to the total virus titer. (total virus = extracellular virus + cell-associated virus)

Table 2.3 The outcome of serial passaging of EAV strain VBS53 in Hela 'Low' and Hela 'High' cell lines*

Hela 'Low' P109	EAV titer (pfu/ml)		
	Mock-Hela	EAV-Hela	EAV-medium
1 h p.i.	0	1.55×10^5	1.12×10^5
P1	0	9.4×10^2	0
P2	0	0.5	0
P3	0	0	0
P4	0	0	0
P5	0	0	0
P6	0	0	0
P7	0	0	0
Hela 'High' P200	EAV titer (pfu/ml)		
	Mock-Hela	EAV-Hela	EAV-medium
1 h p.i.	0	1.14×10^5	1.12×10^5
P1	0	2.05×10^5	0
P2	0	1.65×10^4	0
P3	0	1.70×10^4	0
P4	0	3.5×10^5	0
P5	0	1.5×10^6	0
P6	0	7.2×10^6	0
P7	0	2.1×10^7	0

* Hela 'Low' (P109) and Hela 'High' (P200) cell lines were inoculated with EAV strain VBS53 at an m.o.i of 3 (using an inoculum of 0.3 ml). Mock-inoculated Hela cells were included. The same volume of EAV infective inoculum was added to 10% EMEM medium without cells and included as an additional control. After 1 h of incubation at 37ºC, no washing, 10 ml medium was added to each culture. After 4 days of incubation at 37ºC, the supernatant (P1) (10 ml) was harvested, centrifuged at 1900 rpm for 10 min to remove cell debris. The clarified supernatant was then filtered through a 0.45 µm membrane to ensure removal of cell debris. Filtered supernatant (0.3 ml) from mock-inoculated Hela cells (P1 mock-Hela), EAV-inoculated Hela cells (P1 EAV-Hela), and EAV-inoculated medium (P1 EAV-medium) were added to uninfected Hela cell cultures (P110 or P201) or medium without cells, respectively, and incubated at 37ºC for 1 h. A 10 ml volume of medium was added and the cultures incubated for 4 days at 37ºC to provide 'P2 mock-Hela', 'P2 EAV-Hela', and 'P2 EAV-medium', respectively. The same procedure was followed in attempting to amplify the virus up to P7.

Table 2.4 The results of karyotypic analyses of Hela ‘Low’ (P98) and Hela ‘High’ (P202) cell lines using G-banding technique

Hela ‘Low’ (P98)	81-82, XXX, +der(1;3)(q10;q10), der(1;9)(p10;q10), add(2)(q37), add(3)(q12)×1-2, +der(3;5)(p10;q10), add(5)(p10), +i(5)(p10)×3-5, +add(7)(p11.2), +9, +i(9)(p10), +10, der(10)t(10;?)(p13;?), ?der(11;20)(q10;q10), +der(12)t(3;12)(q21;q21.2), -13, +der(15;15)(q10;q10), +17, add(19)(p13.1), +20, add(22)(q13.3), +1-4mar[cp] chromosome pattern.
Hela ‘High’ (P202)	78-94, XXX, +der(1;3)(q10;q10), der(1;9)(p10;q10), add(2)(q37), i(2)(p10), i(2)(q10), add(3)(q12)×1-2, +der(3;5)(p10;q10), add(5)(p10), +i(5)(p10)×3-5, +6, +add(7)(p11.2), +9, +i(9)(p10), +10, der(10)t(10;?)(p13;?), ?der(11;20)(q10;q10), +der(12)t(3;12)(q21;q21.2), +17, add(19)(p13.1), +20, add(22)(q13.3), +1-14mar, 0-4dmin[cp] chromosome pattern.

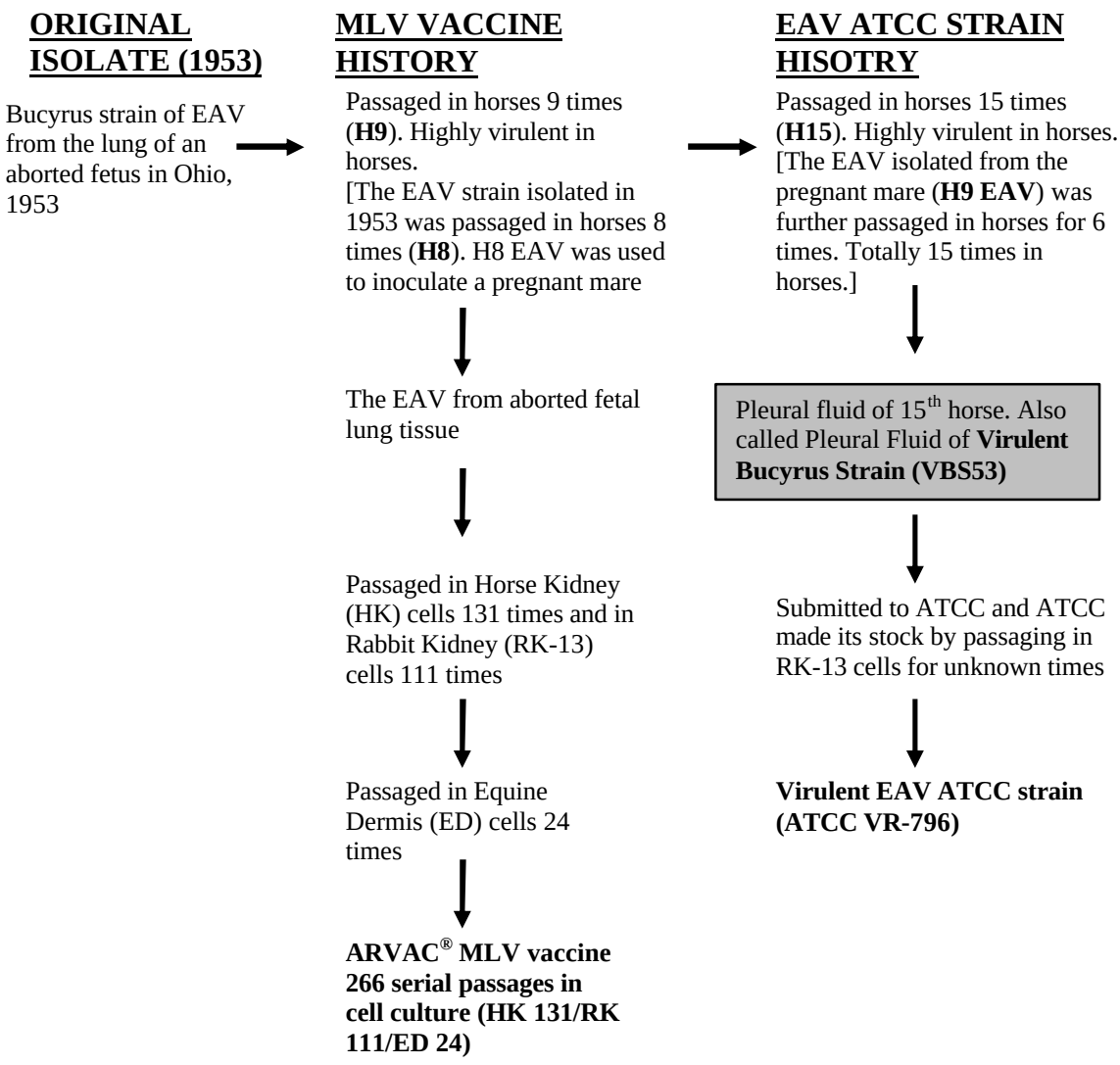


Fig 2.1 Passage history of the original Bucyrus virus to produce the ATCC and MLV vaccine strains of EAV. The EAV strain VBS53 that was used in this study is highlighted.

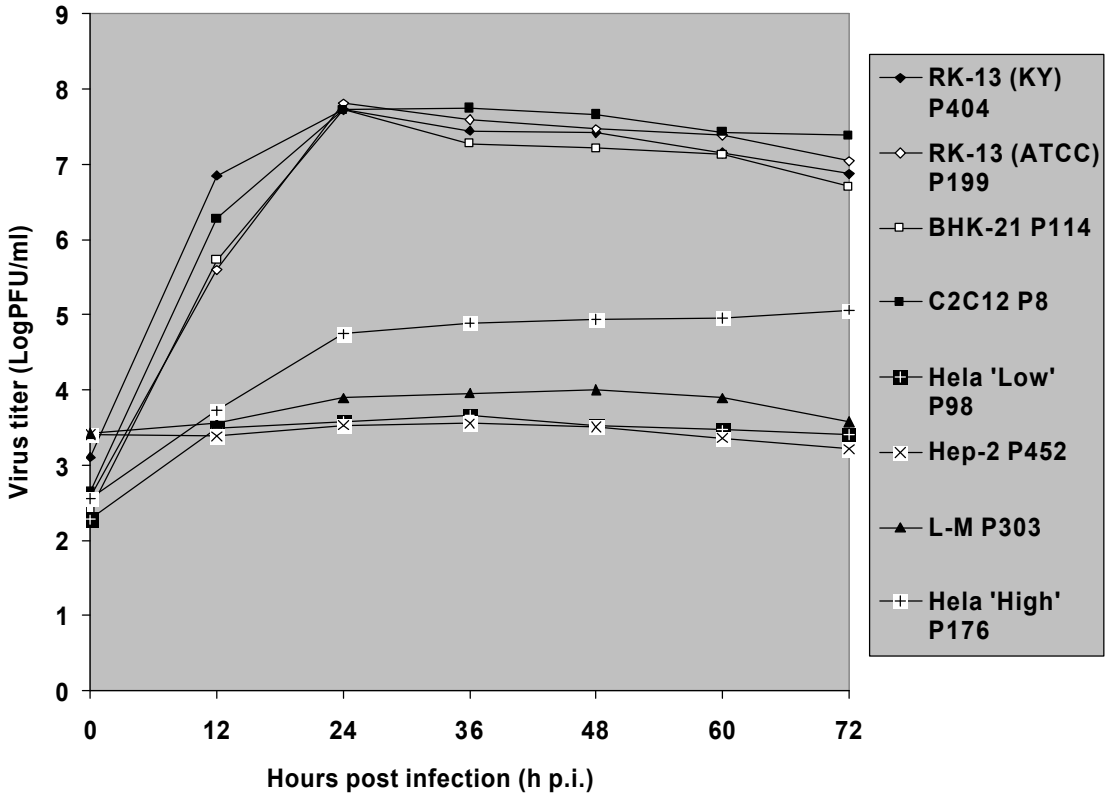


Fig 2.2 Growth of the VBS53 strain of EAV in eight cell lines. Subconfluent monolayers of BHK-21, RK-13 (ATCC), RK-13 (KY), Hela 'High', Hela 'Low', Hep-2, and L-M cell lines were inoculated with EAV strain VBS53 at an m.o.i. of 3 at 37°C for 1 h and then washed three times with PBS to remove unbound virus. The cultures were then supplemented with 10 ml EMEM medium and incubated at 37°C to allow virus growth. This was designated time zero with respect to infection. At 0, 12, 24, 36, 48, 60, and 72 h p.i., both the supernatants and cells were harvested and sonicated three times (20V, 15 sec/time) to fully disrupt the cells and release virus particles. Virus (supernatants and cells together) was titrated by plaque assay on RK-13 (KY) cells as previously described (McCollum *et al.* 1962). These results were confirmed in three separate experiments.

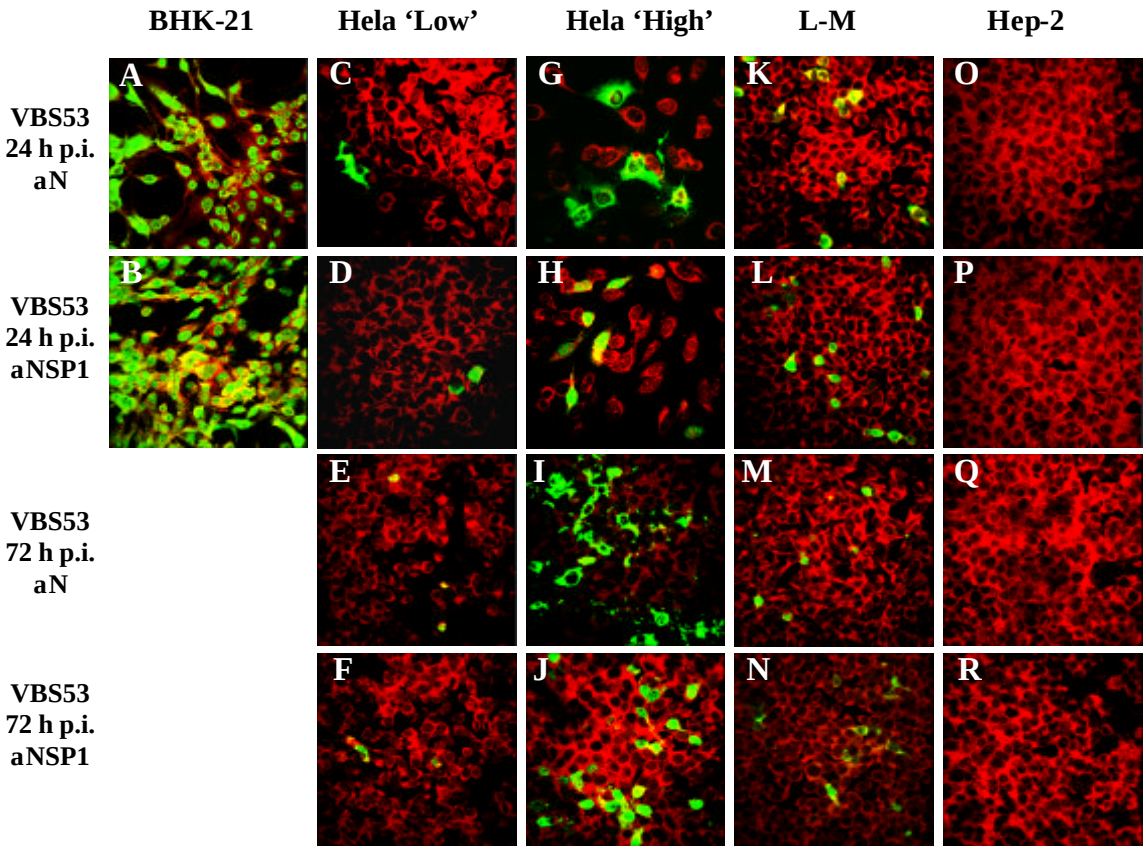


Fig 2.3 Indirect immunofluorescence assays on cells infected with the VBS53 strain of EAV. At 24 and 72 h post infection, EAV-infected cells grown in 8-chamber slides were fixed with cold acetone for 10 min, and then washed three times with PBS plus 10mM glycine. Slides were incubated with MAb 3E2 against EAV nucleocapsid protein (A, C, G, K, O for 24 h p.i., and E, I, M, Q for 72 h p.i.) or MAb 12A4 against EAV NSP1 (B, D, H, L, P for 24 h p.i., and F, J, N, R for 72 h p.i.) followed by FITC-conjugated goat antimouse immunoglobulin. The cells were counterstained by Evans blue. The images were recorded with a LEICA confocal microscope.

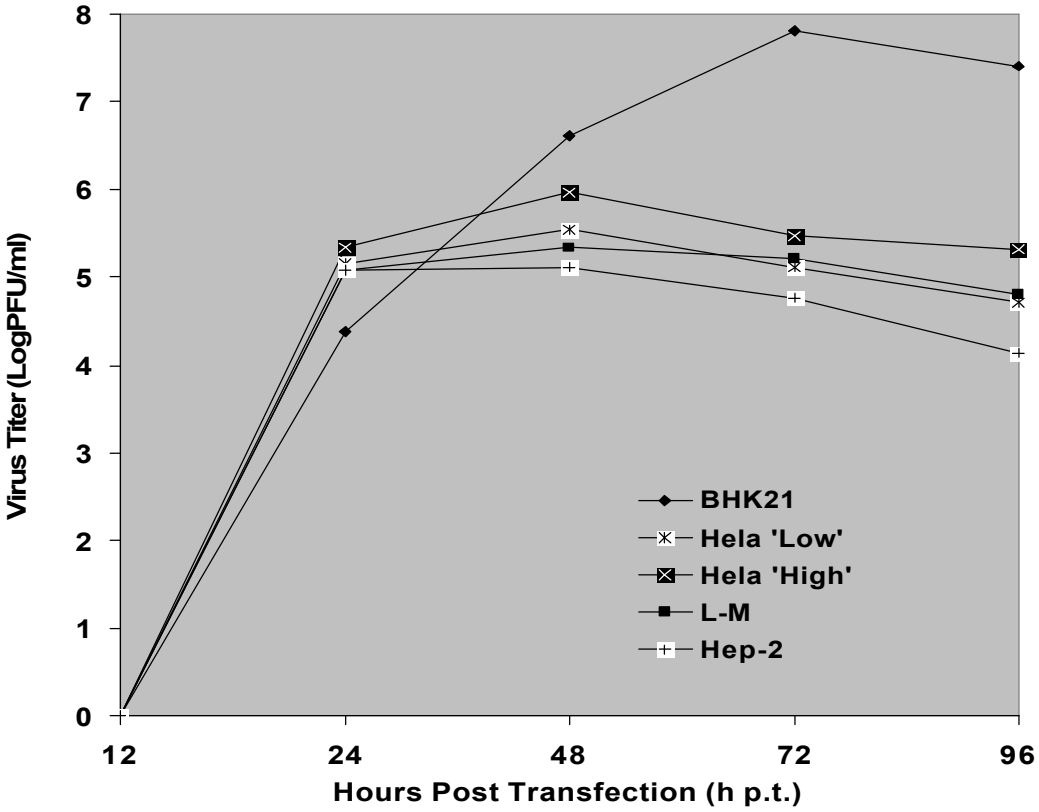


Fig 2.4 Virus yield in cells transfected with viral RNA *in vitro* transcribed from an infectious EAV cDNA clone pEAV2421/211EB. BHK-21, Hela 'Low', Hela 'High', Hep-2, and L-M cell lines were transfected with *in vitro*-transcribed viral RNA by means of electroporation (see Materials and Methods). At 12, 24, 48, 72, and 96 h post transfection, the supernatants were harvested and titrated by plaque assay on RK-13 (KY) cells.

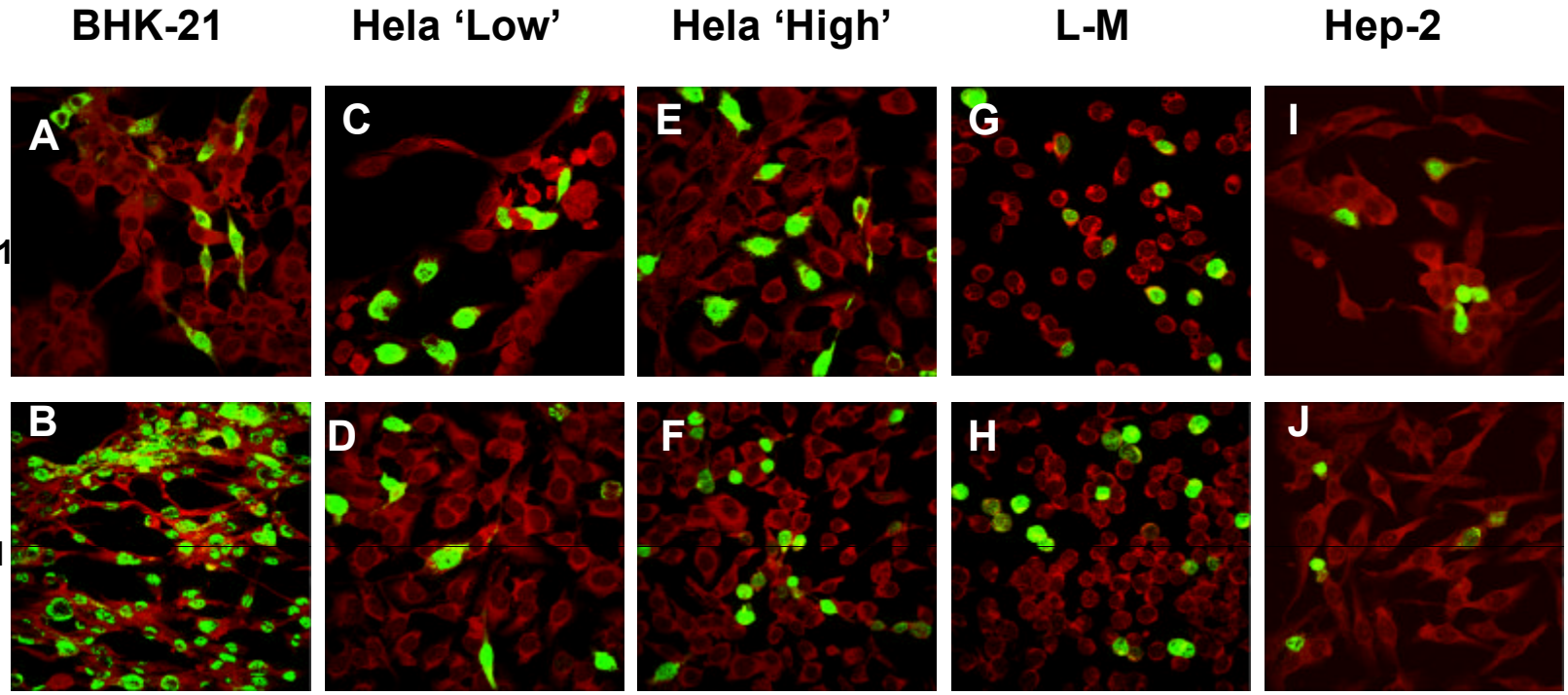


Fig 2.5 Indirect immunofluorescence assays on cells transfected with *in vitro*-transcribed viral RNA. At 24 and 48 h post transfection, viral RNA-transfected cells grown in 8-chamber slides were fixed with cold acetone for 10 min, and then washed three times with PBS plus 10mM glycine. Slides were incubated with MAb 12A4 against EAV NSP1 (A-J) or MAb 3E2 against EAV nucleocapsid protein (data not shown) followed by FITC-conjugated goat antimouse immunoglobulin. The cells were counterstained by Evans blue. The images were recorded with a LEICA confocal microscope.

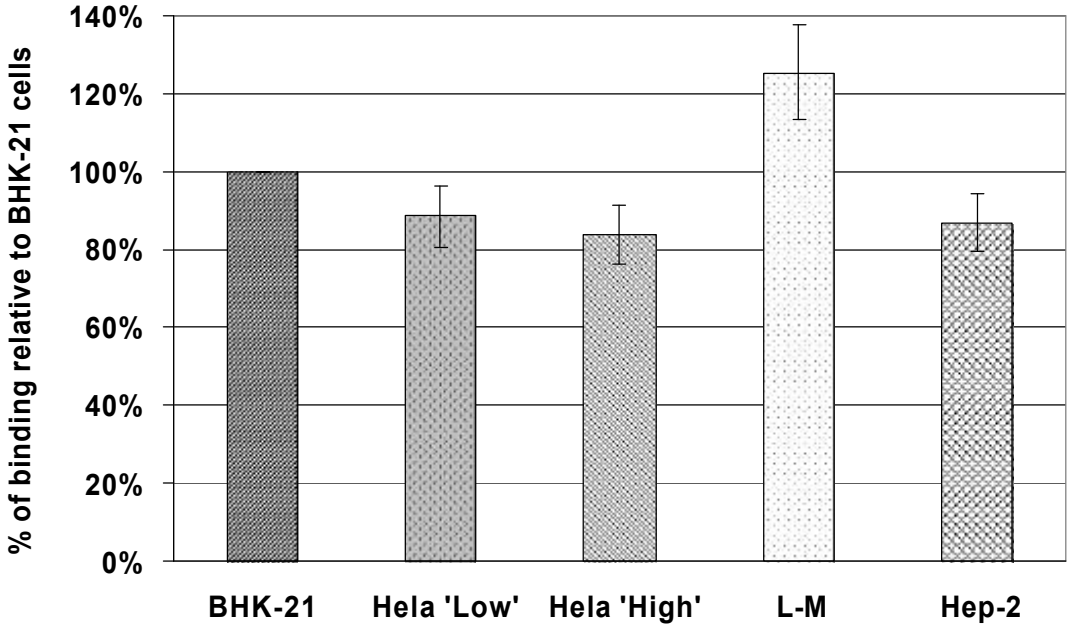
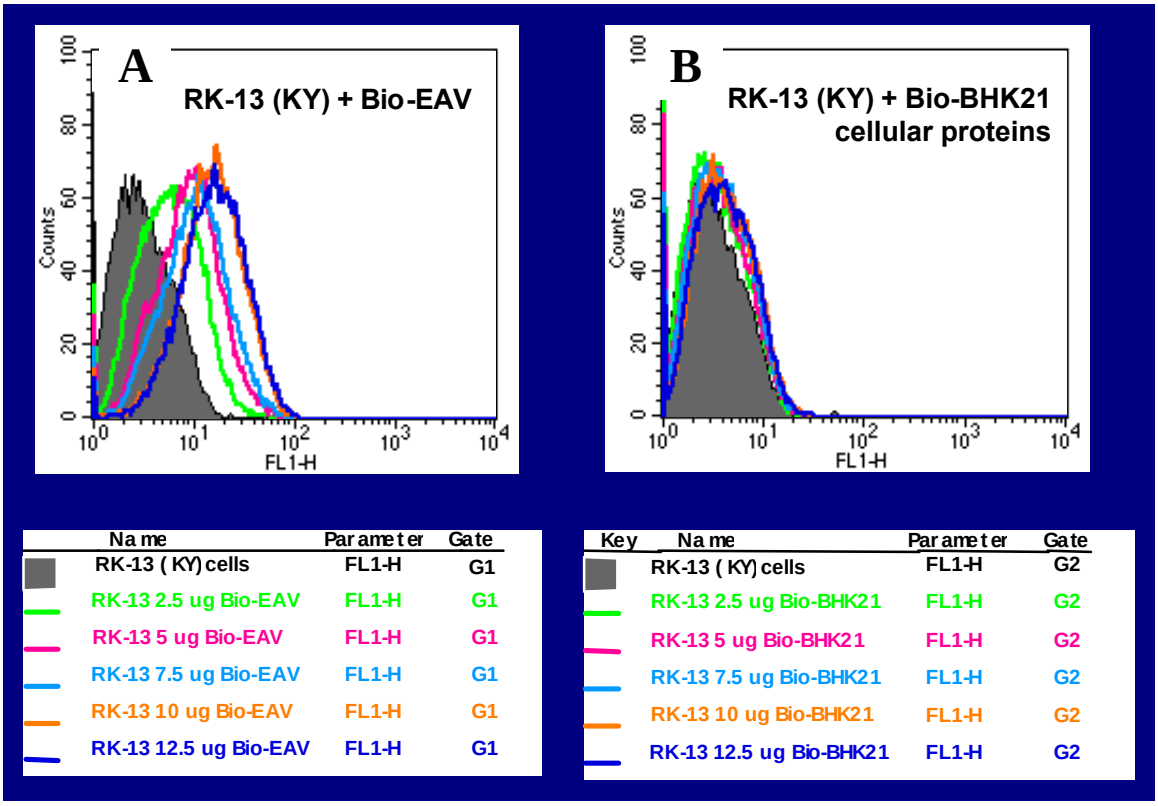
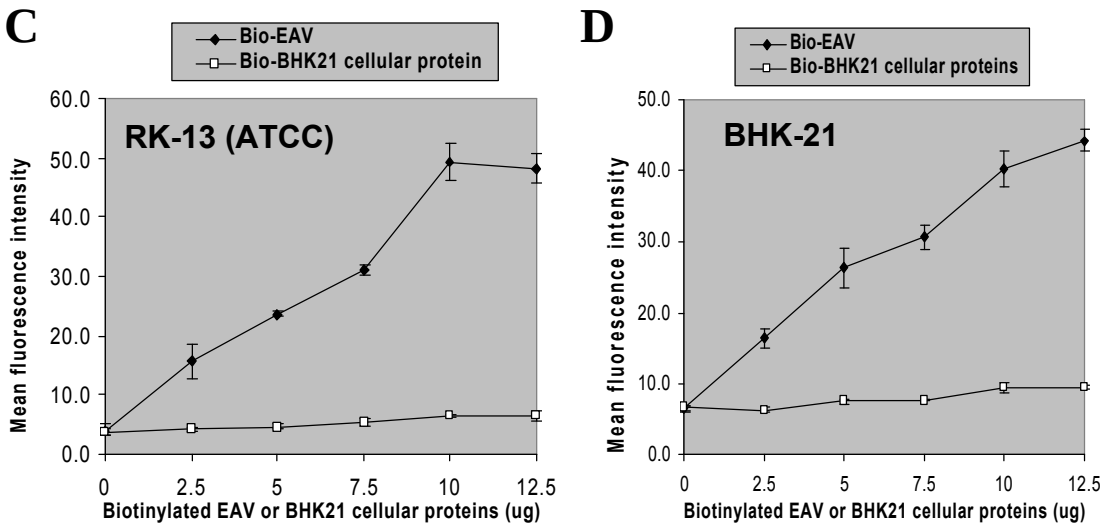


Fig 2.6 Binding of the VBS53 strain of EAV to various cell lines. Subconfluent monolayers of BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cell lines were trypsinized and resuspended in culture medium at a concentration of 2 x 10^6 cells per ml, and cooled down at 4ºC for at least 30 min. For each cell line, 2 x 10^6 PFU of EAV (in 200 µl) were added to duplicates of 1 x 10^6 cells (500 µl) and maintained at 4ºC for 1 h. The cells were washed three times with cold PBS to remove unbound virus and then resuspended in 1 ml of cold culture medium. The cell suspensions were subjected to three cycles of freezing and thawing to release attached viruses, and centrifuged for 10 min at 1900 rpm at 4ºC. The clarified supernatants were titrated by plaque assay on RK-13 (KY) cells. The amount of virus attached to Hela 'High', Hela 'Low', Hep-2, and L-M cells was calculated as percentage of the virus attached to BHK-21 cells. Mean ± Standard Deviation is shown.


A
RK-13 (KY) + Bio-EAV
Counts
FL1-H
B
RK-13 (KY) + Bio-BHK21 cellular proteins
Counts
FL1-H
Name Parameter Gate
RK-13 (KY) cells FL1-H G1
RK-13 2.5 ug Bio-EAV FL1-H G1
RK-13 5 ug Bio-EAV FL1-H G1
RK-13 7.5 ug Bio-EAV FL1-H G1
RK-13 10 ug Bio-EAV FL1-H G1
RK-13 12.5 ug Bio-EAV FL1-H G1
Key Name Parameter Gate
RK-13 (KY) cells FL1-H G2
RK-13 2.5 ug Bio-BHK21 FL1-H G2
RK-13 5 ug Bio-BHK21 FL1-H G2
RK-13 7.5 ug Bio-BHK21 FL1-H G2
RK-13 10 ug Bio-BHK21 FL1-H G2
RK-13 12.5 ug Bio-BHK21 FL1-H G2


C
Bio-EAV
Bio-BHK21 cellular protein
RK-13 (ATCC)
Mean fluorescence intensity
Biotinylated EAV or BHK21 cellular proteins (ug)
D
Bio-EAV
Bio-BHK21 cellular proteins
BHK-21
Mean fluorescence intensity
Biotinylated EAV or BHK21 cellular proteins (ug)

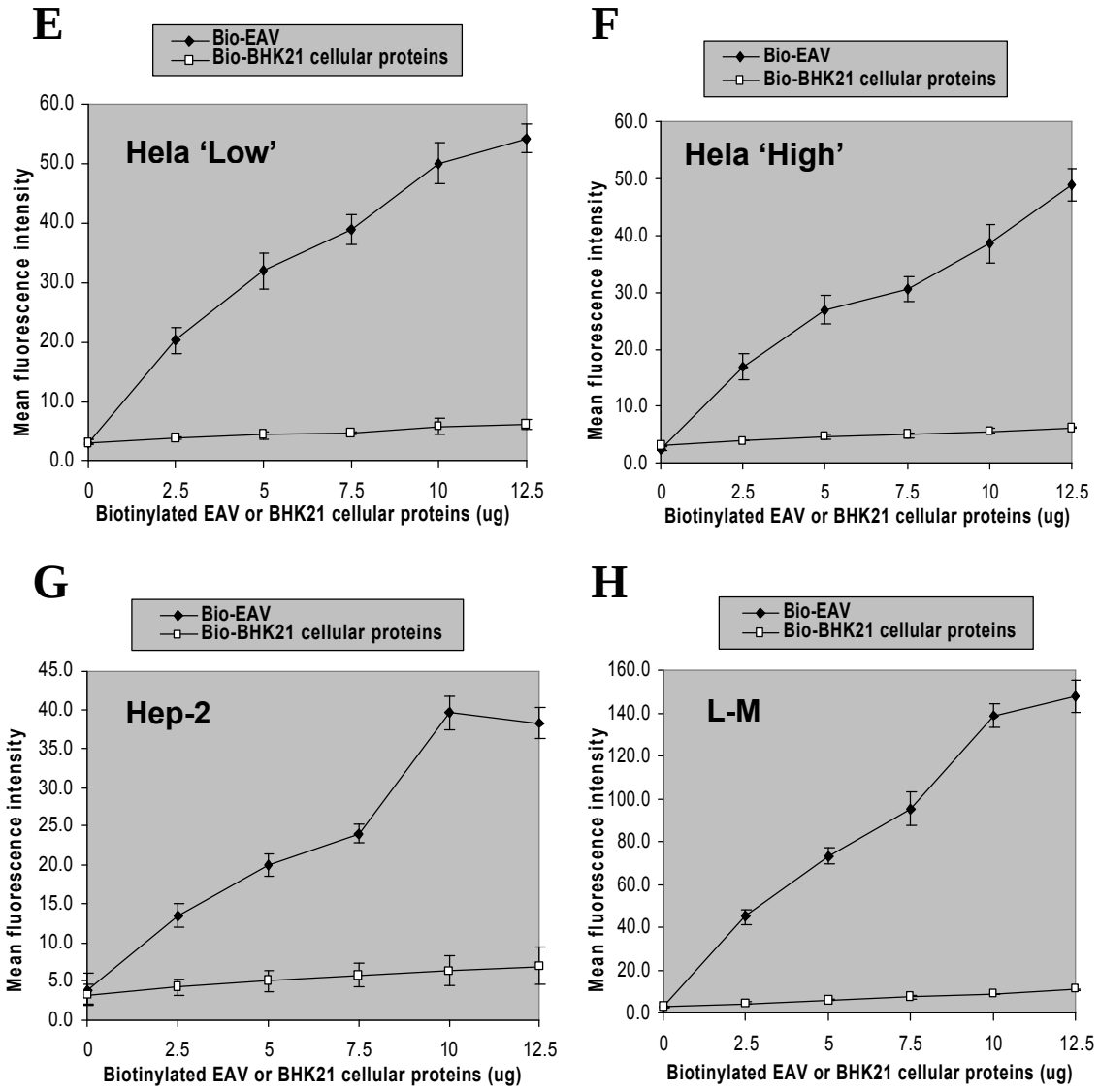


Fig 2.7 Binding of biotinylated EAV strain VBS53 to various cell lines as determined by flow cytometry. Suspensions of RK-13 (KY), RK-13 (ATCC), BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cells were inoculated with different amounts (0, 2.5 µg, 5.0 µg, 7.5 µg, 10.0 µg, and 12.5 µg) of biotinylated EAV or biotinylated BHK-21 cellular proteins (control). Following 1 h of adsorption at 4ºC, cells were washed with PBSA and incubated with streptavidin-fluorescein conjugate for 1 h at 4ºC. The cells were washed and fluorescence intensity of each sample was analyzed by flow cytometry. In the case of each sample, 30,000 cells were counted and dead cells were excluded through propidium iodide staining. Binding of biotinylated EAV or biotinylated BHK-21 cellular proteins to RK-13 (KY) cells were shown in (A) and (B), respectively. The binding of biotinylated EAV or biotinylated BHK-21 cellular proteins to other cell lines were converted to another format as shown in (C-H): RK-13 (ATCC) (C), BHK-21 (D), Hela 'Low' (E), Hela 'High' (F), Hep-2 (G), and L-M (H). In (C-H), Mean ± Standard Deviation is shown.

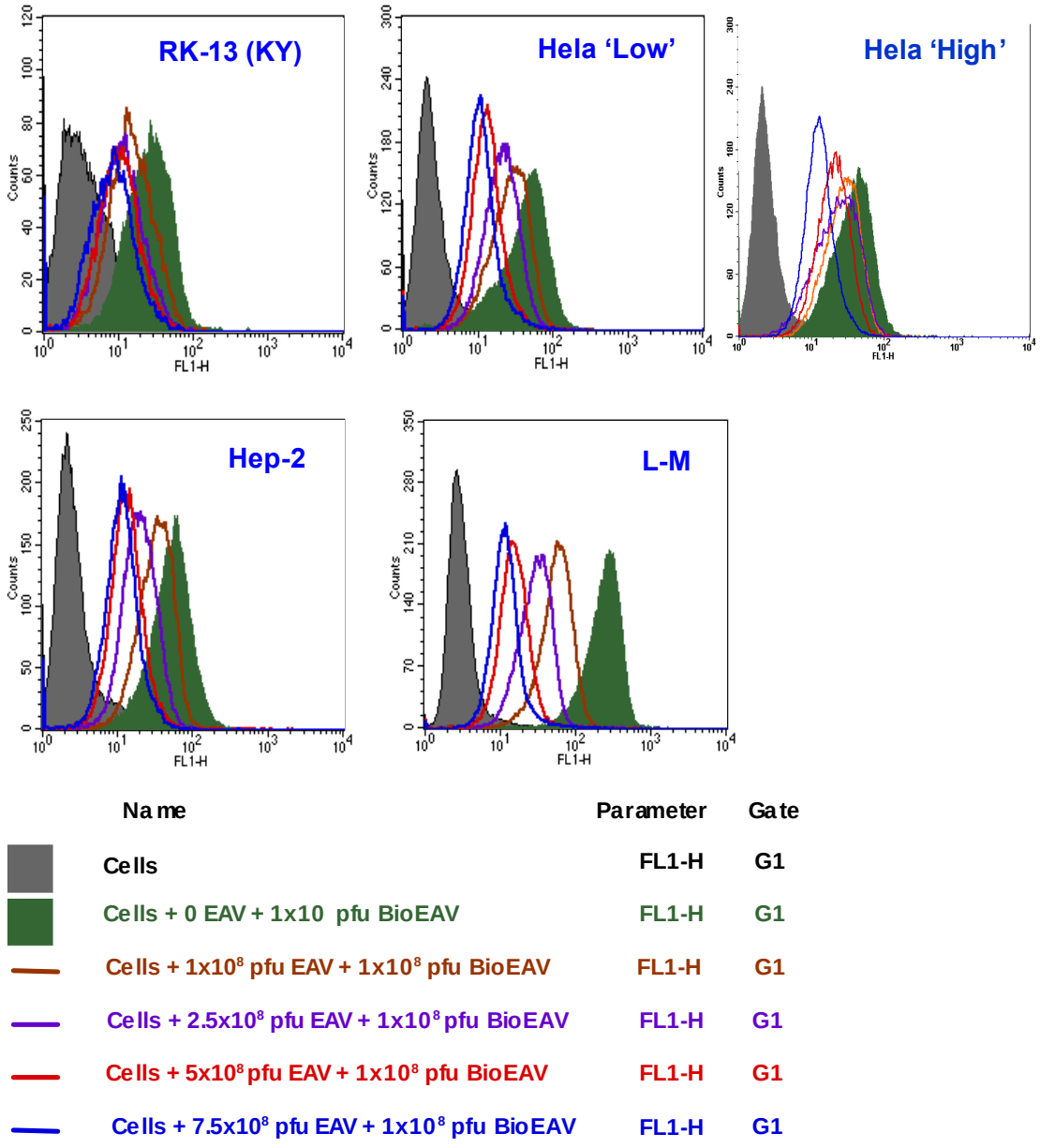


Fig 2.8 Binding of biotinylated EAV strain VBS53 to various cell lines was competitively inhibited by nonbiotinylated EAV. Suspensions of RK-13 (KY), Hela 'High', Hela 'Low', Hep-2, and L-M cells were first incubated at 4ºC for 1 h with different amounts of nonbiotinylated EAV (0, 1×10^8 pfu, 2.5×10^8 pfu, 5×10^8 pfu, 7.5×10^8 pfu) and then incubated with 1×10^8 pfu biotinylated EAV for another 1 h at 4ºC. The amount of biotinylated virus attached to cells was determined by flow cytometry as described above.

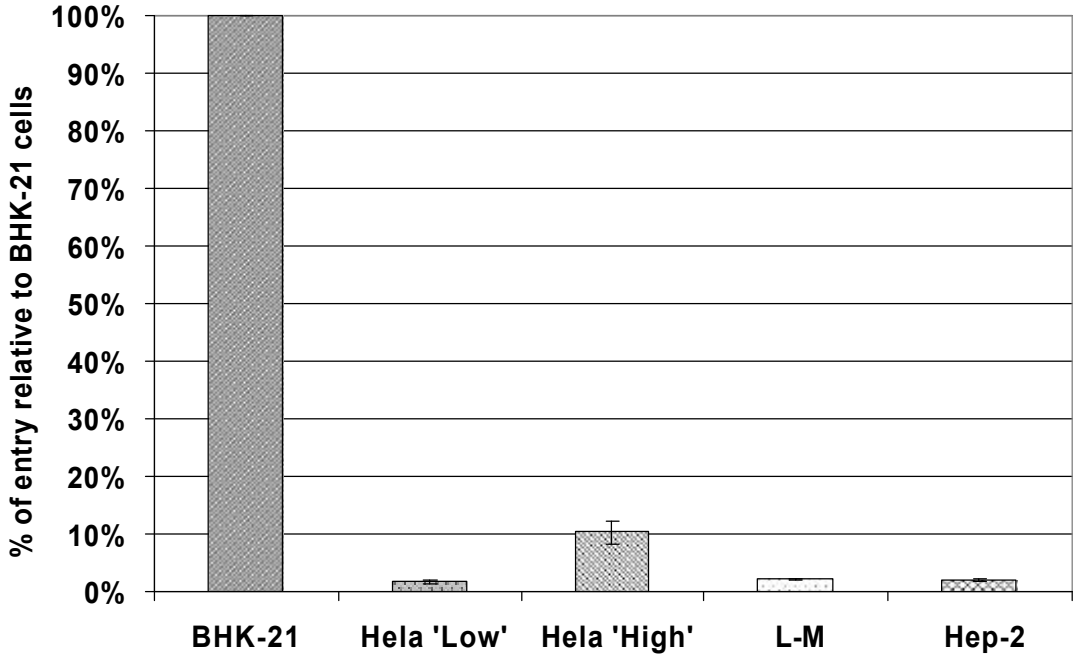


Fig 2.9 Entry of EAV strain VBS53 into various cell lines. Subconfluent monolayers of BHK-21, Hela 'High', Hela 'Low', Hep-2, and L-M cells were trypsinized and resuspended in culture medium at a concentration of 2 x 10^6 cells per ml, and cooled down at 4ºC for at least 30 min. For each cell line, 2 x 10^6 PFU of EAV (in 200 µl) were added to duplicates of 1 x 10^6 cells (500 µl) and maintained at 4ºC for 1 h to allow for virus attachment. The cells were washed three times with cold PBS to remove unbound virus and then resuspended in 1 ml of pre-warmed culture medium and incubated at 37ºC for 1 h with agitation to allow for virus entry. After 1 h of incubation at 37ºC, the cells were treated with 1.0 ml acid glycine (pH 3.0) to inactivate non-internalized virus. Then cells were washed four times with PBS and resuspended in 1 ml of culture medium. The last wash was saved for titration. Serial decimal dilutions of the cell suspensions were made in culture medium and plated onto monolayers of RK-13 (KY) cells. Inoculated cultures were incubated at 37ºC for 1 h. Cell monolayers were overlaid with culture medium containing 0.75% carboxymethyl cellulose and incubated at 37ºC for 4 days. Infectious centers were counted after staining of plates with 10% formalin buffered crystal violet. Efficiency of virus entry into Hela 'High', Hela 'Low', Hep-2, and L-M cells was calculated as a ratio of infectious center counts in each cell line being evaluated to infectious center counts in BHK-21 cells. Mean ± Standard Deviation is shown.

Chapter Three

Establishment and Characterization of Persistent Equine Arteritis Virus Infection in Hela Cells

ABSTRACT

It has been previously demonstrated that BHK-21, RK-13, and C2C12 cell lines are fully susceptible, Hela 'High' cell line (P170-221) is partly susceptible, and Hep-2, L-M, and Hela 'Low' (P95-115) cell lines are non-susceptible, to infection with the VBS53 strain of EAV (Chapter 2). Furthermore, it was shown that the VBS53 strain of EAV was able to establish persistent infection in Hela 'High' cell line, but unable to establish persistent infection in either BHK-21, RK-13, or C2C12 cell lines in which EAV infection was highly cytolytic, with all the cells dead by 48 h post infection. Persistent infection also could not be established in Hep-2, L-M, or Hela 'Low' cell lines in which virus could no longer be detected after two passages. Similarly, transfection with *in vitro*-transcribed viral RNA was capable of initiating persistent infection only in the Hela 'High' cell line but not in BHK-21, Hep-2, L-M, or the Hela 'Low' cell lines. Persistently infected Hela 'High' cell line had the following characteristics: i) The persistently infected cells had been subcultured and maintained for over 3 years. ii) Persistently infected cell monolayers underwent recurrent episodes of partial destruction and subsequent recovery over the period under study. iii) Over the course of the study, the percentage of EAV fluorescent-positive cells in the persistently infected monolayers varied from 1% to 37%. In most passage levels, the percentage of virus-positive cells ranged between 15% and 25%. iv) Infectious virus was continuously produced throughout the course of persistent infection. Virus infectivity titers varied from 10^2 to 10^6 plaque forming units (pfu) per ml but in most passage levels tested, the titer ranged from 10^4 to 10^6 pfu per ml. In all cases, the yield of cell-associated virus was comparable to that of extracellular virus. v) After cryopreservation for 3 years in liquid nitrogen, revived cultures still could produce infectious virus. Virus infectivity titers were similar before and after freezing of the cell line. vi) An attempt to clone a single cell that could continuously produce infectious virus was unsuccessful. vii) Addition of EAV neutralizing antibody-positive equine serum to the medium in which the persistently

infected cell line was cultivated resulted in the elimination of the persistent infection. The viruses recovered from the 35th passage (Hela-EAVP35) and the 80th passage (Hela-EAVP80) of the persistently infected Hela 'High' cell line grew more efficiently than the VBS53 strain of EAV in Hela 'Low' and Hela 'High' cells. EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 grew with the similar efficiency in BHK-21 cells. No significant differences were observed in the growth of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 in Hep-2 and L-M cell lines, respectively. Notwithstanding the fact that the VBS53 strain of EAV can only establish persistent infection in the Hela 'High' cell line but not in the Hela 'Low' cell line, Hela-EAVP35 and Hela-EAVP80 virus strains were able to establish persistent infection in both Hela 'High' and Hela 'Low' cell lines. Neither of these strains, however, was able to establish persistent infection in BHK-21, Hep-2, and L-M cell lines. Entire genome sequencing of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 revealed nucleotide and deduced amino acid changes during the course of persistent infection. The entire genome of each of these three EAV strains was 12,704 nucleotide (nt) in length. The nucleotide differences between EAV strains VBS53 and Hela-EAVP35, VBS53 and Hela-EAVP80, Hela-EAVP35 and Hela-EAVP80 totaled 26 nt, 34 nt, and 16 nt, respectively. The replicase polyprotein and the structural proteins had 15 aa, 17 aa, and 6 aa differences, respectively, between EAV strains VBS53 and Hela-EAVP35, VBS53 and Hela-EAVP80, and Hela-EAVP35 and Hela-EAVP80. The majority of deduced amino acid changes involved EAV structural proteins E, GP2b, GP3-5 encoded by ORFs2a, 2b, and 3-5, respectively. In conclusion, an *in vitro* model of EAV persistence in cell culture was established for the first time. Properties of the model system were characterized. The strain of EAV that evolved during establishment and maintenance of persistent infection in Hela cells was phenotypically and genetically analyzed.

INTRODUCTION

Equine arteritis virus (EAV) is the prototype member of the family *Arteriviridae* in the order *Nidovirales* (Cavanagh 1997). The genome of equine arteritis virus is a single-stranded, positive-sense RNA molecule of 12.7 kb, which includes nine functional open reading frames (ORFs) (Snijder and Meulenberg 1998; Snijder *et al.* 1999). The first two ORFs (1a and 1b) are located at the 5'-terminal three-quarters of the genome and encode viral replicases; the other seven ORFs (2a, 2b, and 3-7) are located at the 3' one-quarter of the genome and encode structural proteins E, GP2b (Gs), GP3, GP4, GP5 (G_L), M, and N, respectively (den Boon *et al.* 1991; Snijder *et al.* 1999). The ORF1a and ORF1b are translated from the genomic RNA into two replicase polyproteins which are proteolytically processed into 12 nonstructural proteins (nsp1-12) and multiple processing intermediates required for viral replication and transcription (den Boon *et al.* 1991; Snijder and Meulenberg 1998; Ziebuhr *et al.* 2000). The structural proteins are expressed from 5'- and 3'-coterminal nested set of six subgenomic mRNAs (de Vries *et al.* 1990).

EAV is the causative agent of equine viral arteritis (EVA), a globally distributed infectious disease of equids. Most EAV infections are subclinical in nature; occasionally infection results in respiratory or systemic illness, abortion/stillbirths in pregnant mares, and interstitial pneumonia, enteritis, or pneumonoenteritis in young foals (Glaser *et al.* 1997; Timoney and McCollum 1993; 1996). After natural infection with EAV, up to 30% to 70% of stallions can subsequently become carriers and these constantly shed the virus in their semen (Timoney and McCollum 1993). Persistently infected stallions are the principal reservoir of EAV and are responsible for perpetuation and dissemination of EAV in equine populations (Balasuriya and MacLachlan 2004c; Timoney and McCollum 1993). Carrier stallions are also thought to be a significant natural source of genetic and phenotypic diversity of EAV (Balasuriya *et al.* 1999a; 2004a; Hedges *et al.* 1999a). There is convincing evidence that establishment and maintenance of the carrier state in the stallion is testosterone-dependent with the detailed mechanism as yet unknown (Little *et al.* 1991; McCollum *et al.* 1994). What host factor(s) (except testosterone) or viral factor(s) contribute to the establishment and maintenance of persistent EAV infection in stallions remain to be determined. Geographically and temporally distinct EAV isolates

can vary markedly in the incidence and severity of the clinical disease they induce and in their abortigenic potentials (Balasuriya *et al.* 1998; 1999b; Balasuriya and MacLachlan 2004c; McCollum and Timoney 1999; Timoney and McCollum 1993). The genetic determinants of virulence have not yet been defined for EAV.

The ability of many viruses to establish persistent infection in cell culture has been documented. Such infections have been used to study virus-host cell interactions. Also, they are a useful tool for investigating virus and cell evolution (Ahmed *et al.* 1981; Colbere-Garapin *et al.* 1989; de la Torre *et al.* 1988a; 1989; Kaplan *et al.* 1989). Persistent infection in cell culture could possibly serve as a model system for elucidating mechanisms of viral persistence *in vivo*. In fact, genetic and phenotypic characterizations of virus mutants and host cell mutants during persistent infection have already greatly helped in the study of virus-receptor interactions (Borzakian *et al.* 1993; Calvez *et al.* 1993; Colbere-Garapin *et al.* 1989; de la Torre *et al.* 1989; Duncan *et al.* 1998; Gosselin *et al.* 2003; Pavio *et al.* 1996; 2000; Pelletier *et al.* 1991; 1998a). Such studies have also served to define virus virulence gene (Couderc *et al.* 1993; 1994; Diez *et al.* 1990a; 1990b), and to identify viral and host cell determinants involved in persistent viral infection (Benton *et al.* 1996; Borzakian *et al.* 1993; Calvez *et al.* 1993; Chen *et al.* 1996; Desforges *et al.* 2001; Dryga *et al.* 1997; Duncan *et al.* 1998; Okada *et al.* 1987; Pelletier *et al.* 1998a).

EAV infection is usually highly cytolytic in cell culture, e.g. BHK-21, RK-13, and C2C12 (Snijder and Meulenberg 1998 and Chapter 2). Recently, it has been found that a Hela 'High' cell line (P170-221) is partly susceptible to infection with the VBS53 strain of EAV, whereas Hep-2, L-M, and Hela 'Low' (P95-115) cell lines are non-susceptible to this virus strain (Chapter 2). The present study further demonstrated that the VBS53 strain of EAV was able to establish persistent infection in Hela 'High' cell line but not in Hela 'Low', Hep-2, or L-M cell lines. Properties of the persistent EAV infection in Hela 'High' cell line were characterized. Genetic and phenotypic variations in the virus during the course of persistent infection were investigated. The mechanism and significance of persistent EAV infection in Hela cells are discussed.

MATERIALS AND METHODS

Cells and viruses. The origin and conditions for the cultivation of BHK-21, RK-13, C2C12, Hep-2, L-M, Hela 'Low', and Hela 'High' cell lines have been described in Chapter 2. The origin and passage history of the VBS53 strain of EAV have been described in Chapter 2. The VBS53 strain of EAV was propagated twice in BHK-21 cells to produce virus stocks which were used in an attempt to establish persistent infection *in vitro*. Hela-EAVP35 and Hela-EAVP80 are the virus strains obtained from the 35th and 80th passage of the persistently infected Hela 'High' cell line, respectively.

Antibodies. The development and characterization of monoclonal antibodies to the nucleocapsid protein (N; MAb 3E2) and the non-structural protein 1 (nsp1; MAb 12A4) of EAV have been previously described (MacLachlan *et al.* 1998; Wagner *et al.* 2003). Both MAbs 3E2 and 12A4 were kindly provided by Drs. Udeni Balasuriya and James MacLachlan at University of California, Davis.

Cell counting and cell viability. Before serially passaging cells, cell culture supernatants containing detached cells were transferred to a tube. Cells were trypsinized and the detached cells pooled together to provide a cell suspension. A 100 µl volume of cell suspension was mixed with 100 µl of 0.4% trypan blue and loaded onto a hemocytometer. Dead cells are stained by trypan blue. Cells were counted and cell viability was calculated.

Attempt to establish persistent EAV infection. Subconfluent monolayers of BHK-21, RK-13 (KY), C2C12, Hela 'High' (P171), Hela 'Low' (P98), Hep-2, and L-M cell lines grown in T-25 flasks were inoculated with the VBS53 strain of EAV at an m.o.i. of 3. Following one-hour absorption at 37ºC, the cells were washed three times with PBS and 10 ml of fresh culture medium was added. Inoculated cultures were incubated at 37ºC. Cultures were microscopically monitored daily for the development of cytopathic effect and subcultured once every four days (1:4 split) if any cells survived. Extracellular and cell-associated viruses were harvested separately, and titrated by routine plaque assay in the RK-13 (KY) cell line. Expression of the viral nucleocapsid (N) protein and non-structural protein 1 (NSP1) was examined using an indirect immunofluorescence assay. Attempts to establish persistent infection in Hela 'Low' (P98), Hep-2, and L-M cell lines with EAV strain VBS53 was repeated once. The attempt to establish persistent infection

in the Hela ‘High’ (P196) cell line was repeated twice. Attempts to establish persistent infection in BHK-21, Hela ‘Low’ (P98), Hela ‘High’ (P196), Hep-2, and L-M cell lines with Hela-EAVP35 or Hela-EAVP80 viruses were performed in accordance with protocols described above.

RT-PCR detection of viral RNA. The aim of the study was to establish persistent infection in the Hela ‘High’ (P171) and Hela ‘Low’ (P98) cell lines with EAV strain VBS53. At selected cell culture passages, supernatants and cells were harvested and sonicated three times (20V, 15 sec/time) to fully disrupt the cells and release virus particles. Cell debris was removed by centrifugation and 140 µl of clarified supernatants were used for viral RNA extraction using the QIAamp viral RNA mini kit (Qiagen). The primers used for one-step RT-PCR were based on amplification of a conserved region of ORF1b (Gilbert *et al.* 1997): forward primer 9299P (nt position 9299-9318: 5’CCTGAGACACTGAGTCGCGT3’) and reverse primer 9464N (nt position 9483-9464: 5’CCTGATGCCACATGGAATGA3’). A 10 µl volume of extracted RNA was used for the one-step RT-PCR in a total volume of 50 µl following the instructions of the one-step RT-PCR kit (Qiagen). The reaction mixtures were incubated at 50ºC for 30 min and at 95ºC for 15 min and then were cycled for 40 times as follows: 94ºC for 30 sec, 53ºC for 30 sec, and 72ºC for 1 min. The reaction was terminated by a single extension step at 72ºC for 10 min. A 10 µl volume of the one-step RT-PCR products were analyzed on a 1.8% agarose gel. The predicted PCR products are 185 bp long. After addition of anti-EAV equine serum to the medium of persistently infected cells, RT-PCR analysis of viral RNA at each cell passage level was carried out in accordance with the protocols previously described.

Transfection of cells with *in vitro* transcribed EAV RNA. *In vitro* transcription of viral RNA from the full-length infectious EAV cDNA clone pEAV2421/211EB and transfection of BHK-21, Hela ‘High’ (P200), Hela ‘Low’ (P102), Hep-2, and L-M cell lines with viral RNA have been described previously (Chapter 2). After transfection, the cultures were microscopically monitored daily for the development of cytopathic effect and subcultured once every four days (1:4 split) if any cells survived. Extracellular viruses were harvested and titrated by routine plaque assay in RK-13 (KY) cells.

Expression of the viral nucleocapsid (N) protein and non-structural protein 1 (NSP1) were determined using an indirect immunofluorescence assay.

Cloning of cells. Subconfluent monolayers of the 26th passage of the persistently infected Hela 'High' cell line initiated with the VBS53 strain of EAV were washed, trypisinized, and resuspended in fresh culture medium. After counting, the cells were diluted to a concentration of 10 cells per ml and then distributed into two 96-well plates in the amount of 0.1 ml per well. Plates were incubated at 37ºC in the presence of 5% CO2. Individual wells were inspected and culture medium was supplemented until the cell monolayer reached confluence. Supernatants from those wells which initially contained a single cell were titrated by plaque assay in RK-13 (KY) cells.

Effect of anti-EAV equine serum on persistent EAV infection. EAV neutralizing Ab-positive equine serum used in the study was the O.I.E affirmed 'medium positive reference serum standard (BL394080, produced in 1993)'. On multiple repeat testing, the reference serum standard had a neutralizing antibody titer of 1:32 when titrated against the attenuated vaccine strain of equine arteritis virus (ARVAC®, FDAH). The 81st serial passage of the persistently infected Hela 'High' cell line initiated with the VBS53 strain of EAV was subcultured (1:4 split ratio) and grown in Eagle's minimum essential medium (EMEM) with 10% ferritin supplemented calf serum (FSCS), and EMEM with 10% EAV-neutralizing antibody-positive equine serum, respectively. Serial subcultivation of the persistently infected Hela cells grown in each medium was carried out once every four days (1:4 split ratio). From the 88th passage, EAV Ab-positive equine serum was removed from the medium of one replicate of the culture which was further serially passed up to the 94th passage. The other replicate of the cell culture continued to be passed in the presence of EAV Ab-positive equine serum up to the 94th passage. At each passage level, extracellular and cell-associated virus containing materials were harvested and titrated by plaque assay. Expression of the viral nucleocapsid (N) protein was investigated using an indirect immunofluorescence assay. Also, RT-PCR analysis of viral RNA was also carried out at each passage level.

Indirect immunofluorescence assay (IFA). The indirect immunofluorescence assay was performed as previously described (Chapter 2).

Growth of different EAV strains in various cells. Growth of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 in BHK-21, Hela 'High' (P198), Hela 'Low' (P100), Hep-2, and L-M cell lines was compared. Subconfluent monolayers of each cell line grown in T-25 flasks were inoculated with the respective viruses at an m.o.i. of 3. Flasks were incubated at 37°C for 1 h. The inoculum was aspirated off, and cell sheets washed three times with PBS to remove unbound virus and the sheets then overlaid with 10 ml of EMEM medium. This was designated time zero with respect to infection. Inoculated cultures were incubated at 37°C. At 0, 24, 48, 72, and 96 h post infection (p.i.), supernatants were harvested and viral infectivity was determined by plaque assay on cultures of the RK-13 (KY) cell line as previously described (McCollum *et al.* 1962).

Isolation of viral RNA, RT-PCR amplification and sequencing. Viral RNA was directly isolated from EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 using the QIAamp viral RNA mini kit (Qiagen). The complete genomes of these three viruses were amplified in nine overlapping fragments (**Appendix 1**). Viral RNA was first reverse transcribed with Superscript II (Invitrogen) into cDNA following the manufacture's instructions. Then, PCR amplification was performed with high-fidelity proof-reading *PfuTurbo* DNA polymerase (Stratagene) following optimized thermocycling protocols (**Appendix 2**). The PCR products were gel-purified using QIAquick gel extraction kit (QIAGEN). Both sense and anti-sense strands were sequenced with a total of 76 primers (**Appendix 3**) using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and Half-Dye Mix (Bioline) following the optimized cycle sequencing protocols (**Appendix 4**). After cycle sequencing, extension products were purified using the ethanol/EDTA precipitation method and then resuspended in template suppression reagent (TSR). The samples were then loaded onto ABI PRISM 310 Genetic Analyzer. Sequence data were analyzed with Vector NTI Suite V.7 software and summarized in **Appendices 5 to 13**.

RESULTS

The VBS53 strain of EAV is capable of establishing persistent infection in Hela 'High' cell line but not in BHK-21, RK-13, C2C12, Hela 'Low', Hep-2 and L-M cell lines. It has been previously demonstrated that BHK-21, RK-13, and C2C12 cell lines are

fully susceptible, the Hela 'High' cell line (P170-221) is partly susceptible, and Hep-2, L-M, and Hela 'Low' (P95-115) cell lines are non-susceptible, to infection with the VBS53 strain of EAV (Chapter 2). EAV infection of BHK-21, RK-13, and C2C12 cell lines is highly cytolytic leading to the death of all cells by 48 h post infection. However, inoculation of Hela 'High', Hela 'Low', Hep-2, and L-M cell lines with the VBS53 strain of EAV did not result in any apparent cytopathic effects (CPE) throughout the course of a one-step growth curve up to 96 h post infection (Chapter 2). In the case of each cell line, inoculated cells appeared to be morphologically indistinguishable from mock-inoculated cells. Surprisingly, it was possible to initiate persistent infection with the VBS53 strain of EAV in Hela 'High' (P171) cells and these continuously produced infectious virus even after 94 serial passages (Fig 3.1). The ability of EAV strain VBS53 to establish persistent infection in the Hela 'High' cell line was confirmed in two further experiments in Hela P196 cells (data not shown). In contrast, after two serial passages, no infectious virus could be detected in Hela 'Low' (P98), Hep-2, and L-M cell lines inoculated with EAV strain VBS53 (Table 3.1), indicating that persistent infection was not established in these cell lines. In order to determine whether any noninfectious virus was present, the Hela 'Low' (P98) cell line inoculated with EAV strain VBS53 and subsequent passages derived from it were examined for the presence of viral proteins and viral RNA. On IFA, few cells were positive for the nucleocapsid protein (Fig 3.2) and non-structural protein 1 (nsp1) (data not shown) at the 1st passage level of EAV-inoculated Hela 'Low' cell line. No viral specific fluorescence was detected from the 2nd to 20th passage of EAV-inoculated Hela 'Low' cell line. In contrast, expression of viral proteins was detected by IFA throughout the course of persistent infection in the Hela 'High' cell line (Fig 3.2), although the percentage of EAV fluorescent-positive cells fluctuated. On RT-PCR analysis for viral RNA, EAV RNA was detected throughout the course of persistent infection in the Hela 'High' cell line (Fig 3.3A), whereas viral RNA was only detected at the 1st and 2nd passages, and not from the 3rd to 20th passages, of the EAV-inoculated Hela 'Low' cell line (Fig 3.3B). Collectively, these findings confirm that the VBS53 strain of EAV is able to establish persistent infection in the Hela 'High' cell line, but unable to do so either in BHK-21, RK-13, C2C12 cell lines in which all the cells

eventually die due to the cytolytic effects of the virus, or in the Hela 'Low', Hep-2, and L-M cell lines in which cells survive without continuous production of virus.

Transfection with viral RNA is capable of initiating persistent infection in the Hela 'High' cell line but not in BHK-21, Hep-2, L-M, and Hela 'Low' cell lines. In order to determine whether failure to establish persistent EAV infection in Hela 'Low', Hep-2, and L-M cell lines was due to the fact that the virus cannot achieve cell entry, various cell lines were transfected with viral RNA *in vitro*-transcribed from an infectious EAV cDNA clone pEAV2421/211EB (Balasuriya *et al.*, unpublished). In the case of transfected BHK-21 cells, cytopathic effects appeared and progressed from 72 h post transfection with all the cells had died by 96 h after transfection. In contrast, no CPE was observed in transfected Hela 'High', Hela 'Low', Hep-2, and L-M cell lines up to 96 h post transfection, although infectious virus production was detected in these transfected cells as shown in Table 3.2. Serial passaging of these transfected cell lines resulted in two distinct outcomes. Serial subculture of transfected Hela 'High' cells led to establishment of persistent infection in which infectious virus was produced throughout the observation period (from passage 1 to passage 10) (Table 3.2). Whereas no apparent CPE was observed from passage 1 to passage 5 in this line, cell destruction of limited extent was observed from passage 6 to passage 10. On the other hand, one or two more subcultivations of transfected Hep-2, L-M, and Hela 'Low' cells resulted in loss of infectious virus production (Table 3.2), indicating that persistent infection was not established in these cell lines. No cell destruction was observed in transfected Hep-2, L-M, and Hela 'Low' cell lines throughout the observation period (from passage 1 to passage 10).

Characterization of persistently infected Hela 'High' cells. Persistently infected Hela 'High' cells initiated with the VBS53 strain of EAV were characterized by the following properties. **i)** The persistently infected cells had been subcultured and maintained for over 3 years. **ii)** Persistently infected cultures underwent recurrent episodes of partial destruction and subsequent recovery (Table 3.3). For example, in passages 1 and 2, no apparent cell destruction was observed; from passage 3 to passage 5, a degree of cell destruction was observed; from passage 6 to passage 9, cells gradually recovered and the extent of cell destruction decreased compared to that observed in

passages 4 and 5; some cell destruction was observed again from passage 10 to passage 14; cells again gradually recovered from passage 15 to passage 18; such recurrences of partial destruction and subsequent recovery of cells continued throughout the observation period. The percentage of dead cells (as determined by trypan blue staining) during periods of cell destruction was higher than that during periods of cell recovery. **iii)** During the course of persistent infection, the percentage of EAV fluorescent-positive cells varied from 1% to 37%. But in most passages of the persistently infected culture, the percentage of virus-positive cells ranged between 15% and 25% (Table 3.3). IFA images of persistently infected Hela 'High' cells at randomly selected passages are shown in Fig 3.2. It should be pointed out that cell death and EAV fluorescent-positive cells were determined randomly for some passages but not every passage of the persistently infected Hela 'High' cell line. **iv)** Infectious virus was continuously produced throughout the course of persistent infection with virus infectivity titers varying from 10^2 to 10^6 pfu per ml. The virus infectivity titers ranged from 10^4 to 10^6 pfu per ml in most passages of the persistently infected culture (Table 3.3; Fig 3.1). The yield of cell-associated virus was comparable (at the same log level) to that of extracellular virus at every passage level tested (Table 3.4). The ratio of extracellular virus to cell-associated virus fluctuated from 30:70 to 55:45 (Table 3.4). **v)** After storage for 3 years in liquid nitrogen, the revived cell culture was still able to produce infectious virus with the virus titer similar to what it was prior to freezing. **vi)** An attempt to clone a single cell that could continuously produce infectious virus was unsuccessful. Cells were cloned from the 26^{th} passage of the persistently infected Hela 'High' cell line by distributing an appropriately diluted cell suspension into 96-well plates. Wells were visually examined and only those wells that received a single cell were used. Eventually, 46 clones were obtained. However, none of them were found to produce infectious virus. **vii)** The effect of EAV neutralizing antibody-positive equine serum on persistently infected cells is shown in Table 3.5. The persistently infected Hela 'High' cells were treated with EAV neutralizing antibody-positive equine serum from the 82^{nd} passage. Before treatment with antiserum, the extracellular and cell-associated virus in the persistently infected Hela 'High' cell line was 5.7×10^5 pfu/ml and 9.5×10^5 pfu/ml, respectively. After two serial passages in the presence of antiserum, no infectious virus was detectable in either the supernatants or the

lysates of the culture. The cell culture treated with antiserum was maintained up to the 94th passage during which no infectious virus could be detected. Interestingly, in the case of antiserum-treated cell cultures, expression of viral nucelocapsid protein was undetectable by the IFA test from the 83rd to the 94th cell culture passage, although viral RNA was still detectable by the RT-PCR from the 83rd to the 85th cell culture passage. This indicated that viral RNA could be present for several additional passages even when infectious virus and viral protein expression were no longer detectable. Following removal of the antiserum from the culture medium at the 88th serial passage, no renewed virus replication, viral antigen expression, or EAV RNA production was detected upon 7 additional passages of Hela 'High' cells. This is evidence that persistently infected Hela 'High' cells could be cured of EAV infection by culturing the cells in the presence of EAV neutralizing antibody-positive equine serum. In contrast, infectious virus, viral protein expression, and viral RNA were consistently detected in the mock-treated cell culture throughout the observation period.

Growth kinetics of persistent viruses in various cell cultures. To determine whether the growth properties of the challenge strain of EAV had changed during the course of persistent infection, growth kinetics of the parental EAV strain VBS53 and viruses recovered from the 35th (Hela-EAVP35) and the 80th (Hela-EAVP80) passage of the persistently infected Hela 'High' cell line were compared in BHK-21, Hela 'High' (P198), Hela 'Low' (P100), Hep-2, and L-M cell lines. In BHK-21 cells (Fig 3.4A), EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 all caused a cytolytic infection. Replication of each virus strain followed a similar growth kinetic curve, with 3-4 $\log_{10}$ increase in infectivity titers within 24-48 h, followed by a gradual decline in titers after 48 h. This indicated that these three EAV strains can cause productive infections and grow with similar efficiency in BHK-21 cells. In both Hep-2 (Fig 3.4B) and L-M (Fig 3.4C) cell lines, the growth of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 was not efficient, having similar growth kinetics for all three strains. On the other hand, in both Hela 'High' (Fig 3.4D) and Hela 'Low' (Fig 3.4E) cell lines, Hela-EAVP35 and Hela-EAVP80 strains grew better and replicated more efficiently than the VBS53 strain of EAV. Hela-EAVP35 and Hela-EAVP80 strains had almost the same replication efficiency in either Hela 'High' (Fig 3.4D) or Hela 'Low' (Fig 3.4E) cells.

Ability of Hela-EAVP35 and Hela-EAVP80 to establish persistent infection in both Hela 'Low' and Hela 'High' cell lines but not in BHK-21, Hep-2, and L-M cell lines. To determine whether virus recovered from persistently infected cells has acquired any new features in establishing persistent infection, an attempt was made to establish persistent infection in BHK-21, Hela 'Low', Hela 'High', Hep-2, and L-M cell lines with Hela-EAVP35 and Hela-EAVP80, respectively. As was the experience with the VBS53 strain of EAV, Hela-EAVP35 and Hela-EAVP80 caused cytolytic infection in BHK-21 cells with all the cells dead by 48 h post inoculation without the establishment of persistent infection. Similar to EAV strains VBS53, Hela-EAVP35 and Hela-EAVP80 were not able to establish persistent infection in Hep-2 and L-M cell lines in which three serial passages led to the loss of virus production (Fig 3.5A). In contrast to the VBS53 strain of EAV, which can only establish persistent infection in the Hela 'High' but not in the Hela 'Low' cell line, Hela-EAVP35 and Hela-EAVP80 were able to establish persistent infections in both Hela 'High' and Hela 'Low' cell lines (Fig 3.5 B and C). This was confirmed by IFA studies (Fig 3.6). In the persistently infected Hela 'High' cell line in which infection was initiated with Hela-EAVP35 or Hela-EAVP80, the virus infectivity titer on 1^{st} passage reached 2×10^6 pfu/ml (Fig 3.5B and C). In contrast, when infection in persistently infected Hela 'High' cell line was initiated with EAV strain VBS53, the virus infectivity titer on 1^{st} passage was less, only reaching 10^4 pfu/ml. It took two or three serial passages for the virus titer to reach 10^6 pfu/ml (Fig 3.1). This confirmed that Hela-EAVP35 and Hela-EAVP80 viruses grow better than EAV strain VBS53 in the Hela 'High' cell line in the course of the initial few passages in cell culture. In Hela 'High' cells persistently infected with Hela-EAVP35 or Hela-EAVP80, apparent cell destruction was observed from the initial passage, whereas cell destruction was not observed until the 6^{th} serial passage in persistently infected Hela 'Low' cells. During the initial number of passages (1^{st} to 5^{th}), virus yields in Hela 'Low' cells were consistently lower than those in Hela 'High' cells persistently infected with the same virus strains (Fig 3.5B and C). Virus strains Hela-EAVP35 and Hela-EAVP80 evidently grow better in Hela 'High' cells than in Hela 'Low' cells over the course of the initial several passages of infection. However, from the 6^{th} and subsequent passages, the level of cell destruction

and virus yields in persistently infected Hela 'Low' cells approximated to those in Hela 'High' cells (Fig 3.5B and C).

Comparative nucleotide and amino acid sequence analysis of the viruses capable of establishing persistent infection in Hela cells. To investigate genetic variation of EAV during persistent infection of Hela cells, the entire genomes of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 were sequenced. Sequences were determined by direct sequencing of RT-PCR fragments and could thus be assumed to reflect the consensus sequence of the virus population at the time of RNA extraction. The full-length nucleotide sequences and the deduced amino acid sequences of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 were compared and the findings summarized in Table 3.6. The entire genomes of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 were all 12,704 nt in length, namely the same length as the published sequence of EAV030. The complete nucleotide (nt) differences between EAV strains VBS53 and Hela-EAVP35, VBS53 and Hela-EAVP80, Hela-EAVP35 and Hela-EAVP80 were 26 nt, 34 nt, and 16 nt, respectively. All of these nucleotide changes occurred at ORFs1a, 1b, 2a, 2b, and 3-5; no nucleotide changes were observed at the 5' non-translated region (NTR 1-224 including 5' leader 1-211), nor in ORFs 6-7 and the 3' NTR (12646-12704). The total number of amino acid differences in the replicase polyprotein and structural proteins between EAV strains VBS53 and Hela-EAVP35, VBS53 and Hela-EAVP80, Hela-EAVP35 and Hela-EAVP80 were 15 aa, 17 aa, and 6 aa, respectively. There were 14 nt, 20 nt, and 10 nt differences in the replicase gene, most of which were silent mutations which only resulted in 3 aa, 4 aa, and 1 aa changes, between EAV strains VBS53 and Hela-EAVP35, VBS53 and Hela-EAVP80, Hela-EAVP35 and Hela-EAVP80, respectively. There were 12 nt, 14 nt, and 6 nt differences in the structural protein genes (ORFs2a, 2b, and 3-5), respectively. Most of these were non-synonymous mutations which resulted in 12 aa, 13 aa, and 5 aa changes between EAV strains VBS53 and Hela-EAVP35, VBS53 and Hela-EAVP80, Hela-EAVP35 and Hela-EAVP80, respectively. The majority of deduced amino acid changes involved EAV structural proteins E, GP2b, GP3-5 which are encoded by ORFs2a, 2b, and 3-5, respectively.

The catalytic dyad (Cys-164 and His-230) of the nsp1 protease, the critical residues (Cys-270, His-332, Cys-319, Cys-349, Cys-354, Cys-344, and Cys-356) of the nsp2

protease, the catalytic triad (His-1103, Asp-1129 and Ser-1184) of the nsp4 protease, the putative substrate-binding region (Thr-1179 and His-1198) of the nsp4 protease, and 11 cleavage sites on the replicase polyprotein 1ab were all conserved in three EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 (Table 3.6; Snijder and Meulenberg 1998; Ziebuhr *et al.* 2000). No potential N-glycosylation site changes were observed with respect to the structural proteins of these three EAV strains.

Five ambiguous nucleotides were observed in EAV strain VBS53: 3707Y, 7021R, 8260Y, 8290Y, and 10722Y (Y is T or C; R is A or G). Because of the existence of degenerate codons, however, the ambiguity of nucleotide at 3707, 7021, 8260, and 8290 did not cause any change to the respective amino acids encoded by them. The ambiguity of nucleotide T or C at 10722 resulted in the ambiguity of amino acid Leu or Ser at position 8 of the GP4 protein. No ambiguous nucleotides were observed in either Hela-EAVP35 or Hela-EAVP80. The degree of homology between Hela-EAVP35 and Hela-EAVP80 (6 aa differences) was higher than that between either of these strains and the VBS53 strain of EAV (15 aa differences between VBS53 and Hela-EAVP35; 17 aa differences between VBS53 and Hela-EAVP80). This would indicate that EAV evolved during the course of persistent infection in the Hela 'High' cell line. Most of the mutations would appear stable. However, reversion of mutations was occasionally observed. For example, the amino acid at position 88 of the GP2b protein of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 was Met, Val, and Met, respectively; the amino acid at position 246 of the GP5 protein was Arg, Lys, and Arg, respectively.

DISCUSSION

Equine arteritis virus infection is usually highly cytolytic in cultured cells, e.g. BHK-21, RK-13, and C2C12 ((Snijder and Meulenberg 1998 and Chapter 2). It has recently been found, however, that Hela 'High' cell line (P170-221) is partly susceptible to infection with the VBS53 strain of EAV, whereas Hep-2, L-M, and Hela 'Low' (P95-115) cell lines are non-susceptible to this virus strain (Chapter 2). In this study the VBS53 strain of EAV was shown to be able to establish persistent infection in a Hela 'High' cell line. To the authors' knowledge, it is the first report of establishment of persistent EAV infection in cell culture.

Characterization of persistent viral infection in cell culture. Persistently infected cell cultures may or may not exhibit virus-specific cytopathic effects despite the fact that they continuously produce infectious virus (Frisk *et al.* 1999; Gercel *et al.* 1985; Kaplan *et al.* 1989; Moscona and Galinski 1990). In this study, it was shown that persistently infected Hela 'High' cells in which infection was initiated with EAV strain VBS53, underwent multiple episodes of partial destruction and subsequent recovery. During the course of persistent infection in the Hela 'High' cell line, the percentage of EAV fluorescent-positive cells varied from 1% to 37% but with the range falling between 15% and 25% in most passages. The observation that the percentage of virus-positive cells can show cyclic variation has also been reported for other persistent systems. For instance, in BHK-21 cells persistently infected with rabies virus, the percentage of cells exhibiting positive immunofluorescence varied from 5% to 100% in cycles of 6-8 subcultures (Wiktor and Clark 1972); in Hela cells persistently infected with human rhinovirus-2, the percentage of cells producing infectious centers varied from 0.03% to 23% (Gercel *et al.* 1985). In contrast to persistent poliovirus infection in Hela cells in which freezing down of cells in liquid nitrogen resulted in the cells losing the ability to produce infectious virus (Kaplan *et al.* 1989), Hela cells persistently infected with EAV were still able to produce infectious virus after being frozen down in liquid nitrogen for 3 years, with the virus titer similar to what it had been prior to freezing (data not shown).

Addition of virus-specific antibodies or anti-viral compounds to the media of persistently infected cells may or may not result in elimination of the persistent infection. It has been shown that cloning or subculture of measles virus/Hela cells in the presence of anti-measles serum in the culture medium led to a state in which no infectious virus was released, whereas the vast majority of these cells were still immunofluorescence positive (Rustigian 1966b), indicating the existence of viral antigen. When human influenza C virus/MDCK cells (Goshima and Maeno 1989) or poliovirus/human erythroblastoid K562 cells (Lloyd and Bovee 1993) were subcultured for several serial passages with growth medium containing homologous virus-neutralizing antibodies, extracellular virus became undetectable. However, upon further passage in the absence of antiserum there was a return of infectious virus yield. In contrast, it was observed that three serial subcultivations of human rhinovirus-2/Hela cells in the presence of antiserum

resulted in a cure (no extracellular virus or infectious centers or viral antigens could be detected) (Gercel *et al.* 1985). In addition, after removal of antiserum, no renewed virus production or viral antigen expression could be detected upon 12 further passages (Gercel *et al.* 1985), indicating that the formerly persistently infected cells were permanently cleared of infection. It was also found that the anti-viral compound ribavirin was able to eliminate foot-and-mouth disease virus (FMDV) from persistently infected BHK-21 cells (de la Torre *et al.* 1987). Addition of EAV neutralizing antibody-positive equine serum to the medium of persistently infected Hela 'High' cells resulted in the elimination of persistent infection. No renewed virus production or viral antigen expression or EAV RNA replication occurred upon 7 further passages in the absence of anti-EAV serum.

Characteristically, persistently infected cells are resistant to superinfection with homologous viruses but are still susceptible to infection with heterologous viruses. For example, Hela cells persistently infected with human rhinovirus-2 (HRV-2) were resistant to superinfection with homologous viruses (HRV-2, HRV-2 TS-1 mutant, and HRV-4) but were susceptible to poliovirus type 2 and vesicular stomatitis virus (VSV) infection (Gercel *et al.* 1985). The virus yields in HRV-2/Hela cells superinfected with HRV-2, TS-1 mutant, or HRV-4 were similar to those produced in HRV-2/Hela cells alone (mock-superinfected) but were less than those produced in normal Hela cells infected with the respective virus. In contrast, virus yields in HRV-2/Hela cells superinfected with poliovirus type 2 or VSV were comparable to those obtained in normal Hela cells infected with the respective virus (Gercel *et al.* 1985). The question whether Hela 'High' cells persistently infected with EAV are resistant to superinfection with EAV was investigated in this study (Table 3.7). Virus yields in persistently infected Hela 'High' cell line which was superinfected with EAV strain VBS53 were comparable to those obtained in persistently infected Hela 'High' cells which were mock-superinfected, but were not less than those produced in normal Hela 'High' cells which were infected with EAV strain VBS53. On the basis of this observation, it was not certain whether persistently infected Hela 'High' cells were resistant to superinfection with EAV. The difference between HRV-2/Hela cells and EAV/Hela 'High' cells is probably caused by the fact that normal Hela cells are fully susceptible to HRV-2 infection, whereas normal Hela 'High' cells are only partly susceptible to infection with EAV strain VBS53.

Virus and cell evolution during persistent infection of cell culture. Equine arteritis virus evolution during persistent infection of the Hela 'High' cell line was observed in this study. Hela-EAVP35 and Hela-EAVP80, isolated from the 35^{th} and 80^{th} passage of persistently infected Hela 'High' cell line, respectively, grew more efficiently than the parental EAV strain VBS53 in Hela 'Low' and Hela 'High' cell lines. Hela-EAVP35 and Hela-EAVP80 were able to establish persistent infections in Hela 'Low' cells, whereas the parental EAV strain VBS53 was unable to do so (Table 3.8). Furthermore, compared to the parental EAV strain VBS53, Hela-EAVP35 and Hela-EAVP80 had different neutralization phenotypes (Chapter 4) and exhibited potential changes in pathogenicity (Chapter 5). Besides these phenotypic changes, direct sequencing of the entire genomes of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 provides clearcut evidence of viral genetic evolution during persistent infection of Hela 'High' cells. It should be pointed out that the entire genome sequences of these three viruses are actually the predominant sequences within the viral quasispecies. In the replicase, amino acid mutations were only observed in nsp1, nsp2, nsp7, and nsp9. In the structural proteins, amino acid changes were found in E, GP2b, GP3, GP4, and GP5 proteins but not in either M or N proteins.

Further studies are needed to determine which amino acid(s) mutations are responsible for the corresponding phenotypic changes. Special attention should be paid to nsp2 Asp-577? Gly, nsp9 Pro-1933? Ser, E Ser-53? Cys, GP2b Leu-15? Ser, GP2b Trp-31? Arg, GP3 Leu-135? Pro, and GP5 Pro-98? Leu.

It is interesting that, compared to the VBS53 strain of EAV, neither Hela-EAVP35 nor Hela-EAVP80 exhibited apparent phenotypic differences (e.g. growth kinetics and the ability to establish persistent infection) in BHK-21, Hep-2, or L-M cell lines (Fig 3.4 and Table 3.8).

The virus-encoded RNA-dependent RNA polymerases (RdRp) are low fidelity enzymes with mutation rates as high as the order of 10^{-3} to 10^{-5} errors per nucleotide per replication cycle (Domingo and Holland 1997; Drake 1993). In addition, RNA viruses have very short replication times and generate very large populations during replication. Therefore, RNA viruses exist not as a single genotype but rather as a heterogeneous mixture of related genomes known as a viral quasi-species (Holland *et al.* 1992). The

quasi-species nature of RNA viruses allows viruses to adapt to environmental changes more rapidly and facilitates the emergence of viral variants (Castro *et al.* 2005). Therefore, it is not surprising that EAV has evolved after long-term persistence in Hela cells. In fact, EAV may also evolve after many passages of cytolytic infection in cell cultures. For example, the parent EAV strain isolated from horses did not infect primary hamster kidney cells; however, when the same parent EAV strain underwent 82 passages of cytolytic infection in horse kidney cells, the obtained virus could be able to infect primary hamster kidney cells (Wilson *et al.* 1962). Also, after many passages of cytolytic EAV infection in cell cultures, the virus had been observed to get attenuated (McCollum 1969).

Cell evolution during persistent EAV infection of Hela cells was not investigated in this study. Previously, it has been demonstrated that the Hela cell line becomes more susceptible to infection with EAV strain VBS53 after extended serial passage (Chapter 2). It is reasonable to postulate that cells would evolve during persistent infection of the Hela 'Low' cell line initiated with Hela-EAVP35 or Hela-EAVP80. The question whether Hela cells cured of persistent infection with anti-EAV serum have different susceptibility from the corresponding passage level of normal Hela cells to EAV infection also needs to be investigated.

Virus and cell evolution have also been observed in other persistent viral infection systems. Genetic and antigenic changes have been demonstrated in hepatitis A virus (HAV) variants arising during persistent infection of BS-C-1 cells (Lemon *et al.* 1991). Foot-and-mouth disease virus isolated from persistently infected BHK-21 cells was more cytolytic than parental FMDV for BHK-21 cells (de la Torre *et al.* 1988a). Further studies revealed amino acid substitutions of the viral capsid protein and antigenicity alteration of FMDV during persistence (Diez *et al.* 1990a). Moreover, FMDV became progressively less virulent for mice and cattle during the course of persistence in BHK-21 cells (Diez *et al.* 1990b). The poliovirus mutants selected from human neuroblastoma IMR-32 cells persistently infected with the Sabin strains could reach 1-3 $\log_{10}$ units higher titer in IMR-32 cells than that in non-neural Hep-2 cells, while parental viruses had similar titers in both cell lines (Colbere-Garapin *et al.* 1989). Correlated with this modified cell tropism, the poliovirus mutants recovered from carrier culture could establish secondary persistent

infection in non-neural Hep-2 cells (Pelletier *et al.* 1991). Cell evolution has been documented in mouse L cells persistently infected with reovirus (Ahmed *et al.* 1981), BHK-21 cells persistently infected with FMDV (de la Torre *et al.* 1988a; 1989; Martin Hernandez *et al.* 1994), and Hela cells (Kaplan *et al.* 1989; Kaplan and Racaniello 1991), human neuroblastoma cells (Pavio *et al.* 2000), and Hep-2 cells (Borzakian *et al.* 1992) persistently infected with poliovirus.

Mechanisms of persistent viral infection in cell culture. A number of studies have shown that various viral and host cell factors could be involved in the establishment and maintenance of persistent infection in cell cultures. For example, temperature-sensitive viral mutants (Preble and Youngner 1972, 1973a, 1973b; Youngner *et al.* 1976), defective interfering viral particles (Ahmed *et al.* 1981; Calain *et al.* 1999; Holland and Villarreal 1974; Holland *et al.* 1976; Kaplan *et al.* 1989; Weiss *et al.* 1980), alteration of viral RNA or protein synthesis (Chen *et al.* 1996; Desforges *et al.* 2001; Dryga *et al.* 1997), and specific amino acid substitutions (Borzakian *et al.* 1993; Calvez *et al.* 1993; Duncan *et al.* 1998; Matloubian *et al.* 1990; Pelletier *et al.* 1991; 1998a; 1998b) have been shown to be involved in different persistent infection systems. Various host factors such as host cell type (Hecht and Summers 1974; Poste *et al.* 1972; Zhang and Racaniello 1997), host cell mutants (Kaplan *et al.* 1989; Kaplan and Racaniello 1991), host cell differentiation stage (Benton *et al.* 1995; 1996; Okada *et al.* 1987) and so on may also be involved in the establishment and maintenance of persistent viral infection. However, the detailed molecular mechanisms of persistent viral infection in cell cultures are not yet well understood and furthermore, the mechanisms may be unique in each system.

The VBS53 strain of EAV is able to establish persistent infection in the Hela ‘High’ but not Hela ‘Low’ cell line, suggesting that some unidentified host cell factors are involved in persistent EAV infection. On the other hand, Hela-EAVP35 and Hela-EAVP80 are able to establish persistent infection in the Hela ‘Low’ cell line, whereas EAV strain VBS53 is unable to do so. This suggests that unidentified viral factors are also involved in the establishment of persistent EAV infection. The fact that EAV strain VBS53 is able to establish persistent infection in the Hela ‘High’ cell line but not in Hela ‘Low’ cell line is consistent with the observation that Hela ‘High’ cells are more susceptible than Hela ‘Low’ cells to the virus infection (Chapter 2). Correlated with the

observation that Hela-EAVP35 and Hela-EAVP80 are able to, whereas EAV strain VBS53 is unable to, establish persistent infection in the Hela 'Low' cell line, is the fact that Hela-EAVP35 and Hela-EAVP80 grow more efficiently than EAV strain VBS53 in the Hela 'Low' cell line. Based on these findings, it is speculated that establishment and maintenance of persistent EAV infection in Hela cells is related to the susceptibility of the cells to EAV infection. It has been previously suggested that higher susceptibility of the Hela 'High' cell line than the Hela 'Low' cell line to infection with the VBS53 strain of EAV is because of higher efficiency of virus entry into Hela 'High' cells than into Hela 'Low' cells (Chapter 2). It would be interesting to investigate whether growth efficiencies of Hela-EAVP35 and Hela-EAVP80 in Hela 'High' and Hela 'Low' cell lines can be correlated with corresponding virus attachment and entry levels. Should this prove to be true, it would suggest that Hela-EAVP35 and Hela-EAVP80 recognize some new cell receptors on Hela cells. It may, thus, be postulated that establishment and maintenance of persistent EAV infection in Hela cells would be predicated on virus-cell receptor interactions. In fact, it has been suggested that interactions between poliovirus and its receptor may be involved in establishment and maintenance of persistent poliovirus infection in host cells (Borzakian *et al.* 1993; Calvez *et al.* 1993; Colbere-Garapin *et al.* 1989; Duncan *et al.* 1998; Pavio *et al.* 1996; 2000; Pelletier *et al.* 1991; 1998a).

In above proposed model, cell heterogeneity is another key factor. If all of the cells in a cell line, such as BHK-21, RK-13, and C2C12, are susceptible to EAV infection, virus infection kills all of the cells and persistent infection cannot be established. If too few cells in a cell line, such as Hep-2 and L-M, are susceptible to EAV infection, the virus is not able to initiate and maintain a persistent infection. Failure to establish persistent infection in the Hela 'Low' cell line with EAV strain VBS53 may be such a case. However, in the case of the Hela 'High' cell line, a certain percentage but not 100% of the cells are susceptible to infection with EAV strain VBS53 or Hela-EAVP35 or Hela-EAVP80; infection of the Hela 'High' cell line with these viruses is not able to kill all of the cells, instead, a persistent infection may be initiated and maintained. Establishment of persistent infection in the Hela 'Low' cell line with either Hela-EAVP35 or Hela-EAVP80 may be due to the same reason. The finding that persistently

infected Hela cells can be cured by treatment with anti-EAV serum would suggest that the persistent EAV infection is maintained by horizontal transmission of the virus released from infected cells to uninfected susceptible cells. It would appear that virus spread from cell to cell through cell fusion or virus spread from parent cells to daughter cells through cell division is not very likely. Failure to establish persistent infection in Hela 'Low', Hep-2, and L-M cell lines upon transfection with EAV RNA (Table 3.2) and failure to obtain a single clone that can continuously produce infectious EAV would support this hypothesis. It has been demonstrated that more than 10^3 cells derived from persistently infected BHK-21 cells are needed to reinitiate a stable, FMDV-producing carrier culture (de la Torre and Domingo 1988b), which would suggest that the persistent infection of cell culture is the result of complex interactions acting at the population level.

In either a one-step growth curve of EAV in Hela cells (Chapter 2) or in persistently infected Hela cells, the percentage of extracellular virus to the total virus present (the sum of extracellular virus and cell-associated virus) was around or below 50%, whereas it was greater than 80% in a one-step growth curve of EAV in BHK-21, Hep-2, or L-M cells. Whether this factor is implicated to the establishment and maintenance of persistent EAV infection in Hela cells remains to be determined.

Whether defective interfering particles of equine arteritis virus are involved in establishment and maintenance of persistent EAV infection in Hela cells also remains to be determined. Whether EAV infection induces production of interferon in Hela cells, and if yes, whether interferon plays a role in the establishment and maintenance of persistent EAV infection in Hela cells is unclear.

Significance of persistent viral infection in cell culture. Persistent viral infections of cell culture have been used to study virus-host cell interactions. They have also been shown to be a useful tool for investigating virus and cell evolutions (Ahmed *et al.* 1981; Colbere-Garapin *et al.* 1989; de la Torre *et al.* 1988a; Kaplan *et al.* 1989). Persistent EAV infection of Hela cells provides a model system to study viral and host cell factors involved in viral persistence. It also provides a tool for elucidating virus-host cell interactions, e.g. EAV-cell receptor interactions. Furthermore, such a system can be used to investigate virus evolution and to study phenotypic (such as neutralization and

virulence) changes. Persistent EAV infection in cell culture may also assist in elucidating the mechanisms of EAV persistence in the carrier stallions.

Table 3.1 The outcome of serial passaging Hela 'Low' (P98), Hep-2, and L-M cell lines inoculated with EAV strain VBS53*

Passage No.	Hela 'Low' cells (P98)		L-M cells (P302)	Hep-2 cells (P460)
	Extracellular virus (pfu/ml)	Cell-associated virus (pfu/ml)	Extracellular virus (pfu/ml)	Extracellular virus (pfu/ml)
1 h p.i.	3.0×10^2	5.0×10^1	5.4×10^2	4.7×10^2
P1	5.7×10^2	2.3×10^2	2.9×10^3	5.5×10^2
P2	5.0	2.0	1.2×10^1	8.0
P3	0	0	0	0
P4	0	0	0	0
P5 – P20	0	0	0	0

* Selected cell lines were inoculated with the VBS53 strain of EAV at an m.o.i. of 3. Inoculated cultures were incubated at 37ºC and subcultured once every four days (1:4 split). Extracellular and cell-associated virus was harvested and titrated by plaque assay on RK-13 (KY) cells. These results were confirmed in two separate experiments.

Table 3.2 Transfection with viral RNA established persistent infection in the Hela 'High' cell line but not in Hela 'Low', Hep-2, and L-M cell lines*

Passage No.	Hela 'Low' cells (P102) Extracellular virus (pfu/ml)	Hela 'High' cells (P200) Extracellular virus (pfu/ml)	L-M cells (P306) Extracellular virus (pfu/ml)	Hep-2 cells (P455) Extracellular virus (pfu/ml)
P1	5.2×10^4	2.1×10^5	6.4×10^4	1.4×10^4
P2	1.3×10^3	6.6×10^3	2.3×10^2	1.1×10^2
P3	1.0×10^1	4.0×10^3	0	0
P4	0	4.4×10^4	0	0
P5	0	1.2×10^5	0	0
P6	0	3.9×10^5	0	0
P7	0	1.3×10^6	0	0
P8	0	3.6×10^6	0	0
P9	0	8.6×10^5	0	0
P10	0	2.6×10^5	0	0

* Selected cell lines were transfected with viral RNA *in vitro*-transcribed from a full-length infectious EAV cDNA clone. Transfected cells were incubated at 37ºC and subcultured once every four days (1:4 split). Extracellular virus was harvested and titrated by plaque assay on RK-13 (KY) cells.

Table 3.3 Some properties of persistently infected Hela 'High' cell line[1] initiated with the VBS53 strain of EAV

Passage level	Extracellular virus titer (pfu/ml)	Cell destruction	cell death[2] (%)	IFA aN[3] positive	IFA aNSP1[4] positive	Passage	Extracellular virus titer (pfu/ml)	Cell destruction	cell death[2] (%)	IFA aN[3] positive	IFA aNSP1[4] positive
1 h p.i.	4.7×10^{2}	no				P27	1.2×10^{6}	cell destuction			
P1	6.0×10^{4}	no	12.5	7.8%	8.2%	P28	8.2×10^{5}	cell destuction			
P2	6.3×10^{4}	no	13.2			P29	2.0×10^{6}	cell destuction		17.8%	17.2%
P3	2.90×10^{5}	partial destuction	16.4			P30	9.6×10^{5}	partial recovery	15.4		
P4	1.79×10^{6}	cell destuction	35.3	22.2%	24.7%	P31	2.1×10^{4}	recovery			
P5	6.00×10^{5}	cell destuction	31.2			P32	2.4×10^{5}	recovery			
P6	1.95×10^{5}	partial recovery	17.8			P33	1.5×10^{6}	partial destuction			
P7	1.0×10^{5}	partial recovery	16.2			P34	2.4×10^{6}	cell destuction	29.3	35.8%	37.4%
P8	4.5×10^{5}	recovery	15.8			P35	4.0×10^{6}	cell destuction			
P9	5.7×10^{5}	recovery				P36	7.9×10^{5}	partial recovery			
P10	1.5×10^{6}	partial destuction				P37	7.7×10^{5}	recovery			
P11	6.8×10^{5}	partial destuction				P38	9.0×10^{4}	recovery		1.4%	2.7%
P12	1.4×10^{6}	cell destuction				P39	1.3×10^{5}	recovery			
P13	9.1×10^{5}	cell destuction				P40	6.5×10^{5}	partial destuction			
P14	9.9×10^{5}	cell destuction				P41	1.2×10^{6}	cell destuction	37.8		
P15	2.9×10^{5}	partial recovery				P42	4.5×10^{5}	partial recovery			
P16	3.0×10^{5}	recovery				P43	6.2×10^{5}	partial recovery			
P17	1.8×10^{5}	recovery				P44	1.8×10^{5}	recovery		5.2%	
P18	1.5×10^{5}	recovery	18.1			P45	1.7×10^{5}	recovery			
P19	1.0×10^{6}	partial destuction				P46	5.1×10^{5}	recovery			
P20	6.3×10^{5}	partial destuction		11.6%	11.0%	P47	1.1×10^{6}	cell destuction			
P21	4.4×10^{5}	cell destuction				P48	1.1×10^{4}	partial recovery			2.7%
P22	1.2×10^{6}	cell destuction				P49	1.2×10^{4}	partial recovery			
P23	2.1×10^{4}	recovery	12.3			P50	6.8×10^{5}	partial recovery			
P24	3.9×10^{5}	recovery				P51	3.1×10^{6}	cell destuction		38.4%	37.1%
P25	1.1×10^{6}	partial destuction		17.5%	20.7%	P52	4.4×10^{4}	partial recovery			
P26	1.4×10^{6}	cell destuction				P53	2.1×10^{4}	partial recovery			

(Table 3.3 continued)

Passage	Extracellular virus titer (pfu/ml)	Cell destruction	cell death[2] (%)	IFA aN[3] positive	IFA aNSP1[4] positive	Passage	Extracellular virus titer (pfu/ml)	Cell destruction	cell death[2] (%)	IFA aN[3] positive	IFA aNSP1[4] positive
P54	9.3×10^3	partial recovery				P75	5.7×10^5	partial destuction			
P55	2.6×10^2	partial recovery		0.4%	0.9%	P76	3.8×10^5	recovery			
P56	5.1×10^2	partial recovery				P77	9.7×10^4	recovery			
P57	3.0×10^4	recovery				P78	5.0×10^5	recovery	12.5%	15.6%	16.8%
P58	1.1×10^6	partial destuction				P79	6.9×10^5	recovery			
P59	2.8×10^6	cell destuction				P80	2.4×10^5	recovery			
P60	2.9×10^4	partial recovery				P81	5.7×10^5	partial destuction			
P61	1.4×10^5	recovery				P82	5.5×10^5	partial destuction			
P62	4.4×10^5	recovery				P83	1.4×10^5	recovery			
P63	4.9×10^5	partial destuction				P84	1.6×10^5	recovery			
P64	6.2×10^5	cell destuction				P85	2.9×10^5	recovery			
P65	6.0×10^5	cell destuction				P86	1.7×10^5	recovery			
P66	7.5×10^5	cell destuction				P87	3.7×10^5	recovery			
P67	3.5×10^5	partial recovery				P88	5.2×10^5	partial destuction			
P68	2.7×10^5	partial recovery				P89	2.8×10^5	recovery			
P69	1.8×10^5	partial recovery				P90	1.1×10^5	recovery			
P70	3.1×10^5	partial recovery				P91	1.1×10^5	recovery			
P71	1.9×10^5	partial recovery				P92	1.8×10^5	recovery			
P72	1.4×10^5	recovery				P93	7.3×10^4	recovery			
P73	3.1×10^5	partial destuction				P94	1.9×10^5	recovery			
P74	5.5×10^5	partial destuction									

Note: [1] Persistent infection was initiated in Hela ‘High’ (P171) cell line with the VBS53 strain of EAV.
[2] Cell death was determined by trypan blue staining as described in Materials and Methods.
[3] aN - monoclonal antibody 3E2 against EAV nucleocapsid protein.
[4] aNSP1 – monoclonal antibody 12A4 against EAV non-structural protein 1.

Table 3.4 Ratio of extracellular to cell-associated virus at selected passage levels of the persistently infected Hela 'High' cell line initiated with the VBS53 strain of EAV

Passage number of persistently infected Hela 'High' cells	Virus titer (pfu/ml)		Ratio of extracellular to cell-associated virus*
	Extracellular virus	Cell-associated virus	
P1	6.0×10^4	5.0×10^4	54.5 : 45.5
P24	3.9×10^5	7.3×10^5	34.8 : 65.2
P50	6.8×10^5	9.6×10^5	41.5 : 58.5
P51	3.1×10^6	4.2×10^6	42.5 : 57.5
P68	2.7×10^5	5.3×10^5	33.8 : 66.2
P74	5.5×10^5	8.1×10^5	40.4 : 59.6
P80	2.4×10^5	5.2×10^5	31.6 : 68.4
P81	5.7×10^5	9.0×10^5	38.8 : 61.2
P82	5.5×10^5	7.6×10^5	42.0 : 58.0
P83	1.4×10^5	2.4×10^5	36.8 : 63.2
P86	1.7×10^5	2.3×10^5	42.5 : 57.5
P88	5.2×10^5	7.1×10^5	42.3 : 57.7

* Determined 4 days after cells were subcultured.

Table 3.5 Effect of EAV neutralizing antibody-positive equine serum on the persistently infected Hela 'High' cell line initiated with the VBS53 strain of EAV*

Passage number	In EMEM with 10% FSCS				In EMEM with 10% EAV Ab-positive equine serum				In EMEM with 10% FSCS (previously in EMEM with 10% EAV Ab-positive equine serum)			
	Extracellular Virus (pfu/ml)	Cell-associated Virus (pfu/ml)	IFA	RT-PCR result	Extracellular Virus (pfu/ml)	Cell-associated Virus (pfu/ml)	IFA	RT-PCR result	Extracellular Virus (pfu/ml)	Cell-associated Virus (pfu/ml)	IFA	RT-PCR result
PI 81	5.7×10^5	9.0×10^5	+	+	5.7×10^5	9.0×10^5	+	+	NA†	NA	NA	NA
PI 82	5.5×10^5	7.6×10^5	+	+	4.0×10^0	1.2×10^3	+	+	NA	NA	NA	NA
PI 83	1.4×10^5	2.4×10^5	+	+	0	2.5×10^0	-	+	NA	NA	NA	NA
PI 84	1.6×10^5	5.6×10^5	+	+	0	0	-	+	NA	NA	NA	NA
PI 85	2.9×10^5	6.9×10^5	+	+	0	0	-	+	NA	NA	NA	NA
PI 86	1.7×10^5	2.3×10^5	ND‡	+	0	0	ND	-	NA	NA	NA	NA
PI 87	3.7×10^5	1.2×10^6	+	+	0	0	-	-	NA	NA	NA	NA
PI 88	5.2×10^5	7.1×10^5	+	+	0	0	-	-	0	0	-	-
PI 89	2.8×10^5	7.6×10^5	ND	+	0	0	ND	-	0	0	-	-
PI 90	1.1×10^5	2.5×10^5	+	+	0	0	-	-	0	0	-	-
PI 91	1.1×10^5	2.3×10^5	ND	+	0	0	ND	-	0	0	-	-
PI 92	1.8×10^5	3.4×10^5	+	+	0	0	-	-	0	0	-	-
PI 93	7.3×10^4	2.8×10^5	ND	+	0	0	ND	-	0	0	-	-
PI 94	1.9×10^5	6.5×10^5	+	+	0	0	-	-	0	0	-	-

* The 81st passage of persistently infected Hela 'High' cells were subcultured and grown in EMEM with 10% ferritin supplemented calf serum (FSCS), and EMEM with 10% EAV-neutralizing Ab-positive equine serum, respectively. Serial subcultivation of the cultures grown in each kind of medium was carried out once every four days (1:4 split ratio). From the 88th passage, EAV Ab-positive equine serum was removed from the medium of one replicate of the culture which was further passed up to the 94th passage. The other replicate of the culture was passed still in the presence of EAV Ab-positive equine serum up to the 94th passage. At each passage, extracellular and cell-associated virus was harvested and titrated by plaque assay. Expression of the viral nucleocapsid (N) protein was examined using IFA. RT-PCR detection of viral RNA at each passage was also carried out.

† NA—Not applicable. ‡ ND—Not done.

Table 3.6. Comparison of nucleotide and amino acid sequences of VBS53 EAV, Hela-EAVP35, and Hela-EAVP80

Leader or ORF	Protein (aa length)	Nucleotide[1]				Modified Amino Acid[2]			
		Position	VBS53 EAV	Hela-EAVP35	Hela-EAVP80	Position	VBS53 EAV	Hela-EAVP35	Hela-EAVP80
5' NTR (1-224)	N/A[3]	-[4]	-	-	-				
5' Leader (1-211)	N/A	-	-	-	-				
ORF1ab (225-9751)	**Nonstructural proteins (nsp)**								
	1ab polyprotein (3175)								
	nsp1: Met1-Gly260 (260)	658	C	T	T	145	Ala	Val	Val
		695	C	C	T				
	nsp2: Gly261-Gly831 (571)	1280	C	C	T				
		1889	T	C	C				
		1954	A	G	G	577	Asp	Gly	Gly
		1979	C	T	T				
		2057	C	T	T				
		2483	A	A	T				
	nsp3: Gly832-Glu1064 (233)	2898	C	C	T				
		3011	G	G	A				
	nsp4: Gly1065-Glu1268 (204)	3707	Y[5]	C	C				
		3797	T	T	C				
	nsp5: Ser1269-Glu1430 (162)	4349	G	A	A				
	nsp6: Gly1431-Glu1452 (22)	-	-	-	-	-	-	-	-
	nsp7: Ser1453-Glu1677 (225)	4900	A	G	G	1559	Lys	Arg	Arg
		4913	T	C	T				
	nsp8/9: Gly1678-Asn1727 (50)/Glu2370(693)	6020	C	C	T	1933	Pro	Pro	Ser
		6752	T	C	C				
		7021	R[5]	A	A				
	nsp10: Ser2371-Gln2837 (467)	8260	Y	C	C				
		8290	Y	C	C				
		8398	T	T	C				
	nsp11: Ser2838-Glu3056 (219)	-	-	-	-	-	-	-	-
	nsp12: Gly3057-Val3175 (119)	9727	T	C	T				

(Table 3.6 continued)

Leader or ORF	Protein (aa length)	Nucleotide[1]				Modified Amino Acid[2]			
		Position	VBS53 EAV	Hela-EAVP35	Hela-EAVP80	Position	VBS53 EAV	Hela-EAVP35	Hela-EAVP80
	Structure proteins								
ORF2a (9751-9954)	E (67)	9867	T	C	C				
		9907	A	A	T	53	Ser	Ser	Cys
		9914	T	C	C	55	Val	Ala	Ala
ORF2b (9824-10507)	GP2b (227)	9867	T	C	C	15	Leu	Ser	Ser
		9907	A	A	T				
		9914	T	C	C	31	Trp	Arg	Arg
		10082	G	G	C	87	Val	Val	Leu
		10085	A	G	A	88	Met	Val	Met
		10157	G	A	A	112	Ala	Thr	Thr
		10189	T	C	C				
ORF3 (10306-10797)	GP3 (163)	10648	A	G	G	115	Ser	Gly	Gly
		10709	T	C	C	135	Leu	Pro	Pro
		10722	Y	T	T				
ORF4 10700-11158)	GP4 (152)	10709	T	C	C	4	Tyr	His	His
		10722	Y	T	T	8	Leu/Ser[6]	Leu	Leu
		11024	A	A	T	109	Ile	Ile	Phe
ORF5 (11146-11913)	GP5 (255)	11171	T	C	C	9	Phe	Ser	Ser
		11438	C	T	T	98	Pro	Leu	Leu
		11475	T	T	C				
		11704	T	C	C				
		11882	G	A	G	246	Arg	Lys	Arg
ORF6 (11901-12389)	M (162)	-	-	-	-	-	-	-	-
ORF7 (12313-12645)	N (110)	-	-	-	-	-	-	-	-
3' NTR (12646-12704)	N/A	-	-	-	-				

Note: [1] Nucleotides are numbered according to the published sequence of EAV030 virus (van Dinten et. al., 1997).

[2] Only missense mutations are shown and silent mutations are not shown. Amino acids of non-structural proteins are numbered according to their location in ORF1ab polyprotein. Amino acids of structural proteins are numbered according to their locations in individual structural protein.

[3] N/A: Not Applicable. [4] - No nucleotide and amino acid changes occurred. [5] Y = T or C; R = A or G. [6] Leu/Ser means leucine or serine.

Table 3.7 Superinfection experiments with the persistently infected Hela 'High' (P171) cell line initiated with the VBS53 strain of EAV

Cell Line	Infection	EAV Titer (pfu/ml)*		
		1 h p.i.	48 h p.i.	96 h p.i.
Hela P243	Mock-infection	0	0	0
Hela P243	Infection with EAV strain VBS53 (moi = 3)	4.5×10^3	2.35×10^4	6.4×10^4
PI 73	Mock-superinfection	4.65×10^3	3.55×10^5	1.65×10^5
PI 73	Superinfection with EAV strain VBS53 (moi = 3)	1.6×10^4	4.45×10^5	1.4×10^5

* Equine arteritis virus titer was determined by plaque assay on RK-13 (KY) cells. pfu/ml – plaque forming unit per ml.

Table 3.8 Summary of the ability of VBS53 EAV, Hela-EAVP35, and Hela-EAVP80 to establish persistent infection in various cell lines

Cell Lines	VBS53 EAV	Hela-EAV P35	Hela-EAV P80
Hela 'High'	+	+	+
Hela 'Low'	-	+	+
Hep-2	-	-	-
L-M	-	-	-
BHK-21	-	-	-

Note: + Able to establish persistent infection
- Unable to establish persistent infection

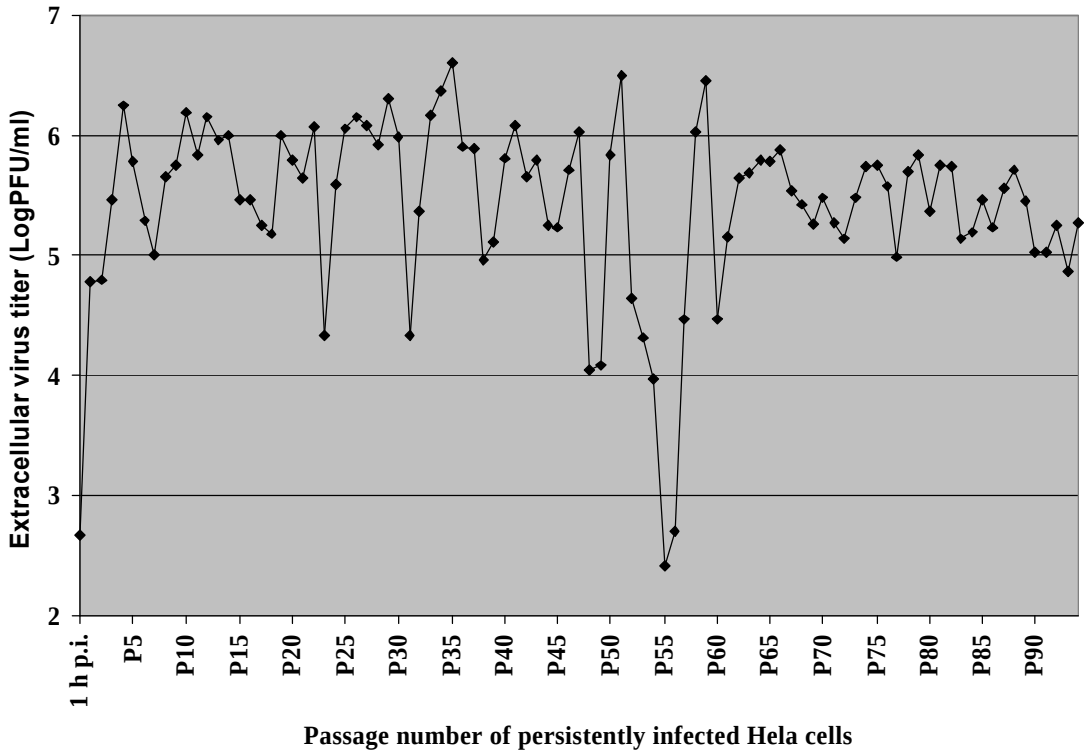


Fig 3.1 Extracellular virus produced in the persistently infected Hela 'High' cell line initiated with the VBS53 strain of EAV. Subconfluent monolayers of Hela 'High' (P171) cells grown in T-25 flasks were inoculated with EAV strain VBS53 at an m.o.i. of 3. Following one-hour absorption at 37ºC, the cells were washed three times with PBS and 10 ml fresh culture medium was added. Inoculated cultures were incubated at 37ºC and subcultured once every four days (1:4 split ratio). Extracellular virus was harvested before subculturing each cell passage. Virus infectivity was titrated by plaque assay on RK-13 (KY) cells. Establishment of persistent infection in Hela 'High' cells with the VBS53 strain of EAV was confirmed in three separate experiments.

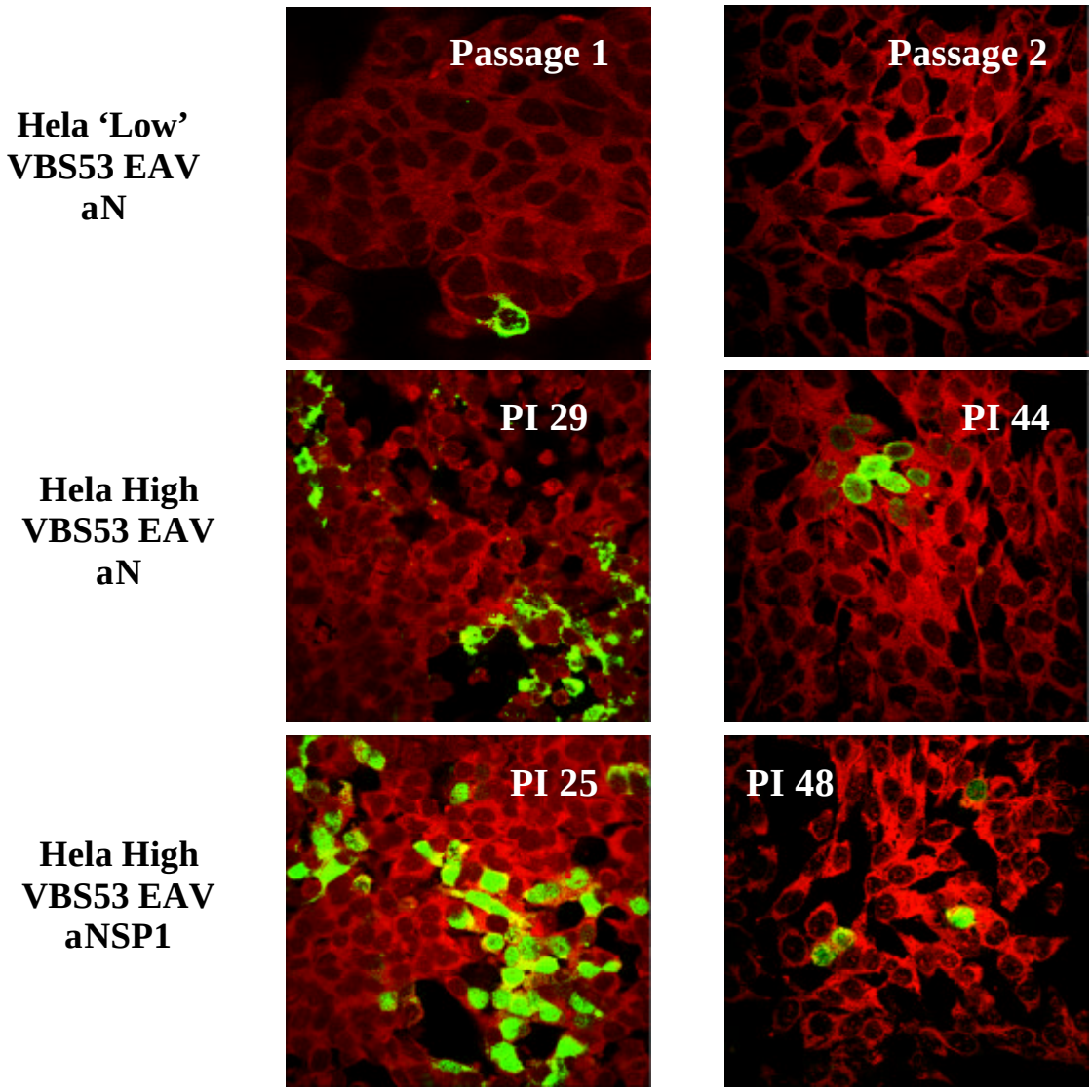


Fig 3.2 Indirect immunofluorescence assay on VBS53 EAV-inoculated Hela 'Low' and persistently infected Hela 'High' cell lines. At indicated passages, cells were fixed with cold acetone for 10 min, and then washed three times with PBS containing 10mM glycine. Slides were incubated with MAb 3E2 against EAV nucleocapsid protein or MAb 12A4 against EAV NSP1 followed by FITC-conjugated goat antimouse immunoglobulin. The cells were counterstained by Evans blue. The images were recorded with a LEICA confocal microscope. The VBS53 strain of EAV failed to establish persistent infection in Hela 'Low' (P98) cells. Passage 1 and passage 2 were shown. The VBS53 strain of EAV initiated persistent infection in Hela 'High' (P171) cells. The persistently infected cells at the 25^{th} (PI 25), 29^{th} (PI 29), 44^{th} (PI 44) and 48^{th} (PI 48) passage were shown.

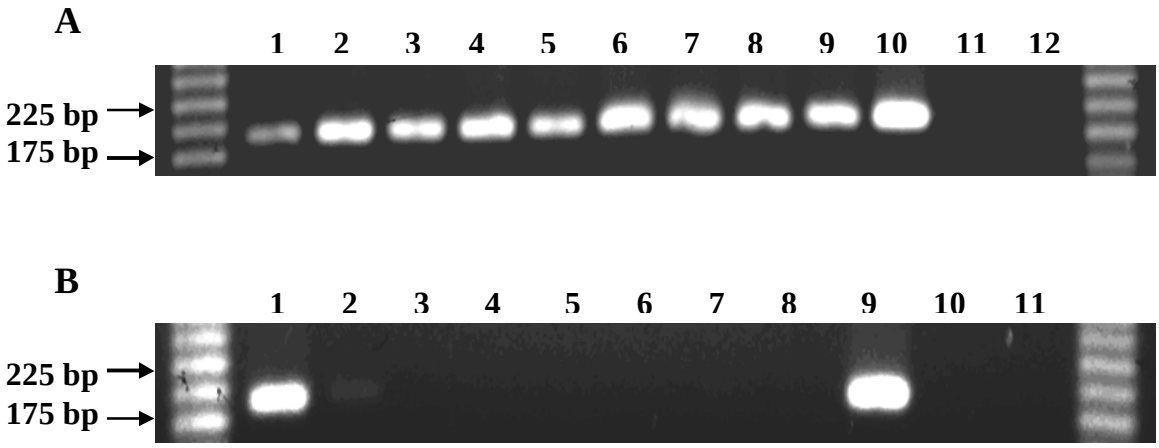


Fig 3.3 RT-PCR detection of viral RNA in persistently infected Hela 'High' cells (A) and VBS53 EAV-inoculated Hela 'Low' cells (B). In (A), the VBS53 strain of EAV established persistent infection in Hela 'High' (P171) cells. Lanes 1-9 correspond to the 1st, 10th, 20th, 30th, 40th, 50th, 60th, 70th, and 80th passage of persistently infected Hela 'High' cells, respectively. In (B), Hela 'Low' (P98) cells inoculated with the VBS53 strain of EAV underwent serial subculturing. Lanes 1-8 correspond to the 1st, 2nd, 3rd, 4th, 5th, 10th, 15th, and 20th passage, respectively. Lane 10 in (A) and lane 9 in (B) are the positive controls with RT-PCR amplification of the parental virus VBS53 EAV. Lane 11 in (A) and lane 10 in (B) are the negative controls of mock-infected Hela 'High' and Hela 'Low' cells. Lane 12 in (A) and lane 11 in (B) are negative controls with water as the template for RT-PCR.

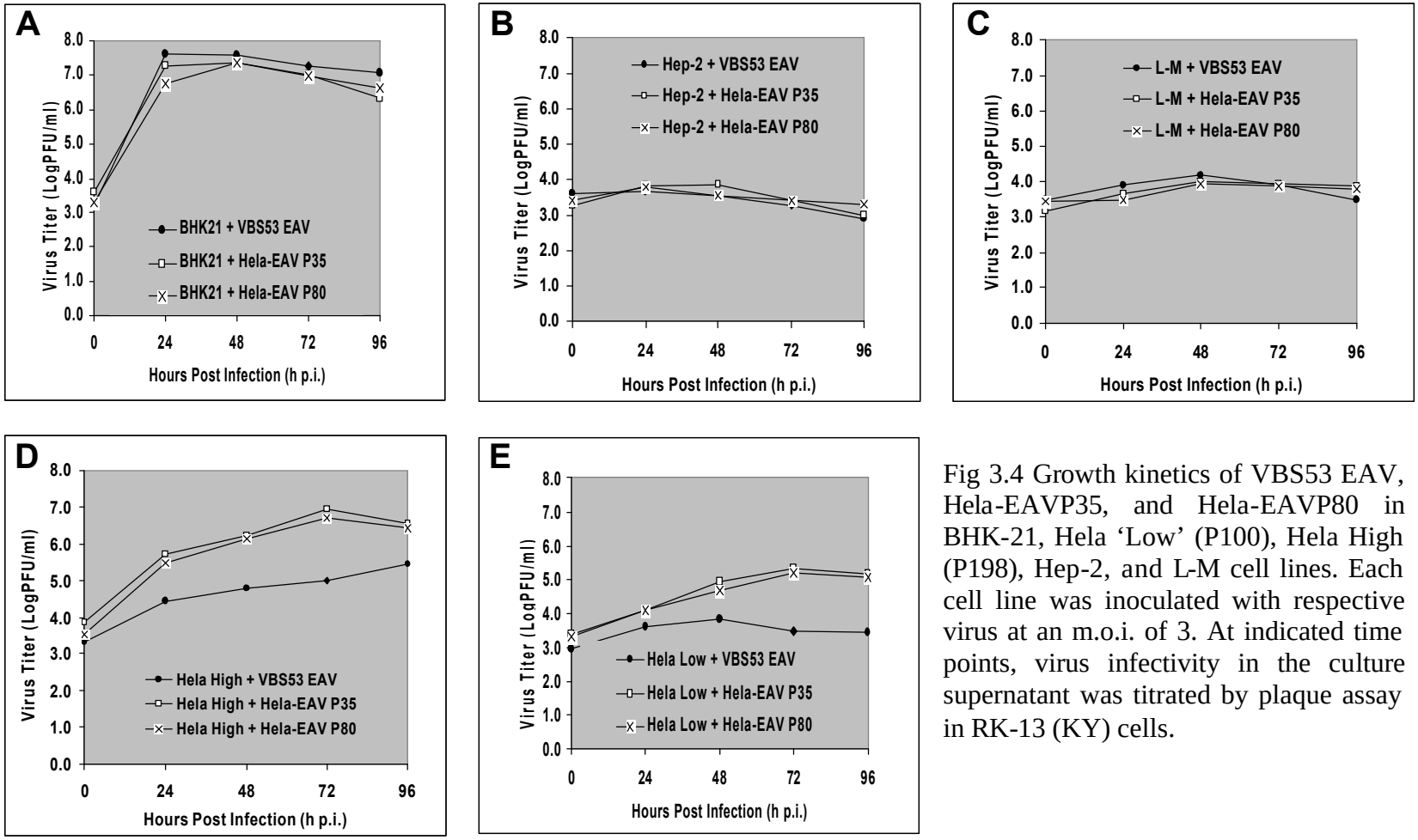
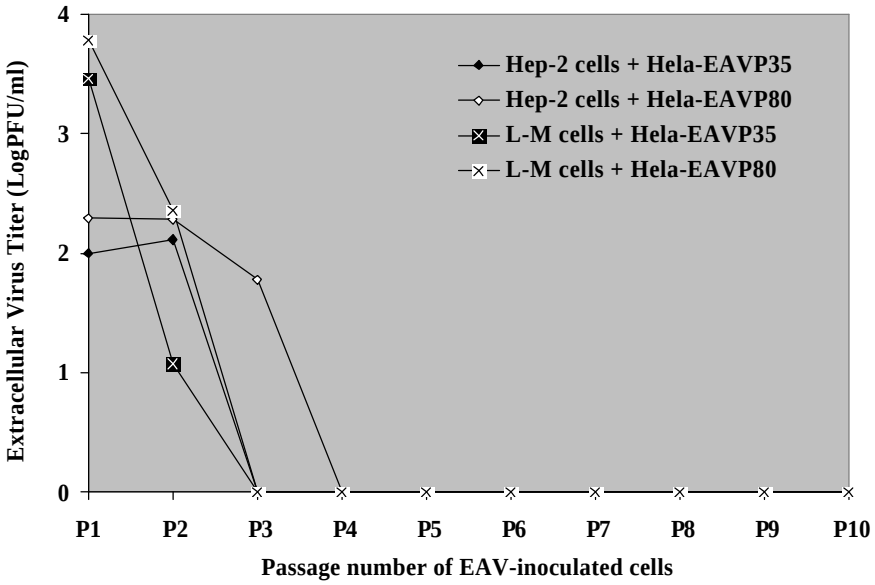
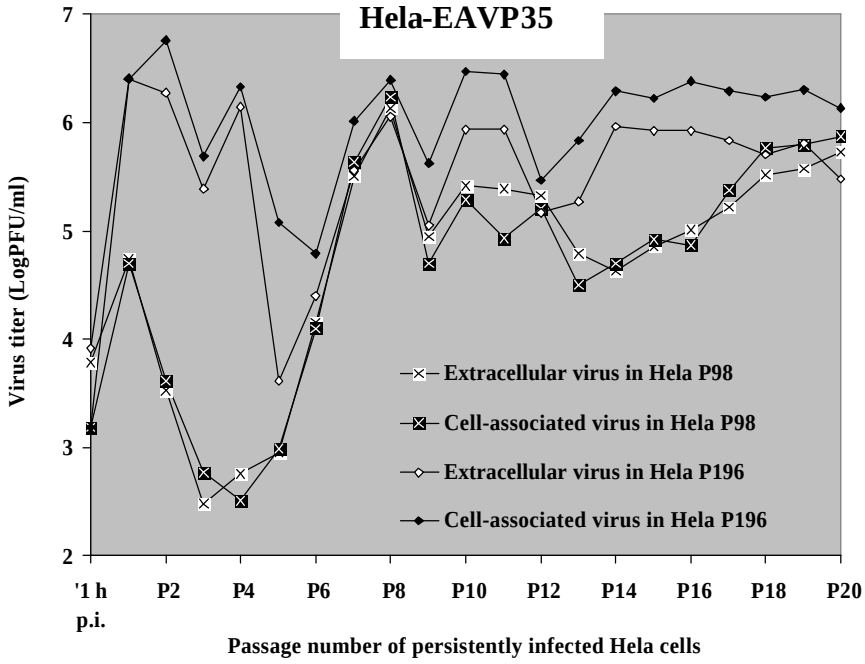


Fig 3.4 Growth kinetics of VBS53 EAV, Hela-EAVP35, and Hela-EAVP80 in BHK-21, Hela 'Low' (P100), Hela High (P198), Hep-2, and L-M cell lines. Each cell line was inoculated with respective virus at an m.o.i. of 3. At indicated time points, virus infectivity in the culture supernatant was titrated by plaque assay in RK-13 (KY) cells.

A


Extracellular Virus Titer (LogPFU/ml)
4
3
2
1
0
Hep-2 cells + Hela-EAVP35
Hep-2 cells + Hela-EAVP80
L-M cells + Hela-EAVP35
L-M cells + Hela-EAVP80
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
Passage number of EAV-inoculated cells

B


Hela-EAVP35
Virus titer (LogPFU/ml)
7
6
5
4
3
2
Extracellular virus in Hela P98
Cell-associated virus in Hela P98
Extracellular virus in Hela P196
Cell-associated virus in Hela P196
'1 h p.i.
P2
P4
P6
P8
P10
P12
P14
P16
P18
P20
Passage number of persistently infected Hela cells

C

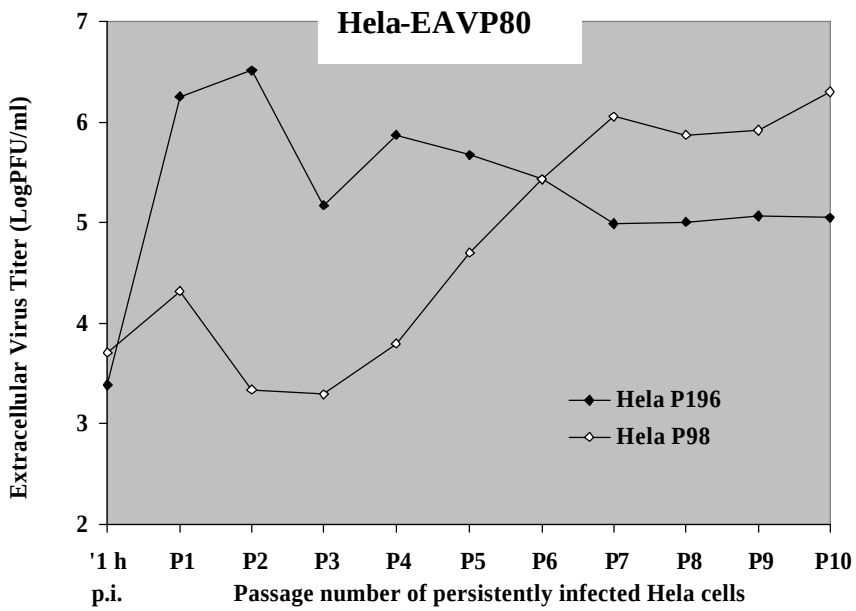


Fig 3.5 Attempts to establish persistent infection in Hela 'Low' (P98), Hela 'High' (P196), Hep-2, and L-M cell lines with Hela-EAVP35 and Hela-EAVP80. In (A), attempt to establish persistent infection in Hep-2 and L-M cells with Hela-EAVP35 and Hela-EAVP80 was performed. In (B), attempt to establish persistent infection in Hela 'Low' and Hela 'High' cells with Hela-EAVP35 was carried out. The infectivity titers of both extracellular and cell-associated viruses at each passage were shown. In (C), attempt to establish persistent infection in Hela 'Low' and Hela 'High' cells with Hela-EAVP80 was conducted.

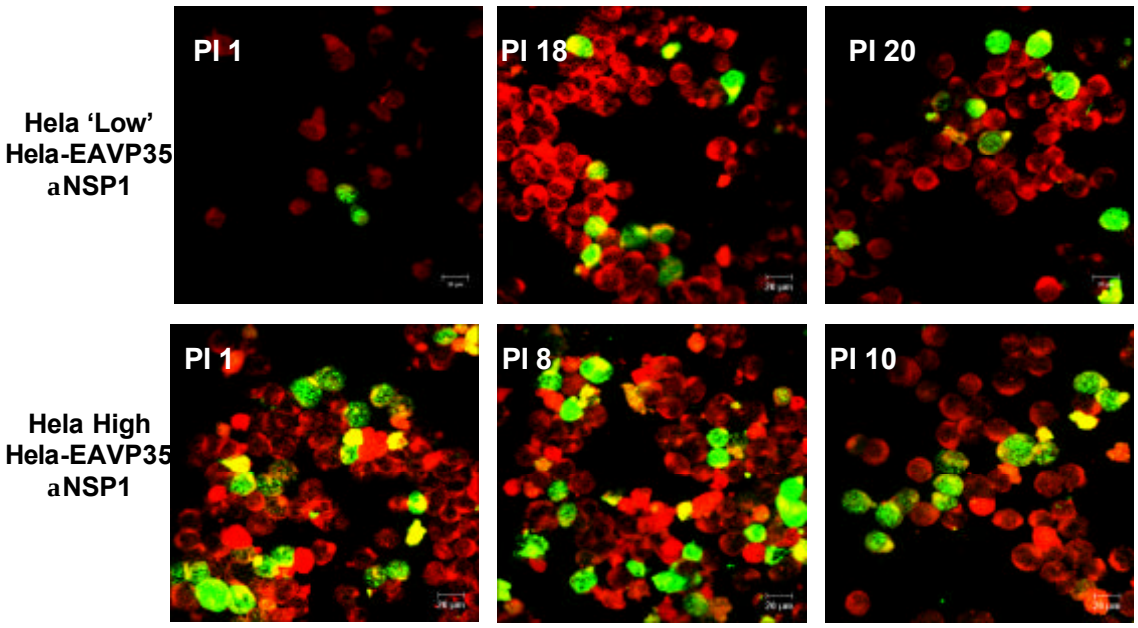


Fig 3.6 Indirect immunofluorescence assay on persistently infected Hela 'Low' and Hela 'High' cell lines initiated with Hela-EAVP35. At indicated passages, cells were fixed with cold acetone for 10 min, and then washed three times with PBS containing 10mM glycine. Slides were incubated with MAb 12A4 against EAV NSP1 followed by FITC-conjugated goat antimouse immunoglobulin. The cells were counterstained by Evans blue. The images were recorded with a LEICA confocal microscope.

Chapter Four

Genetic Characterization of Equine Arteritis Virus during Persistent Infection of Hela Cells and Identification of a New Neutralization Determinant

ABSTRACT

Persistent EAV infection in Hela cells has been recently established (Chapter 3). It has also been shown that EAV evolves during persistent infection with respect to its growth properties and its ability to initiate secondary persistent infection (Chapter 3). In this study, the viruses recovered from the persistently infected Hela cells were characterized for their neutralization phenotypes using EAV-specific monoclonal antibodies and EAV strain-specific polyclonal equine antisera. Neutralization phenotypic differences were observed between these viruses, indicating that viral variants with novel neutralization phenotypes have emerged during persistent infection in Hela cells. Sequencing of the entire structural protein genes (ORFs2a, 2b, and 3-7) revealed nucleotide and deduced amino acid changes among these viruses. In the case of EAV, four neutralization determinant sites have been previously identified within the ectodomain of the GP5 protein (site A: aa 49; site B: aa 61; site C: aa 67-90; and site D: aa 99-106). All of these previously identified neutralization determinants were conserved among the viruses recovered from persistently infected Hela cells, in spite of the fact that neutralization phenotypic differences were found between these viruses. This suggested that some as yet unidentified neutralization determinant(s) exists. An infectious EAV cDNA clone and reverse genetics technology were used to identify additional potential neutralization epitope(s). Five chimeric viruses were generated each of which contained the corresponding ORF5 (which encodes GP5) of the aforementioned viruses recovered from persistently infected Hela cells. The neutralization phenotype of each chimeric virus was compared to that of its parental virus from which the substituted ORF5 was derived. The replacement of ORF5 correlated with the neutralization phenotypic differences determined by most of the EAV-specific monoclonal antibodies, suggesting that the unidentified neutralization determinant(s) recognized by these monoclonal antibodies is located on the EAV GP5 protein. Site-directed mutagenesis further identified that amino acid 98 in the EAV GP5 protein was critical for virus neutralization. Amino acid 98 in

the EAV GP5 protein is a previously unrecognized and undescribed neutralization determinant. It is suggested that amino acid 98 be classified in neutralization site D which is redefined to cover amino acids 98-106.

INTRODUCTION

Equine arteritis virus (EAV) is the prototype virus in the family *Arteriviridae*, genus *Arterivirus* in the order *Nidovirales* (Cavanagh 1997). The family *Arteriviridae* also contains porcine respiratory and reproductive syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (Snijder and Meulenberg 1998). The EAV genome is a single-stranded, positive-sense RNA molecule of 12.7 kb, which includes nine functional open reading frames (ORFs) (Snijder and Meulenberg 1998; Snijder *et al.* 1999). The two ORFs most proximal to the 5' end of the virus (1a and 1b) are approximately 9.5 kb in length and encode two replicase polyproteins (1a and 1ab) (de Vries *et al.* 1997; Snijder and Meulenberg 1998). The remaining seven ORFs (2a, 2b, and 3-7) located at the 3' end of the genome are approximately 2.9 kb in length and encode structural proteins E, GP2b, GP3, GP4, GP5, M, and N, respectively (den Boon *et al.* 1991; Snijder and Meulenberg 1998; Snijder *et al.* 1999).

There is only one serotype of EAV (Fukunaga and McCollum 1977; Golnik *et al.* 1986), and all strains evaluated thus far are neutralized by polyclonal equine sera raised against the virulent Bucyrus strain of EAV (Balasuriya and MacLachlan 2004c). However, EAV strains frequently exhibit neutralization phenotypic differences; e.g., different EAV strains are neutralized to various degrees with different polyclonal antisera and monoclonal antibodies (Balasuriya *et al.* 1997; 2004a; 2004b; Hedges *et al.* 1999a). The EAV GP5 protein has been shown to express neutralization determinants of the virus (Balasuriya *et al.* 1993; 1995b; 1997; 2004b; Chirnside *et al.* 1995; Deregt *et al.* 1994; Glaser *et al.* 1995). The hydropathy profile of the GP5 protein predicts that amino acid residues 1-18 constitute the putative signal sequence, 19-116 constitute the hydrophilic ectodomain of the protein, while the C-terminal half consists of three membrane-spanning domains followed by an endodomain of about 64 amino acids (de Vries *et al.* 1992; Snijder *et al.* 2003). Chirnside *et al.* (1995) has shown that horses developed

EAV-neutralizing antibodies after they were inoculated with either a fusion protein including GP5 aa 55-98 or a synthetic peptide corresponding to GP5 aa 75-97. It was further demonstrated that an *E. coli*-expressed recombinant protein comprising the entire ectodomain (aa 18-122) of EAV GP5 protein induced high neutralizing antibodies in ponies (Castillo-Olivares *et al.* 2001). These data support the hypothesis that the ectodomain (aa 19-116) of EAV GP5 protein is immunodominant. By comparing the sequences of neutralization-sensitive EAV and neutralization-resistant viruses (escape mutants, EM), Glaser *et al.* (1995) identified that amino acid residues at 99 and 100 are critical for virus neutralization. After extensive comparison of GP5 amino acid sequences and neutralization phenotypes of a large number of field and laboratory EAV strains using both neutralizing monoclonal antibodies and EAV strain-specific polyclonal equine antisera, Balasuriya *et al.* (1993; 1995b; 1997) identified four distinct neutralization sites. These sites include amino acids 49 (site A), 61 (site B), 67-90 (site C), and 99-106 (site D) in the GP5 protein. In a recent study, the ORF5 of an infectious EAV clone was replaced with that of different laboratory, field, and vaccine strains of EAV, thus generating various chimeric viruses (Balasuriya *et al.* 2004b). The comparative characterization of the neutralization phenotypes of chimeric viruses and parental viruses has further confirmed the importance of the aforementioned four neutralization sites in the GP5 protein. Taken together, these studies demonstrated that the ectodomain of the GP5 protein contains EAV neutralization determinants. Site D includes several overlapping linear epitopes. The four neutralization sites (A-D) are conformationally interactive (Balasuriya *et al.* 1997; 2004b).

An *in vitro* model of viral persistence involving Hela cell cultures infected with EAV has been recently developed (Chapter 3). In this study, it was shown that viral variants with novel neutralization phenotypes emerged during persistent infection of Hela cells. Sequencing of the entire structural protein genes (ORFs2a, 2b, and 3-7) revealed nucleotide and deduced amino acid changes among these viruses. However, all of the previously identified neutralization determinants (sites A to D) were conserved among the viruses recovered from persistently infected Hela cells, in spite of the fact that neutralization phenotypic differences were found between these viruses. This suggested that some as yet unidentified neutralization determinant(s) exists. An infectious EAV

cDNA clone and reverse genetics technology were used to identify additional potential neutralization epitope(s).

MATERIALS AND METHODS

Cells and viruses. Rabbit kidney (RK-13, KY) and baby hamster kidney (BHK-21) cells were maintained in Eagle's medium (EMEM) with 10% ferritin supplemented calf serum (FSCS), penicillin, streptomycin, fungizone, and sodium bicarbonate. The VBS53 strain of EAV was propagated twice in BHK-21 cells to produce virus stocks which were used to establish persistent infection in the Hela 'High' cell line. Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 are the viruses isolated from the 10^{th}, 35^{th}, 60^{th}, and 80^{th} passage levels of the persistently infected Hela 'High' cell line, respectively.

Monoclonal antibodies and polyclonal anti-sera. The development and characterization of a panel of 12 neutralizing monoclonal antibodies to GP5 (MAbs 5G11, 6D10, 7E5, 9F2, 10F11, 10H4, 1H7, 1H9, 5E8, 6A2, 7D4, and 10B4) and non-neutralizing monoclonal antibodies to the nucleocapsid protein (N; MAb 3E2) and nonstructural protein 1 (nsp1; MAb 12A4) of EAV have been previously described (Table 4.1; Balasuriya *et al.* 1993; 1995b; 1997; 2004b; MacLachlan *et al.* 1998; Wagner *et al.* 2003). The source of EAV strain-specific polyclonal equine antisera (anti-GP5/M, anti-KY53, anti-KY84, anti-KY53+KY84, anti-CA95, anti-ARVAC, anti-NVSL, and anti-EAV030H) and negative control sera (control sera NVSL and OAT PB) have also been previously described (Table 4.1; Balasuriya *et al.* 2002; 2004b; MacLachlan *et al.* 1998; Wagner *et al.* 2003).

Sequencing of structural protein genes of Hela-EAVP10 and Hela-EAVP60. The entire genomes of EAV strain VBS53, Hela-EAVP35, and Hela-EAVP80 have been sequenced (Chapter 3). The structural protein genes (ORFs2a, 2b, and 3-7) of Hela-EAVP10 and Hela-EAVP60 were sequenced following the procedures described for sequencing Hela-EAVP35 and Hela-EAVP80 (Chapter 2). The sequence data and alignments results are summarized in **Appendices 5, and 7 to 13**.

Construction of chimeric full-length EAV cDNA clones containing ORF5 from EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80. EAV infectious cDNA clone pA45 (de Vries *et al.* 2000; Dobbe *et al.* 2001)

served as the backbone for all chimeric constructs used in this study. In the construct pA45, the naturally overlapping ORFs 4 and 5 were separated by a newly introduced 24-nucleotide sequence containing a unique *Afl*II restriction site (Fig 4.1B). The separation did not change the amino acids encoded by ORFs 4 and 5, and did not interfere with virus replication. The progeny viruses stably maintained the mutations that were introduced (de Vries *et al.* 2000). A shuttle vector (pS45) containing a *Bgl*II (10704)- *Xho*I (12869) fragment of the pA45 plasmid was used for cloning of ORF5 from individual EAV strains. To facilitate cloning, a *Not*I restriction site was inserted downstream of the *Afl*II site in the sequence separating ORFs 4 and 5 (Fig 4.1A; Dobbe *et al.* 2001). Both the infectious cDNA clone pA45 and the shuttle vector pS45 were kindly provided by Dr. Eric Snijder, Leiden University, the Netherlands.

The ORF5 sequences were RT-PCR-amplified from EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 using a forward primer 11152SD which contains a *Not*I site upstream of the ORF5 translation initiation codon and a reverse primer 11960N which contains a natural *Xba*I site just downstream of the ORF5 sequence (Table 4.5). The PCR products were digested with restriction enzymes (*Not*I and *Xba*I), and ligated into the pS45 shuttle vector which had been digested with the same restriction enzymes (Fig 4.1). The ligation mixture was transformed into *Eco*li DH5a cells. The colonies were digested with restriction enzymes for screening positive clones. The RT-PCR-derived insert was sequenced to confirm that recombinant shuttles (pS45VBS53, pS45Hela-EAVP10, pS45Hela-EAVP35, pS45Hela-EAVP60, and pS45Hela-EAVP80) contained the correct insert sequences. The *Bgl*II-*Xho*I fragments corresponding to ORFs 4-7 and the genomic 3' end were subcloned from recombinant shuttle vectors into the pA45 plasmid to obtain full-length chimeric EAV cDNA clones containing ORF5 from individual EAV strains (Fig 4.1). The authenticity of the ORF5 inserts in full-length chimeric EAV cDNA clones (5rVBS53, 5rHela-EAVP10, 5rHela-EAVP35, 5rHela-EAVP60, and 5rHela-EAVP80 clones) was confirmed by sequencing.

Site-directed mutagenesis. The site-specific nucleotide substitution of ORF5 11438C? T (which results in GP5 98Pro? Leu) in EAV strain VBS53 was carried out at the PCR step (Fig 4.2). The first-step PCR was performed with positive primer C211156P and negative mutagenic primer 11443N (Table 4.5) using the recombinant

shuttle pS45VBS53 as template. The second-step PCR was performed using the mixture of the 1st mutagenesis PCR product and the template pS45VBS53 together with negative primer 11515N in which the 1st mutagenesis PCR product acted as the positive primer. The 2nd mutagenesis PCR product was the desired fragment containing the site-specific nucleotide substitution (Fig 4.2). The thermacycling conditions for site-directed mutagenesis PCR reactions are shown in **Appendix 14**. The 2nd mutagenesis PCR products were digested with restriction enzymes (*Not*I and *Eco*RI) and cloned into the shuttle pS45VBS53 to obtain site-mutated shuttle pS45VBS53P98L from which the *Bgl*II-*Xho*I fragment was subcloned into the plasmid pA45, thus generating the site-mutated chimeric EAV cDNA clone 5rVBS53P98L. On completion of each cloning step, authenticity of the inserts was confirmed by sequencing.

***In vitro* transcription and generation of chimeric viruses.** Viral RNA transcripts were generated *in vitro* from each chimeric/mutant infectious cDNA clone as previously described (Chapter 2; van Dinten *et al.* 1997). Full-length RNA transcripts were transfected into BHK-21 cells by electroporation in accordance with previously described protocols (Chapter 2; Balasuriya *et al.* 2004b; van Dinten *et al.* 1997). The cells were incubated at room temperature for a 10 min 'recovery period' after electroporation and then resuspended in 15 ml of culture medium which was previously warmed to room temperature. The cells were seeded into T-25 flasks and incubated at 37ºC until cytopathic effects were evident. Meanwhile, an aliquot of electroporated cells was seeded into 8-chamber slides, incubated at 37ºC for 24 h, and then examined by indirect immunofluorescence assay (IFA). Monoclonal antibodies 12A4 and 3E2 specific for EAV nonstructural protein 1 and the nucleocapsid protein (N), respectively, were used to detect virus replication and protein expression according to previously described protocols (Chapter 2). When cytopathic effects were evident (usually 72 h post transfection), tissue culture fluids were harvested from T-25 flasks and centrifuged at 1900 rpm for 10 min at 4ºC. Infectivity titers of chimeric/mutant viruses (5rVBS53, 5rVBS53P98L, 5rHela-EAVP10, 5rHela-EAVP35, 5rHela-EAVP60, and 5rHela-EAVP80) were determined as tissue culture infectious doses ($TCID_{50}$) using RK-13 (KY) cells as the indicator cells. The infectivity titers of these viruses ranged from $10^{4.5}$ to 10^{5} $TCID_{50}$ units per 50 µl. The authenticity of the ORF5 insert in each chimeric/mutant virus

was confirmed by RT-PCR amplification and sequencing as previously described (Chapter 3).

Microneutralization assay. Neutralization titers of various monoclonal antibodies and polyclonal antisera (Table 4.1) against each virus strain were determined by microneutralization assay as previously described (Balasuriya *et al.* 1995b; 1997; 2004b). Briefly, two-fold dilutions of heat-inactivated monoclonal antibodies and polyclonal equine antisera were made in EMEM, with the lowest dilution of 1:32 and 1:8, respectively. Serially diluted monoclonal antibodies (from 1:32 to 1:4096) and polyclonal antisera (from 1:8 to 1:1024) were added to 96-well plates in duplicate for each antibody. Then approximately 200 $TCID_{50}$ of each virus strain was added and mixed with monoclonal antibodies or polyclonal antisera. The plates were incubated at 37ºC for 30 min. A suspension of RK-13 (KY) cells was then added, and cultures were incubated at 37ºC for 60-72 h until cytopathic effect had fully developed in the control wells. Neutralization titers of each antibody against each virus were recorded as the reciprocal of the highest final dilution that provided at least 50% protection of the RK-13 (KY) cell monolayers.

RESULTS AND DISCUSSION

Emergence of viral variants with novel neutralization phenotypes during persistent EAV infection of Hela cells. Persistent EAV infection in Hela cells has been recently established (Chapter 3). It has been shown that EAV evolves during persistent infection of cultured cells with respect to its growth properties and its ability to initiate secondary persistent infection (Chapter 3). In order to determine whether EAV had undergone neutralization phenotypic changes during persistent infection of Hela cells, the parental EAV strain VBS53 and the viruses recovered from the 10^{th} (Hela-EAVP10), 35^{th} (Hela-EAVP35), 60^{th} (Hela-EAVP60), and 80^{th} (Hela-EAVP80) passage levels of the persistently infected Hela 'High' cell line were characterized in term of their neutralization phenotypes using EAV-specific monoclonal antibodies and EAV strain-specific polyclonal equine antisera (Table 4.1). Neutralization phenotypic differences were observed in these viruses. For example, EAV strains VBS53 and Hela-EAVP10 were neutralized by monoclonal antibodies (MAbs) 5G11, 6D10, 7E5, 9F2, 10F11, 10H4,

and 6A2, whereas Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 were not neutralized by any of these MAbs (Table 4.2). On the other hand, EAV strains VBS53 and Hela-EAVP10 were resistant to neutralization by MAb 1H7, whereas Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 were neutralized by this MAb (Table 4.2). Similarly, some of the EAV strain-specific polyclonal equine antisera (e.g. anti-GP5/M and anti-EAV030H) neutralized Hela-EAVP80 to a significantly higher titer than EAV strain VBS53 (Table 4.3). These findings indicate that viral variants with novel neutralization phenotypes emerged during persistent EAV infection in Hela cells. *In vivo*, it has also been repeatedly demonstrated that viruses of different neutralization phenotypes emerge during persistent EAV infection of stallions (Balasuriya *et al.* 1999a; 2001; 2004a; Hedges *et al.* 1999a).

Comparison of the structural protein genes of EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80. In order to further characterize the relationship between EAV structural protein sequences and neutralization phenotypes, EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 were compared for their nucleotide and deduced amino acid sequences of the structural protein genes (ORFs 2a, 2b, and 3-7). The entire genomes of EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 have been sequenced previously (Chapter 3). In this study, the structural protein genes (ORFs 2a, 2b, and 3-7) of Hela-EAVP10 and Hela-EAVP60 were sequenced. As shown in Table 4.4, nucleotide and amino acid changes were observed in the structural proteins E, GP2b, GP3, GP4, and GP5, but not in M and N proteins. The amino acid sequences of structural proteins were 100% identical between EAV strains VBS53 and Hela-EAVP10 except that the amino acid at position 8 of the GP4 protein was Leucine or Serine for VBS53 while Leucine for Hela-EAVP10 (Table 4.4). The degree of amino acid identity among Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 was higher than that between any of these strains and VBS53 or Hela-EAVP10 (Table 4.4).

The EAV GP5 protein has been shown to express neutralization determinants of the virus (Balasuriya *et al.* 1993; 1995b; 1997; 2004b; Chirnside *et al.* 1995; Deregt *et al.* 1994; Glaser *et al.* 1995). To date, four neutralization sites have been identified for EAV and all of them are located within the N-terminal ectodomain of the GP5 protein (site A:

amino acid 49; site B: amino acid 61; site C: amino acids 67-90; and site D: amino acids 99-106) (Balasuriya *et al.* 1993; 1995b; 1997; 2004b). All of these four neutralization sites were conserved among EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 (Table 4.4), although neutralization phenotypic differences were observed among these viruses (Table 4.2). This suggested that some as yet unidentified neutralization determinant(s) exists. Since all of the EAV-neutralizing monoclonal antibodies that have been characterized thus far recognize the GP5 protein as shown by Western blot and/or immunoprecipitation assays (Balasuriya *et al.* 1993; 1995b; 1997; Deregt *et al.* 1994; Glaser *et al.* 1995), it is suspected that the potential neutralization determinant(s) responsible for the neutralization phenotypic differences among EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 is also located on the GP5 protein.

Generation of GP5 chimeric viruses and neutralization phenotypes of these chimeric viruses. To confirm that the potential neutralization determinant(s) responsible for the neutralization phenotypic differences among viruses recovered from persistently infected Hela cells is located on the GP5 protein, the ORF5 of the infectious EAV cDNA clone pA45 was replaced with the respective ORF5 of EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 (Fig 4.2). The neutralization phenotypes of chimeric viruses (5rVBS53, 5rHela-EAVP10, 5rHela-EAVP35, 5rHela-EAVP60, and 5rHela-EAVP80) were determined with EAV-specific MAbs and EAV-strain specific polyclonal equine antisera, and compared to those of the parental viruses from which the substituted ORF5 was derived (Table 4.2 and 4.3). As shown in Table 4.2, chimeric viruses 5rVBS53 and 5rHela-EAVP10 were neutralized by MAbs 5G11, 6D10, 7E5, 9F2, 10F11, 10H4, and 6A2, whereas none of 5rHela-EAVP35, 5rHela-EAVP60, and 5rHela-EAVP80 viruses was neutralized by any of these MAbs. This is consistent with the neutralization results of their respective parental viruses with the same MAbs (Table 4.2). All of the chimeric viruses 5rVBS53, 5rHela-EAVP10, 5rHela-EAVP35, 5rHela-EAVP60, and 5rHela-EAVP80 shared the same backbone as the pA45 virus and the only differences among them were ORF5 sequences. This would suggest that the neutralization phenotypic differences among chimeric viruses detected by above MAbs result from differences in the GP5 proteins. The potential neutralization determinant(s)

recognized by MAbs 5G11, 6D10, 7E5, 9F2, 10F11, 10H4, and 6A2 are located on the EAV GP5 proteins. On the other hand, it was observed that chimeric viruses 5rHela-EAVP35, 5rHela-EAVP60, and 5rHela-EAVP80 were resistant to neutralization by MAb 1H7, whereas their parental viruses Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 were neutralized by this MAb (Table 4.2). These three chimeric viruses have the same ORF5 sequences as their respective parental viruses. This would suggest that neutralization of Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 by MAb 1H7 may involve some other structural proteins. Since the M and N proteins of these viruses did not show any sequence differences (Table 4.4), it is probable that one or some of structural proteins E, GP2b, GP3, and GP4 are involved in neutralization by MAb 1H7. It has been previously demonstrated that MAb 1H7 recognizes amino acid 49 of EAV GP5 protein (Balasuriya *et al.* 1997; 2004b). Furthermore, individual neutralization sites (A-D) interact with one another to form conformation-dependent epitopes (Balasuriya *et al.* 1995b; 1997; 2004b). Therefore, it would appear that the neutralization sites are conformationally dependent and they not only interact with each other but also may interact with other structural proteins.

Generation of mutant virus with site-specific substitution in the GP5 protein and its neutralization phenotype. As has been shown, the potential neutralization determinant(s) recognized by MAbs 5G11, 6D10, 7E5, 9F2, 10F11, 10H4, and 6A2 are located on the EAV GP5 proteins. Comparison of the GP5 protein sequences revealed that there were four amino acid changes (amino acids 9, 98, 202, and 246) among these viruses (Fig 4.3). At position 202, only Hela-EAVP60 and 5rHela-EAVP60 were different from the other viruses; at position 246, only Hela-EAVP35 and 5rHela-EAVP35 were different from the other viruses. Based on the fact that EAV strains VBS53, Hela-EAVP10 and their respective GP5 chimeric viruses were neutralized by MAbs 5G11, 6D10, 7E5, 9F2, 10F11, 10H4, and 6A2, whereas Hela-EAVP35, Hela-EAVP60, Hela-EAVP80 and their respective GP5 chimeric viruses were not neutralized by these MAbs, amino acids 202 and 246 do not appear to be the potential neutralization determinant(s) responsible for neutralization phenotypic differences detected by these MAbs. In contrast, amino acids 9 and 98 would appear to be the possible potential neutralization determinant(s). Since amino acids 1-18 of the GP5 protein are the putative signal

sequence which is cleaved off during transport through the endoplasmic reticulum (de Vries *et al.* 1992; Snijder *et al.* 2003), amino acid 9 is unlikely to be the potential neutralization determinant(s). By exclusion, therefore, amino acid 98 would appear to be the potential neutralization determinant. To unequivocally demonstrate this, amino acid 98 on the GP5 protein of the virus 5rVBS53 was site-specifically mutated from Proline to Leucine (Fig 4.2). The neutralization phenotypes of the mutant virus 5rVBS53P98L were determined and compared to those of the other viruses (Table 4.2 and 4.3). It was shown that 5rVBS53 EAV was neutralized by MAbs 5G11, 6D10, 7E5, 9F2, 10F11, 10H4, and 6A2, whereas the mutant virus 5rVBS53P98L was resistant to neutralization by these MAbs (Table 4.2). This clearly demonstrated that the neutralization phenotypic differences were caused by the exchange of Proline to Leucine in amino acid 98. The neutralization phenotypes of parental EAV strains and chimeric viruses and their respective amino acids at 98 of the GP5 protein are consistent with this finding (Table 4.2 and Fig 4.3). It has been previously demonstrated that MAb 6A2 recognizes amino acid 69 of the EAV GP5 protein (Balasuriya *et al.* 1997; 2004b). This study showed that MAb 6A2 may also recognize amino acid 98 of the EAV GP5 protein, confirming that neutralization epitopes may interact with each other and are conformationally dependent. On the other hand, exchange of Proline to Leucine in amino acid 98 did not result in VBS53 EAV becoming susceptible to neutralization by MAb 1H7, confirming that the neutralization phenotypic differences detected by MAb 1H7 among EAV strains VBS53, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80 were not caused by this site-specific substitution.

In summary, this study demonstrated that viral variants with novel neutralization phenotypes emerged during persistent EAV infection in Hela cells. Previously identified four neutralization sites (A-D) were all conserved among viruses recovered from persistently infected Hela cells, suggesting the existence of some as yet unidentified neutralization determinant(s). Using an infectious EAV cDNA clone and reverse genetics technology, it was identified that amino acid 98 on the EAV GP5 protein is a new neutralization determinant. It is suggested that amino acid 98 be classified in neutralization site D which covers amino acids 98-106.

Table 4.1 Monoclonal antibodies and polyclonal antisera to equine arteritis virus

Antibody	Antibody specificity/epitope location (amino acid position and [site]) recognized by the antibody	Reference
Monoclonal antibodies		
5G11	GP5/99 and 102; [D]	Balasuriya *et al.* 1993; 1995b
6D10	GP5/99 and 102; [D]	Balasuriya *et al.* 1993; 1995b
7E5	GP5/99 and 102; [D]	Balasuriya *et al.* 1993; 1995b
9F2	GP5/99 and 102; [D]	Balasuriya *et al.* 1993; 1995b
10F11	GP5/99 and 102; [D]	Balasuriya *et al.* 1993; 1995b
10H4	GP5/99, 102, and 103; [D]	Balasuriya *et al.* 1993; 1995b
1H7	GP5/49; [A]	Balasuriya *et al.* 1997
1H9	GP5/69; [C]	Balasuriya *et al.* 1997
5E8	GP5/61; [B]	Balasuriya *et al.* 1997
6A2	GP5/69; [C]	Balasuriya *et al.* 1997
7D4	GP5/69; [C]	Balasuriya *et al.* 1997
10B4	GP5/99, 102, and 103; [D]	Balasuriya *et al.* 1997
3E2	N	MacLachlan *et al.* 1998
12A4	nsp1	Wagner *et al.* 2003
polyclonal equine antisera		
anti-GP5/M	GP5 and M proteins	Balasuriya *et al.* 2002
anti-KY53	polyclonal	MacLachlan *et al.* 1998
anti-KY84	polyclonal	MacLachlan *et al.* 1998
anti-KY53+KY84	polyclonal	
anti-CA95	polyclonal	
anti-ARVAC	polyclonal	MacLachlan *et al.* 1998
anti-NVSL	polyclonal	Wagner *et al.* 2003
anti-EAV030H	polyclonal	Commercial serum from National Veterinary Services Laboratory
control serum NVSL	Not applicable	Commercial serum from National Veterinary Services Laboratory
control serum OAT PB	Not applicable	

Table 4.2 Neutralization titers[a] of monoclonal antibodies against parental and chimeric equine arteritis viruses

Virus strain	Monoclonal antibodies													
	5G11	6D10	7E5	9F2	10F11	10H4	1H7	1H9	5E8	6A2	7D4	10B4	3E2[c]	12A4[d]
VBS53 EAV	4096	>4096	1024	512	>4096	>4096	<32	<32	<32	64	<32	<32	<32	<32
5rVBS53 EAV	>4096	>4096	>4096	>4096	>4096	>4096	32	<32	<32	128	<32	<32	<32	<32
5rVBS53P98L	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32
Hela-EAVP10	4096	>4096	2048	1024	>4096	>4096	<32	<32	<32	512	<32	<32	<32	<32
5rHela-EAVP10	4096	>4096	2048	2048	>4096	>4096	<32	<32	<32	512	<32	<32	<32	<32
Hela-EAVP35	<32	<32	<32	<32	<32	<32	2048	<32	<32	<32	<32	<32	<32	<32
5rHela-EAVP35	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32
Hela-EAVP60[b]	<32	<32	<32	<32	<32	<32	2048	<32	<32	<32	<32	<32	<32	<32
5rHela-EAVP60[b]	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32
Hela-EAVP80	<32	<32	<32	<32	<32	<32	>4096	<32	<32	<32	<32	<32	<32	<32
5rHela-EAVP80	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32	<32

[a] Neutralization titers are expressed as the inverse of the antibody dilution providing 50% protection of RK-13 (KY) cell monolayers against 200 $TCID_{50}$ of virus

[b] Neutralization titers are expressed as the inverse of the antibody dilution providing 50% protection of RK-13 (KY) cell monolayers against 100 $TCID_{50}$ of virus

[c] control MAb to N protein of EAV

[d] control Mab to nsp1 of EAV

Table 4.3 Neutralization titers[a] of polyclonal equine antisera against parental and chimeric equine arteritis viruses

Virus strain	Polyclonal equine antisera									
	Anti-GP5/M	Anti-KY53+KY84	Anti-KY84	Anti-CA95	Anti-ARVAC	Anti-EAVNVSL	Anti-EAV030H	Anti-KY53	NVSL NEG[c]	OAT PB[d]
VBS53 EAV	8	>1024	256	512	8	64	64	256	<8	<8
5rVBS53 EAV	64	>1024	1024	>1024	32	256	512	>1024	<8	<8
5rVBS53P98L	128	>1024	>1024	>1024	32	256	1024	>1024	<8	<8
Hela-EAVP10	16	>1024	256	512	<8	128	256	1024	<8	<8
5rHela-EAVP10	128	>1024	1024	1024	64	128	256	1024	<8	<8
Hela-EAVP35	64	>1024	512	1024	16	256	128	512	<8	<8
5rHela-EAVP35	256	>1024	1024	1024	16	512	256	1024	<8	<8
Hela-EAVP60[b]	128	>1024	512	1024	16	512	256	512	<8	<8
5rHela-EAVP60[b]	256	>1024	>1024	>1024	32	256	512	>1024	<8	<8
Hela-EAVP80	1024	>1024	>1024	>1024	32	512	1024	1024	<8	<8
5rHela-EAVP80	128	>1024	>1024	>1024	32	128	1024	1024	<8	<8

[a] Neutralization titers are expressed as the inverse of the antibody dilution providing 50% protection of RK-13 (KY) cell monolayers against 200 $TCID_{50}$ of virus

[b] Neutralization titers are expressed as the inverse of the antibody dilution providing 50% protection of RK-13 (KY) cell monolayers against 100 $TCID_{50}$ of virus

[c,d] Negative control equine serum

Table 4.4 Comparison of structural protein genes of EAV strains VBS53, Hela-EAVP10, -P35, -P60, and -P80

Leader or ORF	Protein (aa length)	Nucleotide location[1]	Amino acid location[2]	VBS53 EAV	Hela-EAVP10	Hela-EAVP35	Hela-EAVP60	Hela-EAVP80
ORF2a (9751-9954)	E (67)	9867	39	T (Val)[3]	C (Val)	C (Val)	C (Val)	C(Val)
		9907	**53**	**A (Ser)**	**A (Ser)**	**A (Ser)**	**T (Cys)**	**T (Cys)**
		9914	**55**	**T (Val)**	**T (Val)**	**C (Ala)**	**C (Ala)**	**C (Ala)**
ORF2b (9824-10507)	GP2b (227)	**9867**	**15**	**T (Leu)**	**T (Leu)**	**C (Ser)**	**C (Ser)**	**C (Ser)**
		9907	28	A (Ala)	A (Ala)	A (Ala)	T (Ala)	T (Ala)
		9914	**31**	**T (Trp)**	**T (Trp)**	**C (Arg)**	**C (Arg)**	**C (Arg)**
		10082	**87**	**G (Val)**	**G (Val)**	**G (Val)**	**C (Leu)**	**C (Leu)**
		10085	**88**	**A (Met)**	**A (Met)**	**G (Val)**	**A (Met)**	**A (Met)**
		10157	**112**	**G (Ala)**	**G (Ala)**	**A (Thr)**	**A (Thr)**	**A (Thr)**
		10189	122	T (Ile)	C (Ile)	C (Ile)	C (Ile)	C (Ile)
		10267	148	T (Tyr)	T (Tyr)	T (Tyr)	C (Tyr)	T (Tyr)
ORF3 (10306-10797)	GP3 (163)	**10648**	**115**	**A (Ser)**	**A (Ser)**	**G (Gly)**	**G (Gly)**	**G (Gly)**
		10709	**135**	**T (Leu)**	**T (Leu)**	**C (Pro)**	**C (Pro)**	**C (Pro)**
		10722	139	Y (Ile)	T (Ile)	T (Ile)	T (Ile)	T (Ile)
ORF4 (10700-11158)	GP4 (152)	**10709**	**4**	**T (Tyr)**	**T (Tyr)**	**C (His)**	**C (His)**	**C (His)**
		10722	**8**	**Y (Leu/Ser)[4]**	**T (Leu)**	**T (Leu)**	**T (Leu)**	**T (Leu)**
		11024	**109**	**A (Ile)**	**A (Ile)**	**A (Ile)**	**T (Phe)**	**T (Phe)**
ORF5 (11146-11913)	GP5 (255)	**11171**	**9**	**T (Phe)**	**T (Phe)**	**C (Ser)**	**C (Ser)**	**C (Ser)**
		11438	**98**	**C (Pro)**	**C (Pro)**	**T (Leu)**	**T (Leu)**	**T (Leu)**
		11475	110	T (Ile)	T (Ile)	T (Ile)	C (Ile)	C (Ile)
		11704	187	T (Leu)	T (Leu)	C (Leu)	C (Leu)	C (Leu)
		11749	**202**	**A (Ile)**	**A (Ile)**	**A (Ile)**	**G (Val)**	**A (Ile)**
		11882	**246**	**G (Arg)**	**G (Arg)**	**A (Lys)**	**G (Arg)**	**G (Arg)**
ORF6 (11901-12389)	M (162)	-[5]	-	-	-	-	-	-
ORF7 (12313-12645)	N (110)	-	-	-	-	-	-	-

Notes to Table 4.4:

[1] Nucleotides are numbered according to the published sequence of EAV030 virus (van Dinten *et al.* 1997).

[2] Amino acids of structural proteins are numbered according to their locations in individual structural protein.

[3] Nucleotide and amino acid changes are shown. Amino acid is shown by three-letter symbols in parenthesis. Missense mutations are shown in bold.

[4] Leu/Ser means leucine or serine.

[5] - No nucleotide and amino acid changes occurred.

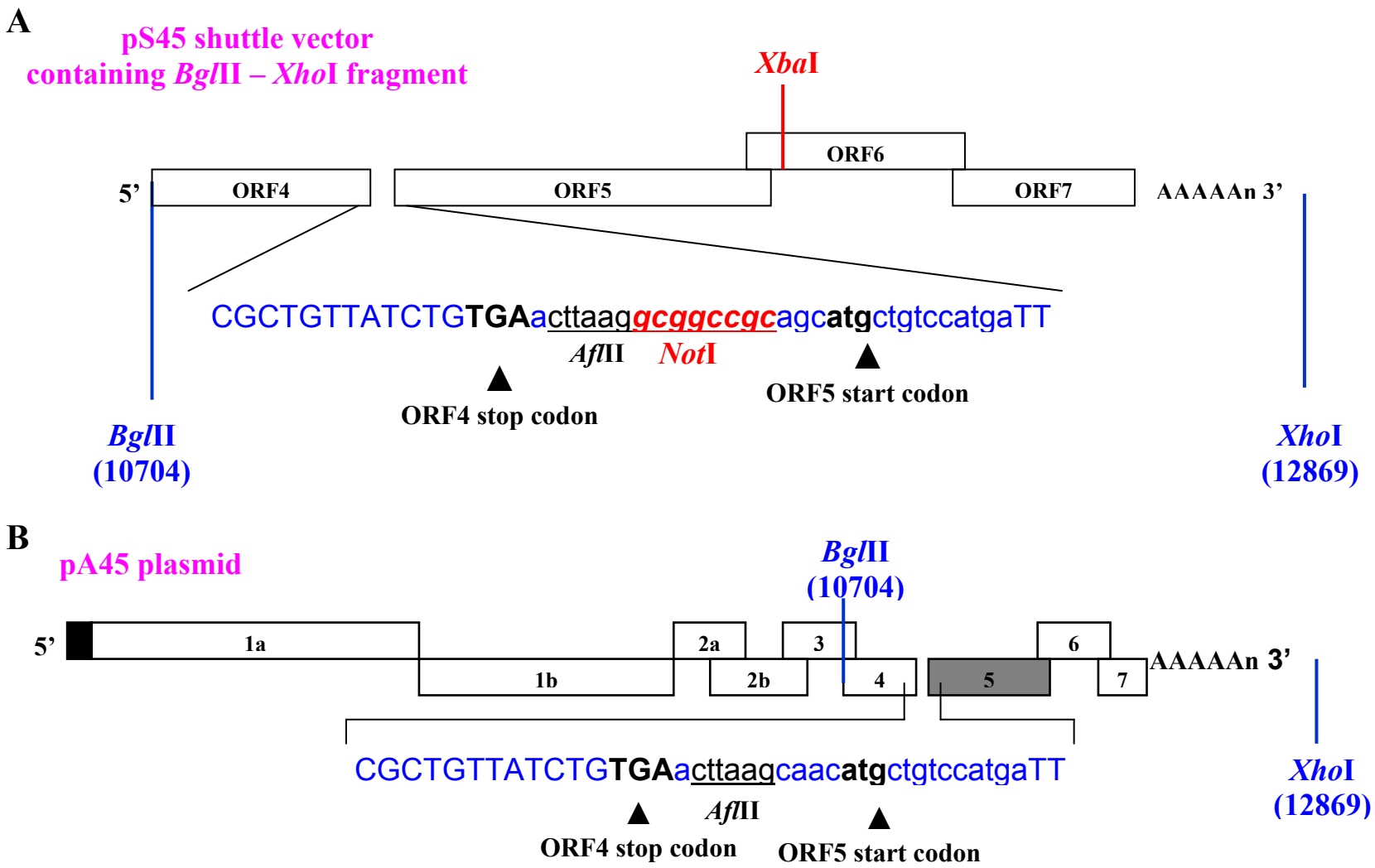
Table 4.5 Oligonucleotide primers used for RT-PCR amplification

Primer ID	Polarity	Sequence (5'-3')	Position (nt)
Primers for cloning entire ORF5 from EAV strains VBS53, Hela-EAVP10, -P35, -P60, and -P80 into the shuttle vector pS45			
11152SD	Positive	TCT GTG AAC TTA AGG CGG CCG CAG C*AT GTT ATC TAT GAT TG*	11152-11176[a]/*11146-11161*[b]
11960N	Negative	AAA GTA A*TC TAG A*TA CTC ACC TAA AAT CCC GTC ACC ACA AAA TGA ATC TA	11960-11911[b]
Primers for site-directed mutagenesis			
C211156P	Positive	GGC TCA ACG CTG TTA TCT GTG AAC	11137-11160[a]
11443N[c]	Negative	TGT AC**A** GTC CAT GCG CCT GTT CCA	11443-11420[b] (nt change at positive sense strand: 11438 C? T; aa change GP5 98 Pro? Leu); mutagenesis of ORF5 from VBS53 EAV
11515N	Negative	ACA CAT CCA ACA CAA CTA TGC C	11515-11494[b]

[a] Nucleotide sequence from the plasmid pS45 and numbered accordingly.

[b] EAV sequence numbered according to the pEAV030 sequence and the *Not*I site in the primer 11152SD is underlined; natural *Xba*I site in the primer 11960N is underlined and italicized.

[c] Mutagenesis primer and the mutated nucleotide is underlined and in bold.


A
pS45 shuttle vector
containing BglII – XhoI fragment
XbaI
ORF6
5'
ORF4
ORF5
ORF7
AAAAAn 3'
CGCTGTTATCTGTGAacttaaggcggccgcagcatgctgtccatgaTT
AflII
NotI
ORF4 stop codon
ORF5 start codon
BglII
(10704)
XhoI
(12869)
B
pA45 plasmid
BglII
(10704)
5'
1a
1b
2a
2b
3
4
5
6
7
AAAAAn 3'
CGCTGTTATCTGTGAacttaagcaacatgctgtccatgaTT
AflII
ORF4 stop codon
ORF5 start codon
XhoI
(12869)

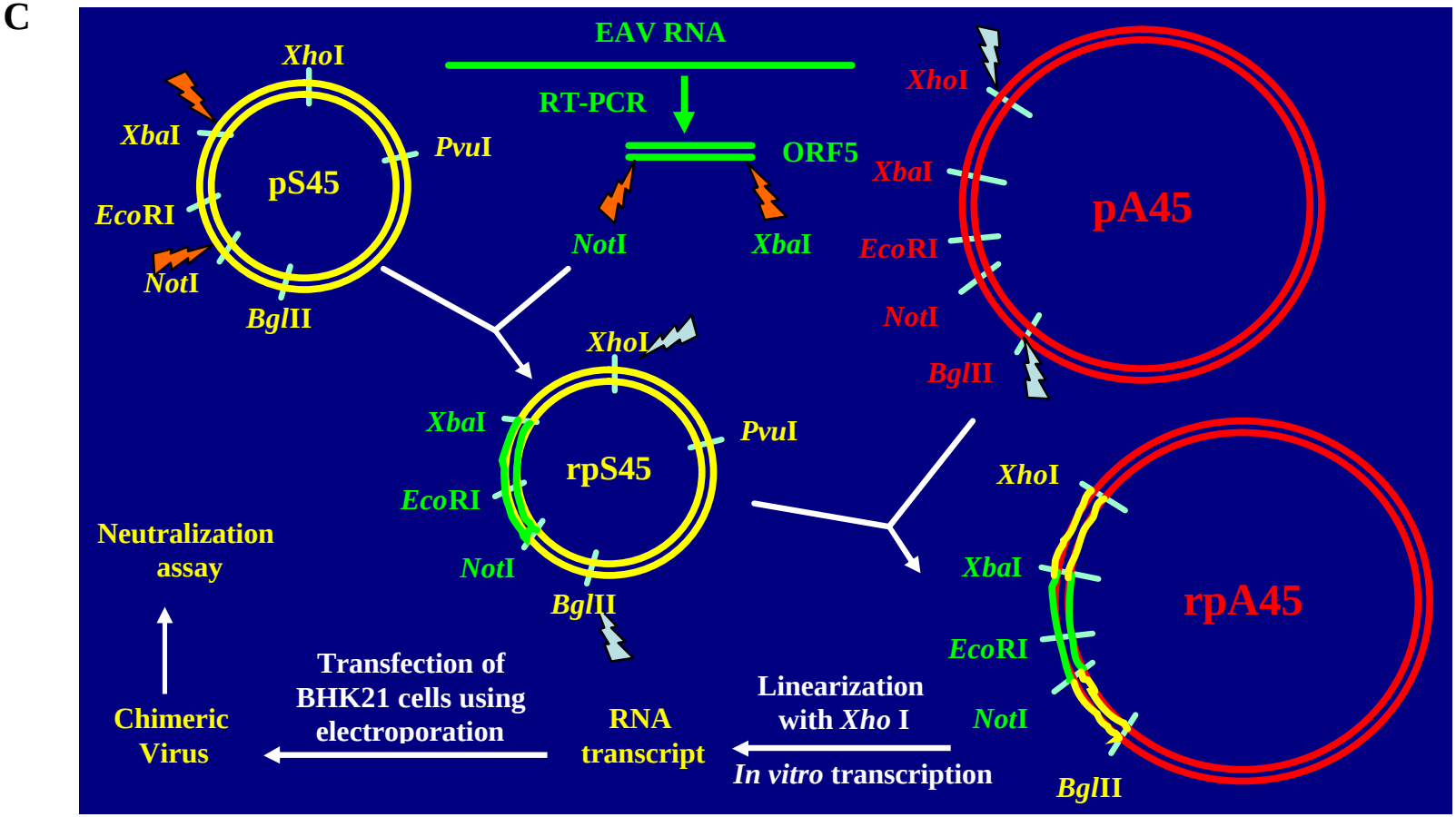


Fig 4.1 Flow diagram outlining construction of the full-length chimeric clone which contains ORF5 from different strains of EAV using the backbone of the infectious cDNA clone pA45 (de Vries *et al.* 2000; Dobbe *et al.* 2001) and generation of chimeric viruses. In A and B, the nucleotides that were inserted to separate ORFs 4 and 5 are shown in lowercase. See Materials and Methods for details.

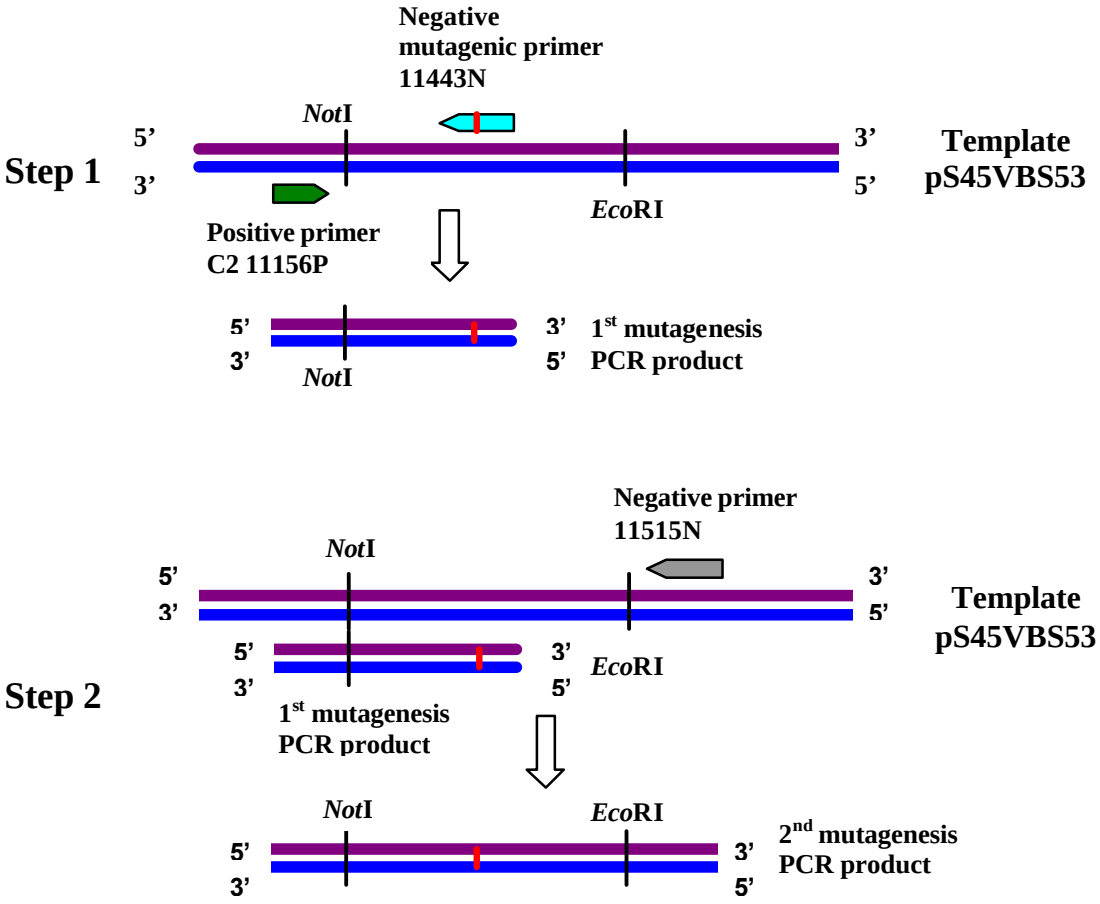


Fig 4.2 Flow diagram outlining the site-directed mutagenesis of EAV ORF5 11438C? T in plasmid pS45VBS53. In step 1, the mutation was introduced to the DNA by performing PCR with positive primer C211156P and negative mutagenic primer 11443N using the template pS45VBS53. In step 2, the 1st mutagenesis PCR product was mixed with the template pS45VBS53 and PCR was performed with positive primer (the 1st mutagenesis PCR product acting as positive primer) and negative primer 11515N. The 2nd mutagenesis PCR product is the desired fragment containing site-specific nucleotide substitution.

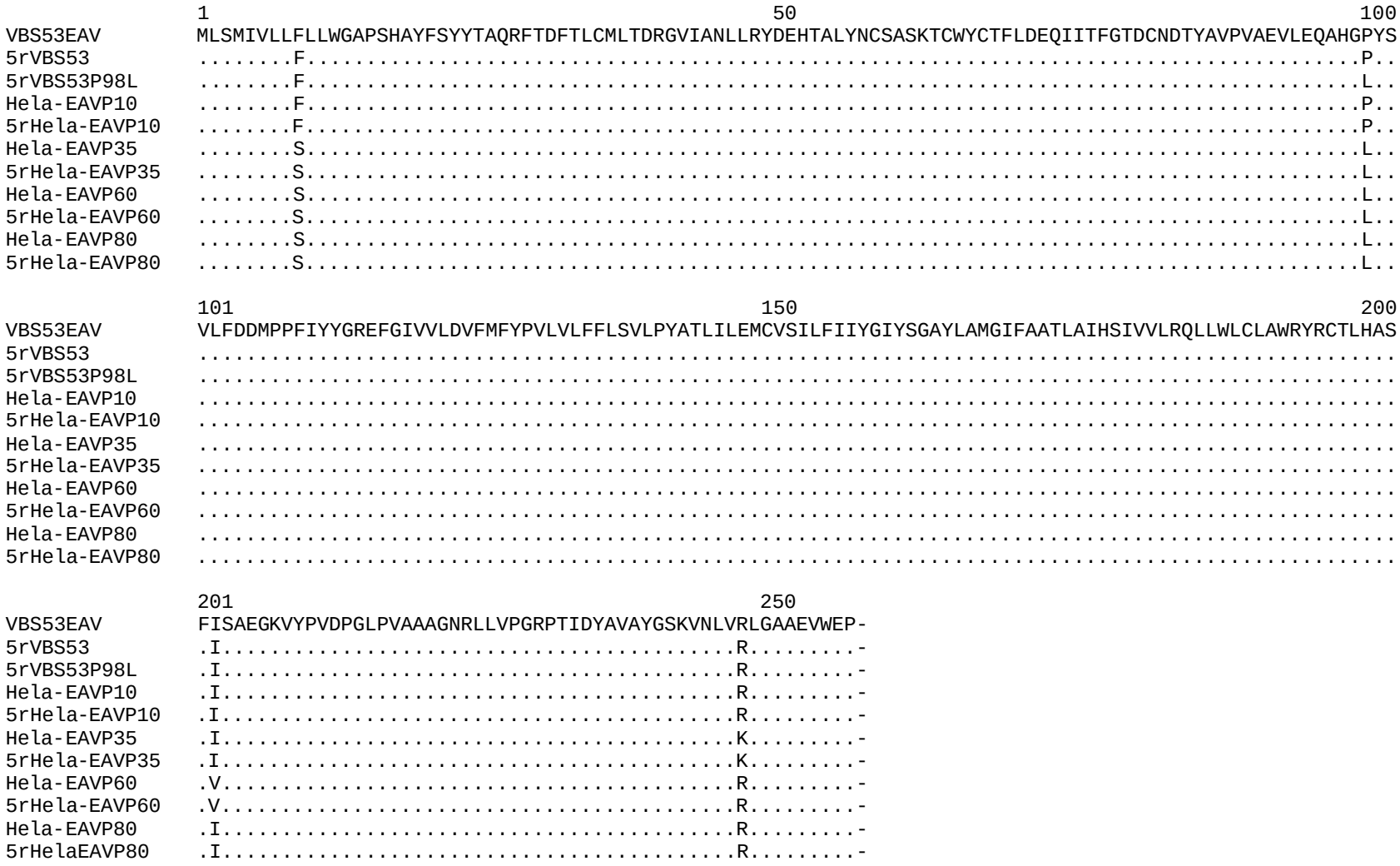


Fig 4.3 Aligned deduced amino acid sequence of the GP5 proteins of parental and chimeric equine arteritis viruses.

Chapter Five

Potential Changes in Pathogenicity of Equine Arteritis Virus During Persistent Infection of Hela Cells

ABSTRACT

A previously described *in vitro* assay has shown that the growth characteristics of different EAV strains in equine endothelial cells can generally be related to the pathogenicity of individual strains of the virus for horses [Moore *et al.* (2002) Virology 298, 39-44; Moore *et al.* (2003) Am J Vet Res 64, 779-84]. Virulent strains of EAV usually replicated more quickly, caused larger plaques, and gave rise to more rapid cell lysis in equine endothelial cells than did avirulent strains of the virus. In the present study, this *in vitro* assay was used to investigate whether virus recovered from Hela cells persistently infected with EAV differed in characteristics from the original strain of EAV used to initiate infection. It was found that viruses recovered from persistently infected Hela cells over time progressively caused smaller and smaller plaques in equine endothelial cells, suggesting that EAV probably became less virulent during the course of persistent infection in Hela cells. Since the actual virulence of different EAV strains can only be definitely determined in horses, the potential changes in pathogenicity of EAV during persistent infection of Hela cells need to be verified by challenge studies in horses.

INTRODUCTION

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), a globally distributed infectious disease of equids (Timoney and McCollum 1993). EAV is a single-stranded, positive sense RNA virus and the prototype member of the family *Arteriviridae* in the order *Nidovirales* (Cavanagh 1997). Only one serotype of EAV has been recognized (Fukunaga and McCollum 1977; Golnik *et al.* 1986), and all strains evaluated thus far are neutralized by polyclonal equine sera raised against the virulent Bucyrus strain of EAV (Balasuriya and MacLachlan 2004c). However, geographically and temporally distinct EAV isolates vary not only antigenically but also in the severity of the clinical disease they induce and in their abortigenic potential (Balasuriya *et al.* 1998; 1999b; Balasuriya and MacLachlan 2004c; McCollum and Timoney 1999;

Timoney and McCollum 1993). While the majority of field strains of EAV cause subclinical infection in horses, some strains can cause the clinical manifestations of EVA. For example, the virulence of certain EAV strains for horses has been defined: highly virulent (VBS53 and ATCC EAV), moderately virulent (KY84, CAN86, AZ87, and IL93), mildly virulent (SWZ64, AUT68, IL94, and CA97), and putatively avirulent (KY63, PA76, KY77, CA95G, and ARVAC vaccine) strains of EAV (MacLachlan *et al.* 1996; McCollum 1981; McCollum and Timoney 1984; McCollum *et al.* 1995; McCollum and Timoney 1999; Moore *et al.* 2003b; Patton *et al.* 1999; Timoney and McCollum 1993).

EAV has a particular tropism for macrophages and vascular endothelium in infected horses, and the clinical manifestations of EVA reflect endothelial injury (Crawford and Henson 1973; Del Piero 2000; Estes and Cheville 1970; MacLachlan *et al.* 1996; McCollum 1981). An *in vitro* assay has recently been developed (Moore *et al.* 2002; 2003b) which has been used to investigate the virulence of various strains of EAV in horses. Comparison of growth characteristics in equine endothelial cells (EECs) of various EAV strains whose virulence had been previously determined in horses revealed that virulent strains of EAV generally replicated more quickly, caused larger plaques, and led to more rapid cell lysis in cultured EECs than the avirulent strains did. However, there was one notable exception. The highly attenuated modified live vaccine strain of EAV caused larger plaques in equine endothelial cells than known virulent strains of the virus (Moore *et al.* 2003b). Based on the findings of the studies, it was concluded that primary equine endothelial cells can serve as a very useful *in vitro* model for characterizing the virulence of EAV (Moore *et al.* 2002; 2003b). It was found that virulent and avirulent strains of EAV induced different quantities of proinflammatory cytokines in alveolar and blood-derived equine macrophages (Moore *et al.* 2003a).

Persistent infection of Hela cells with EAV has been recently established (Chapter 3). It has been shown that EAV evolves during persistent infection with respect to its growth properties, ability to initiate secondary persistent infection (Chapter 3), and its genetic and neutralization phenotypic characteristics (Chapter 4). The objective of the present study was to investigate whether the pathogenicity of EAV had changed in the course of persistent infection in Hela cells. To achieve this goal, the *in vitro* assay

described by Moore *et al.* (2002; 2003b) was used to investigate and estimate the virulence of viruses recovered from persistently infected Hela cells. The actual pathogenicity of these viruses will require challenge studies in horses.

MATERIALS AND METHODS

Cells and viruses. Rabbit kidney (RK-13, KY) cells and baby hamster kidney (BHK-21) cells were maintained in Eagle's medium (EMEM) with 10% ferritin supplemented calf serum (FSCS), penicillin, streptomycin, fungizone, and sodium bicarbonate. The isolation and purification of primary equine endothelial cells (EEC) has been previously described in detail (Hedges *et al.* 2001; Moore *et al.* 2003b). Equine endothelial cells were kindly provided by Drs. Udeni Balasuriya and James MacLachlan at University of California, Davis. Equine endothelial cells were maintained in Dulbecco's modified essential medium (DMEM) with sodium pyruvate, 10% fetal bovine serum, penicillin, streptomycin, non-essential amino acids, and L-glutamine. All experiments were performed on EECs between passages 10 and 15. The VBS53 strain of EAV was propagated twice in BHK-21 cells to produce virus stocks which were used to establish persistent infection in the Hela 'High' cell line. Hela-EAVP10, Hela-EAVP20, Hela-EAVP35, Hela-EAVP50, Hela-EAVP60, and Hela-EAVP80 are the viruses isolated from the 10^{th}, 20^{th}, 35^{th}, 50^{th}, 60^{th}, and 80^{th} passage levels of the persistently infected Hela 'High' cell line, respectively.

Growth of various EAV strains in EECs and RK-13 cells. Each virus (VBS53, Hela-EAVP10, Hela-EAVP20, Hela-EAVP35, Hela-EAVP50, Hela-EAVP60, and Hela-EAVP80) was diluted to approximately 100 pfu per ml and inoculated onto confluent monolayers of EECs or RK-13 cells. Each virus strain was inoculated into six T-25 flasks with an inoculum of 1 ml of diluted virus per flask. After one hour's incubation at 37ºC, cell monolayers were overlaid with EMEM containing 0.75% carboxymethyl cellulose. Inoculated cultures were incubated at 37ºC, and cytopathic effects were checked daily. At each of the following time points, 48, 72, and 87 h post inoculation, duplicates of the inoculated cell cultures were stained with 10% formalin buffered crystal violet. Of the cultures stained at 87 h post inoculation, at least 50 plaques were measured to determine mean plaque size.

RESULTS AND DISCUSSION

Genetic variation and divergent phenotypic properties (e.g. neutralization and virulence) have been repeatedly demonstrated among field isolates and laboratory adapted strains of EAV. Persistently infected stallions (carrier stallions) are thought to be a natural source of genetic and phenotypic diversity of EAV (Balasuriya *et al.* 2004a; Balasuriya and MacLachlan 2004c; Hedges *et al.* 1999a). An *in vitro* model of EAV persistence in Hela cells has been recently established (Chapter 3) to help characterize virus evolution during persistent infection. It has been shown that EAV evolves during persistent infection in cell culture with respect to its growth properties, ability to initiate secondary persistent infection (Chapter 3), and genetic and neutralization phenotypic characteristics (Chapter 4). In order to determine whether the pathogenicity of EAV had changed during persistent infection of Hela cells, an *in vitro* assay described by Moore *et al.* (2002; 2003b) was used to investigate the virulence of viruses recovered from persistently infected Hela cells. All of the EAV strains (VBS53, Hela-EAVP10, Hela-EAVP20, Hela-EAVP35, Hela-EAVP50, Hela-EAVP60, and Hela-EAVP80) caused lytic infections in both RK-13 and primary equine endothelial cells. However, the onset of cytopathic effect (CPE) differed in RK-13 and EECs. In EEC cells, CPE became evident at 48 h post inoculation, and typical plaques could be observed after cells were stained. However, in RK-13 cells, CPE was not apparent even at 72 h post inoculation, and plaques were not clear after cells were stained at either 48 h or 72 h post inoculation. At 87 h post inoculation, CPE in both EECs and RK-13 cells was evident and plaques were clearcut and readily measurable after staining. Therefore, it was decided to determine and compare plaque size of each virus in RK-13 and EECs at 87 h post inoculation.

As shown in Table 5.1 and Fig 5.1, EAV strain VBS53 produced significantly larger plaques in EECs than all of the other EAV strains evaluated (Hela-EAVP10, -P20, -P35, -P50, -P60, and -P80; $P < 0.002$ between VBS53 EAV and Hela-EAVP10; $P < 0.001$ between VBS53 EAV and any other EAV strains); Hela-EAVP10 produced significantly larger plaques in EECs than Hela-EAVP20, -P35, -P50, -P60, and -P80 did ($P < 0.001$); Hela-EAVP20 produced significantly larger plaques in EECs than Hela-EAVP35, -P50, -P60, and -P80 did ($P < 0.001$); Hela-EAVP35 produced significantly larger plaques in EECs than did Hela-EAVP50 ($P < 0.001$), -P60 ($P < 0.002$), and -P80 (P

<0.001); there was no significant difference in plaque size among Hela-EAVP50, -P60, and -P80 strains (P > 0.1). In contrast, there was no significant difference in the size of plaques produced by all seven of these EAV strains in RK-13 cells. If the size of plaques produced by EAV in EECs truly reflects the virulence of a virus strain for horses, these findings would suggest that EAV became progressively less virulent during the course of persistent infection in Hela cells up to the 50^{th} passage in cell culture after which there did not appear to be any further reduction in viral virulence.

However, the actual pathogenicity of these EAV strains needs to be definitively determined by challenge studies in horses. Determination of the pathogenicity of these viruses recovered from persistently infected Hela cells for horses is currently in progress. If EAV, by serial passage in persistently infected Hela cells, should prove to become progressively less virulent for the horse, that is not without precedent. It has been previously demonstrated that foot-and-mouth disease virus became progressively less virulent for mice and cattle during the course of persistence in BHK-21 cells (Diez *et al.* 1990b). So far, the genetic determinants of EAV virulence have not been defined. Alignment of available nucleotide and amino acid sequences of a variety of EAV strains with varying virulence (e.g. EAV strains VBS53, KY84, CAN86, AZ87, IL93, SWZ64, AUT68, IL94, CA97, KY63, PA76, KY77, CA95G, and ARVAC vaccine) failed to identify a unique mutation that could possibly distinguish virulent strains from avirulent strains (data not shown). It would suggest that virulence differences between EAV strains are probably determined by a combination of multiple mutations or by mutation(s) present in the fragment of the genome whose sequences are not available yet for most of the aforementioned EAV strains. Therefore, comparison of the entire genomes of EAV strains that differ in virulence for horses may be the appropriate way to identify the target gene(s) responsible for EAV virulence. At this time, entire genome sequences are only available for a few EAV strains, e.g. the VBS53 strain (Chapter 3), ARVAC vaccine strain (Balasuriya *et al.*, unpublished), EAV030 virus generated from the infectious cDNA clone EAV030 (Balasuriya *et al.* 1999b; van Dinten *et al.* 1997), Hela-EAVP35 and Hela-EAVP80 (Chapter 3). Among these strains, VBS53 EAV has demonstrated virulent properties (MacLachlan *et al.* 1996; McCollum *et al.* 1971); the ARVAC vaccine strain and EAV030 virus have been demonstrated avirulent (Balasuriya *et al.* 1999b;

McCollum 1986); the virulence of Hela-EAVP35 and Hela-EAVP80 for horses has not been established. Determination of the *in vivo* virulence of Hela-EAVP35 and Hela-EAVP80 should help to identify the genetic determinants of EAV virulence.

Table 5.1 Plaque size of different EAV strains in RK-13 (KY) and EEC cells

Virus	Number of plaques measured		Plaque diameter (mm) Mean ± SE	
	RK-13 (KY)	EEC	RK-13 (KY)	EEC
VBS53 EAV	81	59	2.12 ± 0.06	6.17 ± 0.13
Hela-EAVP10	79	63	2.03 ± 0.06	5.67 ± 0.09
Hela-EAVP20	85	60	1.99 ± 0.04	4.88 ± 0.12
Hela-EAVP35	95	98	2.07 ± 0.04	3.92 ± 0.07
Hela-EAVP50	85	83	2.16 ± 0.04	3.33 ± 0.08
Hela-EAVP60	90	55	2.05 ± 0.04	3.53 ± 0.09
Hela-EAVP80	90	58	2.00 ± 0.04	3.36 ± 0.11

RK-13 (KY) = Rabbit Kidney cells (KY); EEC = Equine endothelial cells;
SE = Standard error of the Mean.

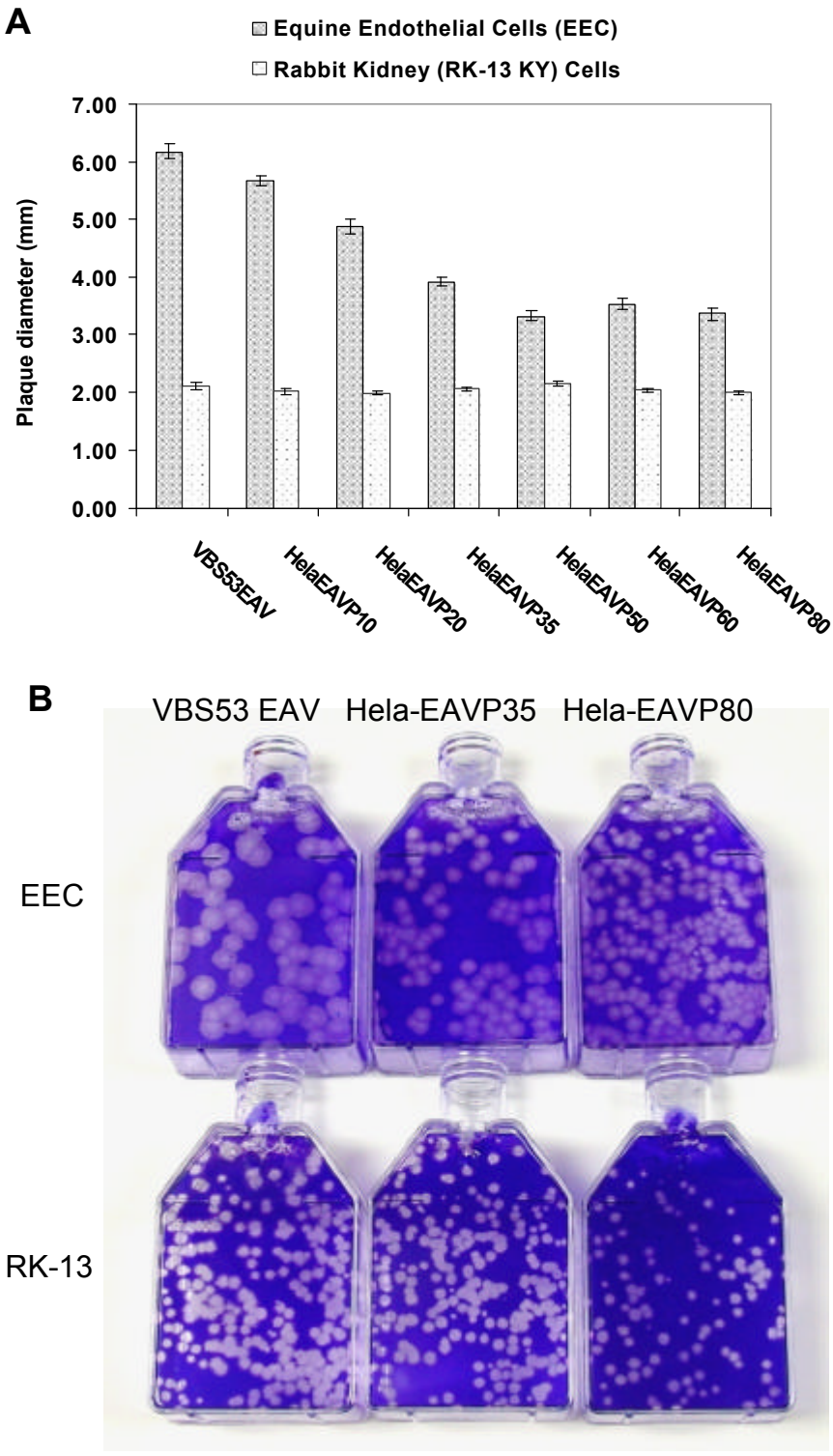


Fig 5.1 Size of plaques produced by EAV strains VBS53, Hela-EAVP10, Hela-EAVP20, Hela-EAVP35, Hela-EAVP50, Hela-EAVP60, and Hela-EAVP80 in equine endothelial cells (EECs) and rabbit kidney (RK-13 KY) cells. Mean ± Standard Error is shown in (A). In (B), pictures of plaques formed by three EAV strains VBS53, Hela-EAVP35, and Hela-EAVP80 in EECs and RK-13 cells are shown.

SUMMARY

Chapter 1 of this dissertation presents an overview of current knowledge of the molecular biology of equine arteritis virus; EAV infection and persistently infected stallions were briefly described; and persistent infection of cell cultures with RNA viruses were reviewed.

Neither a virus attachment molecule nor a specific cell receptor has yet been identified for equine arteritis virus. Identification of the receptor used by EAV for cell attachment and entry has been hampered by the unavailability of a known cell line that lacks the appropriate EAV receptor(s). In Chapter 2, a variety of cell lines of different species and tissue origin were assessed for their permissiveness to infection with the strain VBS53 of EAV and the mechanism that restricts EAV infection in the non-permissive cell lines was investigated. The cell lines BHK-21, RK-13, and C2C12 were found to support productive infection with EAV strain VBS53, whereas Hela, Hep-2, and L-M cell lines exhibited limited susceptibility to infection with the virus. In the course of the study, it was found that the Hela cell line became more susceptible to infection with EAV strain VBS53 after extended serial passage. The respective cell lines were referred to as Hela 'High' (passage 170-221) and Hela 'Low' (passage 95-115) lines. While the Hela 'High' cell line was more susceptible than the Hela 'Low' cell line, it was still considerably less susceptible than the BHK-21 cell line to EAV infection. Subsequent studies demonstrated that infection with EAV strain VBS53 was restricted at the entry step in Hela, Hep-2, and L-M cell lines. The availability of cells with varying susceptibility to EAV infection should help to identify the cellular factor(s) involved in EAV attachment and entry.

Up to 30% to 70% of stallions can subsequently become persistently infected following natural infection with EAV. The detailed mechanism by which EAV establishes persistent infection in stallions is unknown. Carrier stallions are not only a natural reservoir of EAV responsible for maintenance and dissemination of EAV in equine populations, but also a natural source of genetic and phenotypic diversity of EAV. Persistent viral infections in cell culture have been demonstrated as a valuable tool to study virus-host cell interactions, virus and host cell evolutions, and to elucidate the mechanisms of viral persistence. In Chapter 3, an *in vitro* model of EAV persistence,

namely persistently infected cultures of Hela cells, was established and characterized. Virus evolution with respect to virus growth characteristics, ability of the virus to initiate secondary persistent infection, and genetic changes during persistent EAV infection in Hela cells was investigated. In Chapter 4, neutralization phenotypic changes of viruses were observed during the course of persistent EAV infection in Hela cells. Subsequent reverse genetics studies identified that amino acid 98 of the GP5 protein is a new neutralization determinant of EAV. In Chapter 5, using an *in vitro* assay, it was found that EAV probably became progressively less virulent during the course of persistent infection in Hela cells. The potential changes in pathogenicity of EAV during persistent infection of Hela cells need to be verified by inoculation of horses. The results in Chapters 3, 4, and 5 have demonstrated that persistent EAV infection of Hela cells provides a system to characterize genetic and phenotypic evolution of the virus. Persistent EAV infection of Hela cells may also be a useful tool for investigating viral and host cell factors involved in viral persistence and for elucidating virus-host cell interactions, e.g. EAV-cell receptor interactions.

Appendix 1. Primers used for RT-PCR amplification of the genome of EAV

Fragment	Primer ID	Polarity	Nt position	Nucleotide sequence (5' to 3')	Primer Size (bp)
Frag 1	1P	+	1-30	GCT CGA AGT GTG TAT GGT GCC ATA TAC GGC	30
(1559 bp)	1540N	-	1540-1559	TTG AAG TCG CGT GCG CGG TG	20
Frag 2	947P	+	947-966	CTA CGT TTG TGA CAT CTC TG	20
(2191 bp)	3118N	-	3118-3137	GAA CAG ATT CAC AAA AAG CG	20
Frag 3	2610P	+	2610-2629	GAC CAT GCT CTT TAC AAC CG	20
(2010 bp)	4600N	-	4600-4619	GTC ATC ATC AGT GAG GGC AG	20
Frag 4	4357P	+	4357-4376	TCC TAG TGT ACC AGT TCC CC	20
(2240 bp)	6577N	-	6577-6596	TCT CCA GGT CTG TTT CAA GG	20
Frag 5	5802P	+	5802-5821	ACG CTT TTC AAG GGT TCC AC	20
(949 bp)	6731N	-	6731-6750	CCC CCG CGT TTG GTG AAT GC	20
Frag 6	6587P	+	6587-6606	GAC CTG GAG AGT TGT GAT CG	20
(3284 bp)	9850N	-	9850-9870	AGT AAC AAC AGC CAA TGC AAG	21
Frag 7	7391P	+	7391-7411	TGG TTC TGT GGC AAT TGT GTC	21
(2480 bp)	9850N	-	9850-9870	AGT AAC AAC AGC CAA TGC AAG	21
Frag 8	9745P	+	9745-9767	CGT GTG ATG GGC TTA GTG TGG TC	23
(1718 bp)	11442N	-	11442-11462	ATG TCA TCA AAC AGC GCA CTG	21
Frag 9	11097P	+	11097-11117	TTG TGG CTA TAG TTT ATG TTC	21
(1608 bp)	12681N	-	12681-12704	GGT TCC TGG GTG GCT AAT AAC TAC	24

Note: Nucleotides are numbered according to the published sequence of EAV030 virus (van Dinten et al., 1997).

Appendix 2. Optimized thermacycling protocols for PCR amplification of EAV genome

Fragment	Primer	Thermacycling Conditions		
		VBS53 EAV	Hela-EAVP35	Hela-EAVP80
Frag 1	1P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		55ºC 30 sec	55ºC 30 sec	55ºC 30 sec
	1540N	72ºC 2 min	72ºC 2 min	72ºC 2 min
Frag 2	947P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		42ºC 30 sec	42ºC 30 sec	40ºC 30 sec
	3118N	72ºC 3 min	72ºC 3 min	72ºC 3 min
Frag 3	2610P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		46ºC 30 sec	46ºC 30 sec	46ºC 30 sec
	4600N	72ºC 3 min	72ºC 3 min	72ºC 3 min
Frag 4	4357P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		46ºC 30 sec	46ºC 30 sec	46ºC 30 sec
	6577N	72ºC 3 min	72ºC 3 min	72ºC 3 min
Frag 5	5802P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		49ºC 30 sec	49ºC 30 sec	47ºC 30 sec
	6731N	72ºC 2 min	72ºC 2 min	72ºC 2 min
Frag 6	6587P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		46ºC 30 sec	46ºC 30 sec	46ºC 30 sec
	9850N	72ºC 3 min	72ºC 3 min	72ºC 3 min
Frag 7	7391P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		46ºC 30 sec	46ºC 30 sec	46ºC 30 sec
	9850N	72ºC 3 min	72ºC 3 min	72ºC 3 min
Frag 8	9745P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		47ºC 30 sec	45ºC 30 sec	45ºC 30 sec
	11442N	72ºC 3 min	72ºC 3 min	72ºC 3 min
Frag 9	11097P	95ºC 30 sec	95ºC 30 sec	95ºC 30 sec
		42ºC 30 sec	45ºC 30 sec	45ºC 30 sec
	12681N	72ºC 3 min	72ºC 3 min	72ºC 3 min

Note:
1. PCR amplification of each fragment of each strain follows: 95ºC 2 min; 35 cycles of amplification; 72ºC 10 min; 4ºC forever. Above table only shows the thermacycling conditions for 35 cycles of amplification.

Appendix 3. EAV sequencing primers list

Note: Nucleotides are numbered according to the published sequence of EAV030 virus (van Dinten et al., 1997)

Fragment 1	Positve	Sequence (5'-3')	Nt Position	Size (bp)
(1-1559)	1P	GCT CGA AGT GTG TAT GGT GCC ATA TAC GGC	1--30	30
	457P	GGT ATC GAG CTG CCA AAG TC	457--476	20
	833P	AGA CCT GGG TTT GGG CAT CA	833--852	20
	947P	CTA CGT TTG TGA CAT CTC TG	947--966	20
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	233N	CAA ATC CAG TAG CGG AGA AG	233--252	20
	548N	CTT GCC TCT TCA ATG GCT AAC	548--568	21
	880N	AGG CGA ACC CCT CAT AAT GT	880-899	20
	1270N	ATT GCA GAT GCC CCT GAA AC	1270--1289	20
	1540N	TTG AAG TCG CGT GCG CGG TG	1540--1559	20

Fragment 2	Positive	Sequence (5'-3')	Nt Position	Size (bp)
(947-3137)	1524P	CAG GCG CCC ATC CCA GCA CC	1524--1543	20
	1891P	TGG GCA ATA ATG TTG TTC TG	1891--1910	20
	2296P	TCG ATG TTG TGG GCA TGG C	2296--2314	19
	2610P	GAC CAT GCT CTT TAC AAC CG	2610--2629	20
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	1540N	TTG AAG TCG CGT GCG CGG TG	1540--1559	20
	1836N	AAA GCA AGA GAT GGC CAT AT	1836--1855	20
	2106N	ACA GTG CAA GGA ACA TAA GC	2106--2125	20
	2508N	TCA CAA CAG CTC CTG TTT GTT C	2508--2529	22
	3118N	GAA CAG ATT CAC AAA AAG CG	3118-3137	20

Fragment 3	Positive	Sequence (5'-3')	Nt Position	Size (bp)
(2610-4619)	2963P	GAT CTT GTT GGT CTG CCA CC	2963--2982	20
	3331P	ATT CAC TGC TTG GAG TGG CT	3331-3350	20
	3668P	TTT CGC CCA ACC AAC AAC CG	3668--3687	20
	4002P	CTG ATT GAT GGC TTA TCC AA	4002--4021	20
	4357P	TCC TAG TGT ACC AGT TCC CC	4357--4376	20
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	3485N	TGT TCC TGG TCC ACA CTG AC	3485--3504	20
	3832N	CAC GTA GGC AAC ACC ACT TG	3832--3851	20
	4472N	AAG TCC TGT CAA AGC GAC CT	4472--4491	20
	4600N	GTC ATC ATC AGT GAG GGC AG	4600--4619	20

Fragment 4	Positive	Sequence (5'-3')	Nt Position	Size (bp)
(4357-6596)	4357P	TCC TAG TGT ACC AGT TCC CC	4357--4376	20
	4718P	CAT CCT TAA TGC ATC CTT GC	4718--4737	20
	5072P	TGC GGT CTC GAA TGA TTA TG	5072--5091	20
	5436P	TGG GGC GTC GGA CCT TTG CT	5436--5455	20
	5802P	ACG CTT TTC AAG GGT TCC AC	5802--5821	20
	6198P	GGG CGT ACT TAA AAG AGG AG	6198--6217	20
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	4828N	CAG ACC GTC GAT AAC CAC AA	4828--4847	20
	5198N	CTT CCT GAT GCT CCA CGT AA	5198--5217	20
	5569N	ACC CAA GCA GTG TGT CTT TT	5569--5588	20
	5969N	GTA ACG GCC TCA CAT CCA GC	5969--5988	20
	6363N	CAT GGT GGC GGT TTG TAG AT	6363--6382	20
	6577N	TCT CCA GGT CTG TTT CAA GG	6577--6596	20

Fragment 5	Positive	Sequence (5'-3')	Nt Position	Size (bp)
(5802-6750)	6449P	ATT GGC CTA GGT TTG CGT GCC TGC	6449--6472	24
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	6731N	CCC CCG CGT TTG GTG AAT GC	6731--6750	20

Fragment 6	Positive	Sequence (5'-3')	Nt Position	Size (bp)
(6587-9870)	6587P	GAC CTG GAG AGT TGT GAT CG	6587--6606	20
	6950P	ACA CCC AAC CAG CAT TAC GC	6950--6969	20
	7391P	TGG TTC TGT GGC AAT TGT GT	7391--7411	21
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	7111N	GTG TAA CGC GGT CCT GAA GA	7111--7130	20
	7468N	AAG TGG AGC GGT ACA TGA TG	7468--7487	20

Fragment 7	Positive	Sequence (5'-3')	Nt Position	Size (bp)
(7391-9870)	7720P	CAT CGC AGT ACC ACT TCA GG	7720--7739	20
	8107P	AGT CAA GGG TTA CGG TGA TT	8107--8126	20
	8493P	ACA TCT ACG ACC CCT TTG AT	8493--8512	20
	8842P	GCC CGT AGT GTC CAA TGA TA	8842--8861	20
	9206P	GCC CTG GTA GGT GCT TCA TT	9206--9225	20
	9570P	CAC CTG CTG GTT ATG CGA TC	9570--9589	20
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	7831N	GCA CAT CTT TGA CCA GGT GA	7831--7850	20
	8177N	AGG CTG ACA AAA TGT CGG AG	8177--8196	20
	8543N	AAG TCC TGC GCC TCT GCT TC	8543--8562	20
	8907N	CGC TGA GCA CGG TTT ACT GA	8907--8926	20
	9299N	ACG CGA CTC AGT GTC TCA GG	9299--9318	20
	9614N	ACT TCT GTT GAG CTG AGG AG	9614--9633	20
	9850N	AGT AAC AAC AGC CAA TGC AAG	9850--9870	21

Fragment 8	Positive	Sequence (5'-3')	Nt Position	Size (bp)
9745P-11442N	9745P	CGT GTG ATG GGC TTA GTG TGG TC	9745--9767	23
	10142P	GAG TCT TCT AGC TAT GCT CC	10142--10161	20
	10511P	ACA CGG GTT ATG AAT ATG CC	10511--10530	20
	10845P	GGT GTA GGA ATT TTA TTA ATG	10845--10865	21
	11097P	TTG TGG CTA TAG TTT ATG TTC	11097--11117	21
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	10128N	AAG ACT CCA GAT GGT CAA AGC	10128--10148	21
	10517N	GAC CCC GGC ATA TTC ATA AC	10517--10536	20
	10780N	TAA AAT TAC GAG CCT CTG CAG CG	10780--10802	23
	11235N	CGT CAG CAT ACA CAA GGT GAA G	11235--11256	22

Fragment 9	Positive	Sequence (5'-3')	Nt Position	Size (bp)
11097P-12681N	11272P	GCC AAT TTG CTG CGA TAT GAT G	11272--11293	22
	11557P	CTA CCC TAT GCT ACG CTT ATT C	11557--11578	22
	11850P	AGT GGC CTA CGG CAG CAA AGT C	11850--11871	22
	12069P	TTT GTT ATA GTT GGA AGA GC	12069--12088	20
	12481P	GAT TTA AAT CAA CAG GAG CG	12481--12500	20
	Negative	Sequence (5'-3')	Nt Position	Size (bp)
	11442N	ATG TCA TCA AAC AGC GCA CTG	11442--11462	21
	11691N	CAG GCA TAA CCA CAG TAA TTG G	11691--11712	22
	11995N	CTA ACC CAG ATG CTA CAT ACC	11995--12015	21
	12370N	TCA TTG TAG CTT GTA GGC TG	12370--12389	20
	12681N	GGT TCC TGG GTG GCT AAT AAC TAC	12681--12704	24

Appendix 4. Protocols of cycle sequencing

Reagent	Volume Needed
BigDye Ready Reaction Mix (2.5X)	1.5 µl
Half-Dye mix	1.5 µl
EAV Sequencing Primer (3.2 pmol/µl)	1 µl
Template	Variable, depends on template size
Nuclease-free water	Variable
Final Volume	**15 µl**

Note: Perform cycle sequencing following the program below:

(1) Place the tubes in a thermal cycler and set to the correct volume.

(2) Perform an initial denaturation.

   a. Rapid thermal ramp (1°C/second) to 96°C
   b. 96°C for 1 min

(3) Repeat the following for 25 cycles:

- Rapid thermal ramp to 96°C
- 96°C for 10 seconds.
- Rapid thermal ramp to 50°C
- 50°C for 5 seconds.
- Rapid thermal ramp to 60°C
- 60°C for 4 minutes.

(4) Rapid thermal ramp to 4°C and hold until ready to purify.

Appendix 5. Alignment of nucleotide sequences of VBS53 EAV (entire genome), Hela-EAVP10 (ORFs 2-7), Hela-EAVP35 (entire genome), Hela-EAVP60 (ORFs 2-7) and Hela-EAVP80 (entire genome)

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             1                                                  50                                                 100
VBS53 EAV    GCTCGAAGTGTGTATGGTGCCATATACGGCTCACCACCATATACACTGCAAGAATTACTATTCTTGTGGGCCCCTCTCGGTAAATCCTAGAGGGCTTTCC
Hela-EAVP10
Hela-EAVP35  ..................................................................................................
Hela-EAVP60
Hela-EAVP80  ..................................................................................................

             101                                                150                                                200
VBS53 EAV    TCTCGTTATTGCGAGATTCGTCGTTAGATAACGGCAAGTTCCCTTTCTTACTATCCTATTTTCATCTTGTGGCTTGACGGGTCACTGCCATCGTCGTCGA
Hela-EAVP10
Hela-EAVP35  ..................................................................................................
Hela-EAVP60
Hela-EAVP80  ..................................................................................................

             201                                                250                                                300
VBS53 EAV    TCTCTATCAACTACCCTTGCGACTATGGCAACCTTCTCCGCTACTGGATTTGGAGGGAGTTTTGTTAGGGACTGGTCCCTGGACTTACCCGACGCTTGTG
Hela-EAVP10
Hela-EAVP35  ..................................................................................................
Hela-EAVP60
Hela-EAVP80  ..................................................................................................

             301                                                350                                                400
VBS53 EAV    AGCATGGCGCGGGATTGTGCTGTGAAGTGGACGGCTCCACCTTATGCGCCGAGTGTTTTCGCGGTTGCGAAGGAGTGGAGCAATGTCCTGGCTTGTTCAT
Hela-EAVP10
Hela-EAVP35  ..................................................................................................
Hela-EAVP60
Hela-EAVP80  ..................................................................................................

             401                                                450                                                500
VBS53 EAV    GGGACTGTTAAAACTGGCTTCGCCAGTTCCAGTGGGACATAAGTTCCTGATTGGTTGGTATCGAGCTGCCAAAGTCACCGGGCGTTACAATTTCCTTGAG
Hela-EAVP10
Hela-EAVP35  ..................................................................................................
Hela-EAVP60
Hela-EAVP80  ..................................................................................................
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            501                                                 550                                                 600
VBS53 EAV   CTGTTGCAACACCCTGCTTTCGCCCAGCTGCGTGTGGTTGATGCTAGGTTAGCCATTGAAGAGGCAAGTGTGTTTATTTCCACTGACCACGCGTCTGCTA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            601                                                 650                                                 700
VBS53 EAV   AGCGTTTCCCTGGCGCTAGATTTGCGCTGACACCGGTGTATGCTAGCGCTTGGGTTGCGAGCCCGGCTGCTAACAGTTTGATAGTGACCATTGACCAGGA
Hela-EAVP10
Hela-EAVP35 ..........................................................T.....................................C.....
Hela-EAVP60
Hela-EAVP80 ..........................................................T.....................................T.....

            701                                                 750                                                 800
VBS53 EAV   ACAAGATGGGTTCTGCTGGTTAAAACTTTTGCCACCTGACCGCCGTGAGGCTGGTTTGCGGTTGTATTACAACCATTACCGCGAACAAAGGACCGGGTGG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            801                                                 850                                                 900
VBS53 EAV   CTGTCTAAAACAGGACTTCGCTTATGGCTTGGAGACCTGGGTTTGGGCATCAATGCGAGCTCTGGAGGGCTGAAATTCCACATTATGAGGGGTTCGCCTC
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            901                                                 950                                                1000
VBS53 EAV   AGCGAGCTTGGCATATCACAACACGCAGCTGCAAGCTGAAGAGCTACTACGTTTGTGACATCTCTGAAGCAGACTGGTCCTGTTTGCCTGCTGGCAACTA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................
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            1001                                                1050                                                1100
VBS53 EAV   CGGCGGCTACAATCCACCAGGGGACGGAGCTTGCGGTTACAGGTGCTTGGCCTTCATGAATGGCGCCACTGTTGTGTCGGCTGGTTGCAGTTCTGACTTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            1101                                                1150                                                1200
VBS53 EAV   TGGTGTGATGATGAGTTGGCTTATCGAGTCTTTCAATTGTCACCCACGTTCACGGTTACCATCCCAGGTGGGCGAGTTTGTCCGAATGCCAAGTACGCAA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            1201                                                1250                                                1300
VBS53 EAV   TGATTTGTGACAAGCAGCACTGGCGCGTCAAACGTGCAAAGGGCGTCGGCCTGTGTCTCGATGAAAGCTGTTTCAGGGGCACCTGCAATTGCCAACGCAT
Hela-EAVP10
Hela-EAVP35 ..........................................................................C.....................
Hela-EAVP60
Hela-EAVP80 ..........................................................................T.....................

            1301                                                1350                                                1400
VBS53 EAV   GAGTGGACCACCACCTGCACCCGTGTCAGCCGCCGTGTTAGATCACATACTGGAGGCGGCGACGTTTGGCAACGTTCGCGTGGTTACACCTGAAGGGCAG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            1401                                                1450                                                1500
VBS53 EAV   CCACGCCCCGTACCAGCGCCGCGAGTTCGTCCCAGCGCCAACTCTTCTGGAGATGTCAAAGATCCGGCGCCCGTTCCGCCAGTACCAAAACCAAGGACCA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................
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            1501                                                1550                                                1600
VBS53 EAV   AGCTTGCCAAACCGAACCCAACTCAGGCGCCCATCCCAGCACCGCGCACGCGACTTCAAGGGGCCTCAACACAGGAGCCACTGGCGAGTGCAGGAGTTGC
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            1601                                                1650                                                1700
VBS53 EAV   TTCTGACTCGGCACCTAAATGGCGTGTGGCCAAAACTGTGTACAGCTCCGCGGAGCGCTTTCGGACCGAACTGGTACAACGTGCTCGGTCCGTTGGGGAC
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            1701                                                1750                                                1800
VBS53 EAV   GTTCTTGTTCAAGCGCTACCGCTCAAAACCCCAGCAGTGCAGCGGTATACCATGACTCTGAAGATGATGCGTTCACGCTTCAGTTGGCACTGCGACGTGT
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            1801                                                1850                                                1900
VBS53 EAV   GGTACCCTTTGGCTGTAATCGCTTGTTTGCTCCCTATATGGCCATCTCTTGCTTTGCTCCTTAGCTTTGCCATTGGGTTGATACCCAGTGTGGGCAATAA
Hela-EAVP10
Hela-EAVP35 ............................................................................C...........
Hela-EAVP60
Hela-EAVP80 ............................................................................C...........

            1901                                                1950                                                2000
VBS53 EAV   TGTTGTTCTGACAGCGCTTCTGGTTTCATCAGCTAATTATGTTGCGTCAATGGACCATCAATGTGAAGGTGCGGCTTGCTTAGCCTTGCTGGAAGAAGAA
Hela-EAVP10
Hela-EAVP35 ..................................................G.......................T...................
Hela-EAVP60
Hela-EAVP80 ..................................................G.......................T...................
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            2001                                                2050                                                2100
VBS53 EAV   CACTATTATAGAGCGGTCCGTTGGCGCCCGATTACAGGCGCGCTGTCGCTTGTGCTCAATTTACTGGGGCAGGTAGGCTATGTAGCTCGTTCCACCTTTG
Hela-EAVP10
Hela-EAVP35 ..................................................T.....................................................
Hela-EAVP60
Hela-EAVP80 ..................................................T.....................................................

            2101                                                2150                                                2200
VBS53 EAV   ATGCAGCTTATGTTCCTTGCACTGTGTTCGATCTTTGCAGCTTTGCTATTCTGTACCTCTGCCGCAATCGTTGCTGGAGATGCTTCGGACGCTGTGTGCG
Hela-EAVP10
Hela-EAVP35 ..................................................T.....................................................
Hela-EAVP60
Hela-EAVP80 ..................................................T.....................................................

            2201                                                2250                                                2300
VBS53 EAV   AGTTGGGCCTGCCACGCATGTTTTGGGTTCCACCGGGCAACGAGTTTCCAAACTGGCGCTCATTGATTTGTGTGACCACTTTTCAAAGCCCACCATCGAT
Hela-EAVP10
Hela-EAVP35 ..................................................T.....................................................
Hela-EAVP60
Hela-EAVP80 ..................................................T.....................................................

            2301                                                2350                                                2400
VBS53 EAV   GTTGTGGGCATGGCAACTGGTTGGAGCGGATGTTACACAGGAACCGCCGCAATGGAGCGTCAGTGTGCCTCTACGGTGGACCCTCACTCGTTCGACCAGA
Hela-EAVP10
Hela-EAVP35 ..................................................T.....................................................
Hela-EAVP60
Hela-EAVP80 ..................................................T.....................................................

            2401                                                2450                                                2500
VBS53 EAV   AGAAGGCAGGAGCGATTGTTTACCTCACCCCCCCTGTCAACAGCGGGTCAGCGCTGCAGTGCCTCAATGTCATGTGGAAGCGACCAATTGGGTCCACTGT
Hela-EAVP10
Hela-EAVP35 ..................................................A.....................................................
Hela-EAVP60
Hela-EAVP80 ..................................................T.....................................................
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            2501                                                2550                                                2600
VBS53 EAV   CCTTGGGGAACAAACAGGAGCTGTTGTGACGGCGGTCAAGAGTATCTCTTTCTCACCTCCCTGCTGCGTCTCTACCACTTTGCCCACCCGACCCGGTGTG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            2601                                                2650                                                2700
VBS53 EAV   ACCGTTGTCGACCATGCTCTTTACAACCGGTTGACTGCTTCAGGGGTCGATCCCGCTTTATTGCGTGTTGGGCAAGGTGATTTTCTAAAACTTAATCCGG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            2701                                                2750                                                2800
VBS53 EAV   GGTTCCGGCTGATAGGTGGATGGATTTATGGGATATGCTATTTTGTGTTGGTGGTTGTGTCAACTTTTACCTGCTTACCTATCAAATGTGGCATTGGCAC
Hela-EAVP10
Hela-EAVP35 ............................................................................................
Hela-EAVP60
Hela-EAVP80 ............................................................................................

            2801                                                2850                                                2900
VBS53 EAV   CCGCGACCCTTTCTGCCGCAGAGTGTTTTCTGTACCCGTCACCAAGACCCAAGAGCACTGCCATGCTGGAATGTGTGCTAGCGCTGAAGGCATCTCTCTG
Hela-EAVP10
Hela-EAVP35 ............................................................................................C..
Hela-EAVP60
Hela-EAVP80 ............................................................................................T..

            2901                                                2950                                                3000
VBS53 EAV   GACTCTCTGGGGTTAACTCAGTTACAAAGTTACTGGATCGCTGCCGTCACTAGCGGATTAGTGATCTTGTTGGTCTGCCACCGCCTGGCCATCAGCGCCT
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................
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            3001                                                 3050                                                 3100
VBS53 EAV   TGGACTTGTTGACTCTAGCTTCCCCTTTAGTGTTGCTTGTGTTCCCTTGGGCATCTGTGGGGCTTTTACTTGCTTGCAGTCTCGCTGGTGCTGCTGTGAA
Hela-EAVP10
Hela-EAVP35 ..........G.....................................................................................
Hela-EAVP60
Hela-EAVP80 ..........A.....................................................................................

            3101                                                 3150                                                 3200
VBS53 EAV   AATACAGTTGTTGGCGACGCTTTTTGTGAATCTGTTCTTTCCCCAAGCTACCCTTGTCACTATGGGATACTGGGCGTGCGTGGCGGCTTTGGCCGTTTAC
Hela-EAVP10
Hela-EAVP35 .....................................................................................
Hela-EAVP60
Hela-EAVP80 .....................................................................................

            3201                                                 3250                                                 3300
VBS53 EAV   AGTTTGATGGGCTTGCGAGTGAAAGTGAATGTGCCCATGTGTGTGACACCTGCCCATTTTCTGCTGCTGGCGAGGTCAGCTGGACAGTCAAGAGAGCAGA
Hela-EAVP10
Hela-EAVP35 .....................................................................................
Hela-EAVP60
Hela-EAVP80 .....................................................................................

            3301                                                 3350                                                 3400
VBS53 EAV   TGCTCCGGGTCAGCGCTGCTGCCCCCACCAATTCACTGCTTGGAGTGGCTCGTGATTGTTATGTCACAGGCACAACTCGGCTGTACATACCCAAGGAAGG
Hela-EAVP10
Hela-EAVP35 .....................................................................................
Hela-EAVP60
Hela-EAVP80 .....................................................................................

            3401                                                 3450                                                 3500
VBS53 EAV   CGGGATGGTGTTTGAAGGGCTATTCAGGTCACCGAAGGCGCGCGGCAACGTCGGCTTCGTGGCTGGTAGCAGCTACGGCACAGGGTCAGTGTGGACCAGG
Hela-EAVP10
Hela-EAVP35 .....................................................................................
Hela-EAVP60
Hela-EAVP80 .....................................................................................
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            3501                                                 3550                                                 3600
VBS53 EAV   AACAACGAGGTCGTCGTACTGACAGCGTCACACGTGGTTGGCCGCGCTAACATGGCCACTCTGAAGATCGGTGACGCAATGCTGACTCTGACTTTCAAAA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            3601                                                 3650                                                 3700
VBS53 EAV   AGAATGGCGACTTCGCCGAGGCAGTGACGACACAGTCCGAGCTCCCAGGCAATTGGCCACAGTTGCATTTCGCCCAACCAACAACCGGGCCCGCTTCATG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            3701                                                 3750                                                 3800
VBS53 EAV   GTGCACYGCCACAGGAGATGAAGAAGGCTTGCTCAGTGGCGAGGTTTGTCTGGCGTGGACTACTAGTGGCGACTCTGGATCAGCAGTGGTTCAGGGTGAC
Hela-EAVP10
Hela-EAVP35 ......C.....................................................................T...
Hela-EAVP60
Hela-EAVP80 ......C.....................................................................C...

            3801                                                 3850                                                 3900
VBS53 EAV   GCTGTGGTAGGGGTCCACACCGGTTCGAACACAAGTGGTGTTGCCTACGTGACCACCCCAAGCGGAAAACTCCTTGGCGCCGACACCGTGACTTTGTCAT
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            3901                                                 3950                                                 4000
VBS53 EAV   CACTGTCAAAGCATTTCACAGGCCCTTTGACATCAATCCCGAAGGACATCCCTGACAACATCATTGCCGATGTTGATGCTGTTCCTCGTTCTCTGGCCAT
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................
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            4001                                                4050                                                4100
VBS53 EAV   GCTGATTGATGGCTTATCCAATAGAGAGAGCAGCCTTTCTGGACCTCAGTTGTTGTTAATTGCTTGTTTTATGTGGTCTTATCTTAACCAACCTGCTTAC
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            4101                                                4150                                                4200
VBS53 EAV   TTGCCTTATGTGCTGGGCTTCTTTGCCGCTAACTTCTTCCTGCCAAAAAGTGTTGGCCGCCCTGTGGTCACTGGGCTTCTATGGTTGTGCTGCCTCTTCA
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            4201                                                4250                                                4300
VBS53 EAV   CACCGCTTTCCATGCGCTTGTGCTTGTTCCATCTGGTCTGTGCTACCGTCACGGGAAACGTGATATCTTTGTGGTTCTACATCACTGCCGCTGGCACGTC
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            4301                                                4350                                                4400
VBS53 EAV   TTACCTTTCTGAGATGTGGTTCGGAGGCTATCCCACCATGTTGTTTGTGCCACGGTTCCTAGTGTACCAGTTCCCCGGCTGGGCTATTGGCACAGTACTA
Hela-EAVP10
Hela-EAVP35 ..............................................A.....................................................
Hela-EAVP60
Hela-EAVP80 ..............................................A.....................................................

            4401                                                4450                                                4500
VBS53 EAV   GCGGTATGCAGCATCACCATGCTGGCTGCTGCCCTCGGTCACACCCTGTTACTGGATGTGTTCTCCGCCTCAGGTCGCTTTGACAGGACTTTCATGATGA
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................
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            4501                                                4550                                                4600
VBS53 EAV   AATACTTCCTGGAGGGAGGAGTGAAAGAGAGTGTCACCGCCTCAGTCACCCGCGCTTATGGCAAACCAATTACCCAGGAGAGTCTCACTGCAACATTGGC
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            4601                                                4650                                                4700
VBS53 EAV   TGCCCTCACTGATGATGACTTCCAATTCCTCTCTGATGTGCTTGACTGTCGGGCCGTCCGATCGGCAATGAATCTGCGTGCCGCTCTCACAAGTTTTCAA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            4701                                                4750                                                4800
VBS53 EAV   GTGGCGCAGTATCGTAACATCCTTAATGCATCCTTGCAAGTCGATCGTGACGCTGCTCGTAGTCGCAGACTAATGGCAAAACTGGCTGATTTTGCGGTTG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            4801                                                4850                                                4900
VBS53 EAV   AACAAGAAGTAACAGCTGGAGACCGTGTTGTGGTTATCGACGGTCTGGACCGCATGGCTCACTTCAAAGACGATTTGGTGCTGGTTCCTTTGACCACCAA
Hela-EAVP10
Hela-EAVP35 ................................................................................................G
Hela-EAVP60
Hela-EAVP80 ................................................................................................G

            4901                                                4950                                                5000
VBS53 EAV   AGTAGTAGGCGGTTCTAGGTGCACCATTTGTGACGTCGTTAAGGAAGAAGCCAATGACACCCCAGTTAAGCCAATGCCCAGCAGGAGACGCCGCAAGGGC
Hela-EAVP10
Hela-EAVP35 ............C.......................................................................................
Hela-EAVP60
Hela-EAVP80 ............T.......................................................................................
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            5001                                                5050                                                5100
VBS53 EAV   CTGCCTAAAGGTGCTCAGTTGGAGTGGGACCGTCACCAGGAAGAGAAGAGGAACGCCGGTGATGATGATTTTGCGGTCTCGAATGATTATGTCAAGAGAG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            5101                                                5150                                                5200
VBS53 EAV   TGCCAAAGTACTGGGATCCCAGCGACACCCGAGGCACGACAGTGAAAATCGCCGGCACTACCTATCAGAAAGTGGTTGACTATTCAGGCAATGTGCATTA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            5201                                                5250                                                5300
VBS53 EAV   CGTGGAGCATCAGGAAGATCTGCTAGACTACGTGCTGGGCAAGGGGAGCTATGAAGGCCTAGATCAGGACAAAGTGTTGGACCTCACAAACATGCTTAAA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            5301                                                5350                                                5400
VBS53 EAV   GTGGACCCCACGGAGCTCTCCTCCAAAGACAAAGCCAAGGCGCGTCAGCTTGCTCATCTGCTGTTGGATCTGGCTAACCCAGTTGAGGCAGTGAATCAGT
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            5401                                                5450                                                5500
VBS53 EAV   TAAACTGAGAGCGCCCCACATCTTTCCCGGCGATGTGGGGCGTCGGACCTTTGCTGACTCTAAAGACAAGGGTTTCGTGGCTCTACACAGTCGCACAATG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................
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            5501                                                5550                                                5600
VBS53 EAV   TTTTTAGCTGCCCGGGACTTTTTATTTAACATCAAATTTGTGTGCGACGAAGAGTTCACAAAGACCCCAAAAGACACACTGCTTGGGTACGTACGCGCCT
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            5601                                                5650                                                5700
VBS53 EAV   GCCCTGGTTACTGGTTTATTTTCCGTCGTACGCACCGGTCGCTGATTGATGCATACTGGGACAGTATGGAGTGCGTTTACGCGCTTCCCACCATATCTGA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            5701                                                5750                                                5800
VBS53 EAV   TTTTGATGTGAGCCCAGGTGACGTCGCAGTGACGGGTGAGCGATGGGATTTTGAATCTCCCGGAGGAGGCCGTGCAAAACGTCTCACAGCTGATCTGGTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            5801                                                5850                                                5900
VBS53 EAV   CACGCTTTTCAAGGGTTCCACGGAGCCTCTTATTCCTATGATGACAAGGTGGCAGCTGCTGTCAGTGGTGACCCGTATCGGTCGGACGGCGTCTTGTATA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            5901                                                5950                                                6000
VBS53 EAV   ACACCCGTTGGGGCAACATTCCATATTCTGTCCCAACCAATGCTTTGGAAGCCACAGCTTGCTACCGTGCTGGATGTGAGGCCGTTACCGACGGGACCAA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................
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            6001                                                6050                                                6100
VBS53 EAV   CGTCATCGCAACAATTGGGCCCTTCCCGGAGCAACAACCCATACCGGACATCCCAAAAAGCGTGCTTGACAACTGCGCTGACATCAGCTGTGACGCTTTC
Hela-EAVP10
Hela-EAVP35 ..................C.....................................................................................
Hela-EAVP60
Hela-EAVP80 ..................T.....................................................................................

            6101                                                6150                                                6200
VBS53 EAV   ATAGCGCCCGCTGCAGAGACAGCCCTGTGTGATGATTTAGAGAAATACAACCTATCCACGCAGGGTTTTGTGTTGCCTAGTGTTTTCTCCATGGTGCGGG
Hela-EAVP10
Hela-EAVP35 ..................C.....................................................................................
Hela-EAVP60
Hela-EAVP80 ..................T.....................................................................................

            6201                                                6250                                                6300
VBS53 EAV   CGTACTTAAAAGAGGAGATTGGAGACGCTCCACCACTCTACTTGCCATCTACTGTACCATCTAAAAATTCACAAGCCGGAATTAACGGCGCTGAGTTTCC
Hela-EAVP10
Hela-EAVP35 ..................C.....................................................................................
Hela-EAVP60
Hela-EAVP80 ..................T.....................................................................................

            6301                                                6350                                                6400
VBS53 EAV   TACAAAGTCTTTACAGAGCTACTGTTTGATTGATGACATGGTGTCACAGTCCATGAAAAGCAATCTACAAACCGCCACCATGGCGACTTGTAAACGGCAG
Hela-EAVP10
Hela-EAVP35 ..................C.....................................................................................
Hela-EAVP60
Hela-EAVP80 ..................T.....................................................................................

            6401                                                6450                                                6500
VBS53 EAV   TACTGTTCCAAATACAAGATTAGGAGCATTCTGGGCACCAACAATTACATTGGCCTAGGTTTGCGTGCCTGCCTTTCGGGGGTTACGGCCGCATTCCAAA
Hela-EAVP10
Hela-EAVP35 ..................C.....................................................................................
Hela-EAVP60
Hela-EAVP80 ..................T.....................................................................................
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            6501                                                6550                                                6600
VBS53 EAV   AAGCTGGAAAGGATGGGTCACCGATTTATTTGGGCAAGTCAAAATTCGACCCGATACCAGCTCCTGACAAGTACTGCCTTGAAACAGACCTGGAGAGTTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            6601                                                6650                                                6700
VBS53 EAV   TGATCGCTCCACCCCGGCTTTGGTGCGTTGGTTCGCTACTAATCTTATTTTTGAGCTAGCTGGCCAGCCCGAGTTGGTGCACAGCTACGTGTTGAATTGC
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            6701                                                6750                                                6800
VBS53 EAV   TGTCACGATCTAGTTGTGGCGGGTAGTGTAGCATTCACCAAACGCGGGGGTTTGTCATCTGGAGACCCTATCACTTCCATTTCCAATACCATCTATTCAT
Hela-EAVP10
Hela-EAVP35 ..................................................C...............................................
Hela-EAVP60
Hela-EAVP80 ..................................................C...............................................

            6801                                                6850                                                6900
VBS53 EAV   TGGTGCTGTACACCCAGCACATGTTGCTATGTGGACTTGAAGGCTATTTCCCAGAGATTGCAGAAAAATATCTTGATGGCAGCCTGGAGCTGCGGGACAT
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            6901                                                6950                                                7000
VBS53 EAV   GTTCAAGTACGTTCGAGTGTACATCTACTCGGACGATGTGGTTCTAACCACACCCAACCAGCATTACGCGGCCAGCTTTGACCGCTGGGTCCCCCACCTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................
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            7001                                                 7050                                                 7100
VBS53 EAV   CAGGCGCTGCTAGGTTTCAARGTTGACCCAAAGAAAACTGTGAACACCAGCTCCCCTTCCTTTTTGGGCTGCCGGTTCAAGCAAGTGGACGGCAAGTGTT
Hela-EAVP10
Hela-EAVP35 ..................A...................................................................................
Hela-EAVP60
Hela-EAVP80 ..................A...................................................................................

            7101                                                 7150                                                 7200
VBS53 EAV   ATCTAGCCAGTCTTCAGGACCGTGTTACACGCTCTCTGTTATACCACATTGGTGCAAAGAATCCCTCAGAGTACTATGAAGCTGCTGTTTCCATCTTTAA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7201                                                 7250                                                 7300
VBS53 EAV   GGACTCCATTATCTGCTGTGATGAAGACTGGTGGACGGACCTCCATCGACGTATCAGTGGCGCTGCGCGTACTGACGGAGTTGAGTTCCCCACCATTGAA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7301                                                 7350                                                 7400
VBS53 EAV   ATGTTAACATCCTTCCGCACCAAGCAGTATGAGAGTGCCGTGTGCACAGTTTGTGGGGCCGCCCCCGTGGCCAAGTCTGCTTGTGGAGGGTGGTTCTGTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7401                                                 7450                                                 7500
VBS53 EAV   GCAATTGTGTCCCGTACCACGTGGGTCATTGTCACACAACCTCGCTCTTCGCCAACTGCGGGCACGACATCATGTACCGCTCCACTTACTGCACAATGTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................
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            7501                                                 7550                                                 7600
VBS53 EAV   TGAGGGTTCCCCAAAACAGATGGTACCAAAAGTGCCTCACCCGATCCTGGATCATTTGCTGTGCCACATTGATTACGGCAGTAAAGAGGAACTAACTCTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7601                                                 7650                                                 7700
VBS53 EAV   GTAGTGGCGGATGGTCGAACAACATCACCGCCCGGGCGCTACAAAGTGGGTCACAAGGTAGTCGCCGTGGTTGCAGATGTGGGAGGCAACATTGTGTTTG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7701                                                 7750                                                 7800
VBS53 EAV   GGTGCGGTCCTGGATCACACATCGCAGTACCACTTCAGGATACGCTCAAGGGCGTGGTGGTGAATAAAGCTCTGAAGAACGCCGCCGCCTCTGAGTACGT
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7801                                                 7850                                                 7900
VBS53 EAV   GGAAGGACCCCCTGGGAGTGGGAAGACTTTTCACCTGGTCAAAGATGTGCTAGCCGTGGTCGGTAGCGCGACCTTGGTTGTGCCCACCCACGCGTCCATG
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................

            7901                                                 7950                                                 8000
VBS53 EAV   CTGGACTGCATCAACAAGCTCAAACAAGCGGGCGCCGATCCATACTTTGTGGTGCCCAAGTATACAGTTCTTGACTTTCCCCGGCCTGGCAGTGGAAACA
Hela-EAVP10
Hela-EAVP35 ..................................................................................................
Hela-EAVP60
Hela-EAVP80 ..................................................................................................
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            8001                                                8050                                                8100
VBS53 EAV   TCACAGTGCGACTGCCACAGGTCGGAACCAGTGAGGGAGAAACCTTTGTGGATGAGGTGGCCTACTTCTCACCAGTGGATCTGGCGCGCATTTTAACCCA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            8101                                                8150                                                8200
VBS53 EAV   GGGTCGAGTCAAGGGTTACGGTGATTTAAATCAGCTCGGGTGTGTCGGACCCGCGAGCGTGCCACGTAACCTTTGGCTCCGACATTTTGTCAGCCTGGAG
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            8201                                                8250                                                8300
VBS53 EAV   CCCTTGCGAGTGTGCCATCGATTCGGCGCTGCTGTGTGTGATTTGATCAAGGGCATTTAYCCTTATTATGAGCCAGCTCCACATACCACYAAAGTGGTGT
Hela-EAVP10
Hela-EAVP35 ..........................................................C.....................C.........
Hela-EAVP60
Hela-EAVP80 ..........................................................C.....................C.........

            8301                                                8350                                                8400
VBS53 EAV   TTGTGCCAAATCCAGACTTTGAGAAAGGTGTAGTCATCACCGCCTACCACAAAGATCGCGGTCTTGGTCACCGCACAATTGATTCAATTCAAGGCTGTAC
Hela-EAVP10
Hela-EAVP35 ................................................................................................T..
Hela-EAVP60
Hela-EAVP80 ................................................................................................C..

            8401                                                8450                                                8500
VBS53 EAV   ATTCCCTGTTGTGACTCTTCGACTGCCCACACCCCAATCACTGACGCGCCCGCGCGCAGTTGTGGCGGTTACTAGGGCGTCTCAGGAATTATACATCTAC
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................
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            8501                                                 8550                                                 8600
VBS53 EAV   GACCCCTTTGATCAGCTTAGCGGGTTGTTGAAGTTCACCAAGGAAGCAGAGGCGCAGGACTTGATCCATGGCCCACCTACAGCATGCCACCTGGGCCAAG
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            8601                                                 8650                                                 8700
VBS53 EAV   AAATTGACCTTTGGTCCAATGAGGGCCTCGAATATTACAAGGAAGTCAACCTGCTGTACACACACGTCCCCATCAAGGATGGTGTAATACACAGTTACCC
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            8701                                                 8750                                                 8800
VBS53 EAV   TAATTGTGGCCCTGCCTGTGGCTGGGAAAAGCAATCCAACAAAATTTCGTGCCTCCCGAGAGTGGCACAAAATTTGGGCTACCACTATTCCCCAGACTTA
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            8801                                                 8850                                                 8900
VBS53 EAV   CCAGGATTTTGCCCCATACCAAAAGAACTCGCTGAGCATTGGCCCGTAGTGTCCAATGATAGGTACCCGAATTGCTTGCAAATCACCTTACAGCAAGTAT
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................

            8901                                                 8950                                                 9000
VBS53 EAV   GTGAACTCAGTAAACCGTGCTCAGCGGGCTATATGGTTGGACAATCTGTTTTCGTGCAGACGCCTGGTGTGACATCTTACTGGCTTACTGAATGGGTCGA
Hela-EAVP10
Hela-EAVP35 ....................................................................................................
Hela-EAVP60
Hela-EAVP80 ....................................................................................................
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            9001                                                 9050                                                 9100
VBS53 EAV   CGGCAAAGCGCGTGCTCTACCAGATTCCTTATTCTCGTCCGGTAGGTTCGAGACTAACAGCCGCGCTTTCCTCGATGAAGCCGAGGAAAAGTTTGCCGCC
Hela-EAVP10
Hela-EAVP35 ................................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................................

            9101                                                 9150                                                 9200
VBS53 EAV   GCTCACCCTCATGCCTGTTTGGGAGAAATTAATAAGTCCACCGTGGGAGGATCCCACTTCATCTTTTCCCAATATTTACCACCATTGCTACCCGCAGACG
Hela-EAVP10
Hela-EAVP35 ................................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................................

            9201                                                 9250                                                 9300
VBS53 EAV   CTGTTGCCCTGGTAGGTGCTTCATTGGCTGGGAAAGCTGCTAAAGCTGCTTGCAGCGTCGTTGACGTCTATGCTCCATCATTTGAACCTTATCTGCACCC
Hela-EAVP10
Hela-EAVP35 ................................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................................

            9301                                                 9350                                                 9400
VBS53 EAV   TGAGACACTGAGTCGCGTGTACAAGATTATGATCGATTTCAAGCCGTGTAGGCTTATGGTGTGGAGAAACGCGACCTTTTATGTCCAAGAGGGTGTTGAT
Hela-EAVP10
Hela-EAVP35 ................................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................................

            9401                                                 9450                                                 9500
VBS53 EAV   GCAGTTACATCAGCACTAGCAGCTGTGTCCAAACTCATCAAAGTGCCGGCCAATGAGCCTGTTTCATTCCATGTGGCATCAGGGTACAGAACCAACGCGC
Hela-EAVP10
Hela-EAVP35 ................................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................................
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            9501                                                9550                                                9600
VBS53 EAV   TGGTAGCGCCCCAGGCTAAAATTTCGATTGGAGCCTACGCCGCCGAGTGGGCACTGTCAACTGAACCGCCACCGGCTGGTTATGCGATCGTGCGGCGATA
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            9601                                                9650                                                9700
VBS53 EAV   TATTGTAAAGAGGCTCCTCAGCTCAACAGAAGTGTTCTTGTGCCGCAGGGGTGTTGTGTCTTCCACCTCAGTGCAGACCATTTGTGCACTAGAGGGATGT
Hela-EAVP10
Hela-EAVP35 ................................................................................................
Hela-EAVP60
Hela-EAVP80 ................................................................................................

            9701                                                9750                                                9800
VBS53 EAV   AAACCTCTGTTCAACTTCTTACAAATTGGTTCAGTCATTGGGCCCGTGTGATGGGCTTAGTGTGGTCACTGATTTCAAATTCTATTCAGACTATTATTGC
Hela-EAVP10                                                 ..................................................
Hela-EAVP35 ........................C.......................................................................
Hela-EAVP60                                                 ..................................................
Hela-EAVP80 ........................T.......................................................................

            9801                                                9850                                                9900
VBS53 EAV   TGATTTTGCTATTTCTGTGATTGATGCAGCGCTTTTCTTTCTCATGCTACTTGCATTGGCTGTTGTTACTGTGTTTCTTTTCTGGCTCATTGTTGCCATC
Hela-EAVP10 ..........................................................T.....................................
Hela-EAVP35 ..........................................................C.....................................
Hela-EAVP60 ..........................................................C.....................................
Hela-EAVP80 ..........................................................C.....................................

            9901                                                9950                                               10000
VBS53 EAV   GGCCGCAGCTTGGTGGCGCGGTGTTCACGAGGTGCGCGTTACAGACCTGTTTAAGGATTTGCAGTGCGACAACCTGCGCGCGAAAGATGCCTTCCCGAGT
Hela-EAVP10 ......A......T.....................................................................................
Hela-EAVP35 ......A......C.....................................................................................
Hela-EAVP60 ......T......C.....................................................................................
Hela-EAVP80 ......T......C.....................................................................................
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            10001                                                 10050                                                 10100
VBS53 EAV   CTGGGATATGCTCTGTCGATTGGCCAGTCGAGGCTATCGTATATGCTGCAGGATTGGTTGCTTGCTGCGCACCGCAAGGAAGTTATGCCCTCCAATATCA
Hela-EAVP10 ..................................................................G..A.............
Hela-EAVP35 ..................................................................G..G.............
Hela-EAVP60 ..................................................................C..A.............
Hela-EAVP80 ..................................................................C..A.............

            10101                                                 10150                                                 10200
VBS53 EAV   TGCCTATGCCCGGTCTTACTCCTGATTGCTTTGACCATCTGGAGTCTTCTAGCTATGCTCCATTTATCAATGCCTATCGGCAGGCAATTTTGAGTCAATA
Hela-EAVP10 ..........................................................G.................................C...........
Hela-EAVP35 ..........................................................A.................................C...........
Hela-EAVP60 ..........................................................A.................................C...........
Hela-EAVP80 ..........................................................A.................................C...........

            10201                                                 10250                                                 10300
VBS53 EAV   CTCACAAGAGCTCCTGCTCGAAGCCATCAACTGTAAATTGCTTGCTGTGGTTGCACCGGCATTGTATCATAATTACCATCTAGCCAATTTGACCGGACCG
Hela-EAVP10 ..................................................................T.................................
Hela-EAVP35 ..................................................................T.................................
Hela-EAVP60 ..................................................................C.................................
Hela-EAVP80 ..................................................................T.................................

            10301                                                 10350                                                 10400
VBS53 EAV   GCCACATGGGTCGTGCCTACAGTGGGCCAGTTGCACTATTATGCTTCTTCCTCTATTTTTGCTTCATCTGTGGAAGTGTTGGCAGCAATAATACTACTAT
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            10401                                                 10450                                                 10500
VBS53 EAV   TTGCATGCATACCACTAGTGACACGAGTGTACATCTCTTTTACGCGGCTAATGTCACCTTCCCGTCGCACTTCCAGCGGCACTTTGCCGCGGCGCAAGAT
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................
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            10501                                                 10550                                                 10600
VBS53 EAV   TTTGTAGTGCACACGGGTTATGAATATGCCGGGGTCACTATGTTAGTGCACTTGTTTGCCAACTTGGTTCTGACATTTCCGAGCTTAGTTAATTGTTCCC
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            10601                                                 10650                                                 10700
VBS53 EAV   GCCCTGTGAATGTCTTTGCTAATGCTTCTTGCGTGCAAGTGGTTTGTAGTCATACCAACTCAACTACTGGCTTGGGTCAACTTTCTTTTTCCTTTGTAGA
Hela-EAVP10 ...............................................A..................................................
Hela-EAVP35 ...............................................G..................................................
Hela-EAVP60 ...............................................G..................................................
Hela-EAVP80 ...............................................G..................................................

            10701                                                 10750                                                 10800
VBS53 EAV   TGAAGATCTACGGCTGCATATYAGGCCTACTCTTATTTGTTGGTTTGCCTTGTTGTTGGTGCACTTTCTACCCATGCCACGCTGCAGAGGCTCGTAATTT
Hela-EAVP10 ........T............T..............................................................................
Hela-EAVP35 ........C............T..............................................................................
Hela-EAVP60 ........C............T..............................................................................
Hela-EAVP80 ........C............T..............................................................................

            10801                                                 10850                                                 10900
VBS53 EAV   TACTTACATTAGTCATGGATTGGGCCACGTGCACGGTCATGAGGGGTGTAGGAATTTTATTAATGTCACTCATTCTGCATTTCTTTATCTTAATCCCACC
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            10901                                                 10950                                                 11000
VBS53 EAV   ACTCTCACTGCGCCGGCTATAACTCATTGTTTACTTCTGGTTCTGGCAGCCAAAATGGAACACCCAAACGCTACTATCTGGCTGCAGCTGCAGCCGTTTG
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................
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            11001                                                 11050                                                 11100
VBS53 EAV   GGTATCATGTGGCTGGCGATGTCATTGTCAACTTGGAAGAGAATAAGAGGCATCCTTACTTTAAACTTTTGAGAGCGCCGGCTTTACCGCTTGGTTTTGT
Hela-EAVP10 ......................A.....................................................................................
Hela-EAVP35 ......................A.....................................................................................
Hela-EAVP60 ......................T.....................................................................................
Hela-EAVP80 ......................T.....................................................................................

            11101                                                 11150                                                 11200
VBS53 EAV   GGCTATAGTTTATGTTCTTTTACGACTGGTACGTTGGGCTCAACAATGTTATCTATGATTGTATTGCTATTCTTGCTTTGGGGTGCGCCATCACATGCTT
Hela-EAVP10 ..................................................................T.....................................
Hela-EAVP35 ..................................................................C.....................................
Hela-EAVP60 ..................................................................C.....................................
Hela-EAVP80 ..................................................................C.....................................

            11201                                                 11250                                                 11300
VBS53 EAV   ACTTCTCATACTACACCGCTCAGCGCTTCACAGACTTCACCTTGTGTATGCTGACGGATCGCGGCGTTATTGCCAATTTGCTGCGATATGATGAGCACAC
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................

            11301                                                 11350                                                 11400
VBS53 EAV   TGCTTTGTACAATTGTTCCGCCAGTAAAACCTGTTGGTATTGCACATTCCTGGACGAACAGATTATCACGTTTGGAACCGATTGTAATGACACCTACGCG
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................

            11401                                                 11450                                                 11500
VBS53 EAV   GTCCCAGTTGCTGAGGTCCTGGAACAGGCGCATGGACCGTACAGTGTGCTGTTTGATGACATGCCCCCTTTTATTTACTATGGCCGTGAATTCGGCATAG
Hela-EAVP10 ..................................C.....................................T.....................
Hela-EAVP35 ..................................T.....................................T.....................
Hela-EAVP60 ..................................T.....................................C.....................
Hela-EAVP80 ..................................T.....................................C.....................
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            11501                                                 11550                                                 11600
VBS53 EAV   TTGTGTTGGATGTGTTTATGTTCTATCCCGTTTTAGTTCTGTTTTTCTTATCAGTACTACCCTATGCTACGCTTATTCTTGAAATGTGTGTATCTATTCT
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            11601                                                 11650                                                 11700
VBS53 EAV   GTTTATAATCTATGGCATTTACAGCGGGGCCTACTTGGCCATGGGCATATTTGCGGCCACGCTTGCTATACATTCAATTGTGGTCCTCCGCCAATTACTG
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            11701                                                 11750                                                 11800
VBS53 EAV   TGGTTATGCCTGGCTTGGCGATACCGCTGTACGCTTCACGCGTCCTTTATATCAGCTGAGGGGAAAGTGTACCCCGTAGACCCCGGACTCCCGGTTGCCG
Hela-EAVP10 ...T..........................................A.....................................................
Hela-EAVP35 ...C..........................................A.....................................................
Hela-EAVP60 ...C..........................................G.....................................................
Hela-EAVP80 ...C..........................................A.....................................................

            11801                                                 11850                                                 11900
VBS53 EAV   CCGCGGGCAATCGGTTGTTAGTCCCAGGTAGGCCCACTATCGATTATGCAGTGGCCTACGGCAGCAAAGTCAACCTTGTGAGGTTGGGGGCAGCTGAGGT
Hela-EAVP10 ..................................................................G.................
Hela-EAVP35 ..................................................................A.................
Hela-EAVP60 ..................................................................G.................
Hela-EAVP80 ..................................................................G.................

            11901                                                 11950                                                 12000
VBS53 EAV   ATGGGAGCCATAGATTCATTTTGTGGTGACGGGATTTTAGGTGAGTATCTAGATTACTTTATTCTGTCCGTCCCACTCTTGCTGTTGCTTACTAGGTATG
Hela-EAVP10 ..................................................................................................
Hela-EAVP35 ..................................................................................................
Hela-EAVP60 ..................................................................................................
Hela-EAVP80 ..................................................................................................
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            12001                                                 12050                                                 12100
VBS53 EAV   TAGCATCTGGGTTAGTGTATGTTTTGACTGCCTTGTTCTATTCCTTTGTATTAGCAGCTTATATTTGGTTTGTTATAGTTGGAAGAGCCTTTTCTACTGC
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................

            12101                                                 12150                                                 12200
VBS53 EAV   TTATGCTTTTGTGCTTTTGGCTGCTTTTCTGTTATTAGTAATGAGGATGATTGTAGGTATGATGCCTCGTCTTCGGTCCATTTTCAACCATCGCCAACTG
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................

            12201                                                 12250                                                 12300
VBS53 EAV   GTGGTAGCTGATTTTGTGGACACACCTAGTGGACCTGTTCCCATCCCCCGCTCAACTACTCAGATAGTGGTTCGCGGCAACGGGTACACCGCAGTTGGTA
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................

            12301                                                 12350                                                 12400
VBS53 EAV   ACAAGCTTGTCGATGGCGTCAAGACGATCACGTCCGCAGGCCGCCTCTTTTCGAAACGGGCGGCGGCGACAGCCTACAAGCTACAATGACCTACTGCGTA
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................

            12401                                                 12450                                                 12500
VBS53 EAV   TGTTTGGTCAGATGCGGGTCCGCAAACCGCCCGCGCAACCCACTCAGGCTATCATTGCAGAGCCTGGAGACCTTAGGCATGATTTAAATCAACAGGAGCG
Hela-EAVP10 ......................................................................................................
Hela-EAVP35 ......................................................................................................
Hela-EAVP60 ......................................................................................................
Hela-EAVP80 ......................................................................................................
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            12501                                                  12550                                                  12600
VBS53 EAV   CGCCACCCTTTCGTCGAACGTACAACGGTTCTTCATGATTGGGCATGGTTCACTCACTGCAGATGCCGGAGGACTCACGTACACCGTCAGTTGGGTTCCT
Hela-EAVP10 ..........................................................................................................
Hela-EAVP35 ..........................................................................................................
Hela-EAVP60 ..........................................................................................................
Hela-EAVP80 ..........................................................................................................

            12601                                                  12650                                                  12700
VBS53 EAV   ACCAAACAAATCCAGCGCAAAGTTGCGCCTCCAGCAGGGCCGTAAGACGTGGATATTCTCCTGTGTGGCGTCATGTTGAAGTAGTTATTAGCCACCCAGG
Hela-EAVP10 ..........................................................................................................
Hela-EAVP35 ..........................................................................................................
Hela-EAVP60 ..........................................................................................................
Hela-EAVP80 ..........................................................................................................

            12701
VBS53 EAV   AACC
Hela-EAVP10 ....
Hela-EAVP35 ....
Hela-EAVP60 ....
Hela-EAVP80 ....
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Note:

1. The entire genome sequences (1-12704) of VBS53 EAV, Hela-EAVP35, and Hela-EAVP80 were shown. The nucleotide sequences (9751-12704, corresponding to ORFs 2-7 plus 3' non-translated region) of Hela-EAVP10 and Hela-EAVP60 were shown.
2. Y = T or C; R = A or G.

Appendix 6. Alignment of amino acid sequences of polyprotein 1ab of VBS53 EAV, Hela-EAVP35, and Hela-EAVP80

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            1                                                  50                                                 100
VBS53 EAV   MATFSATGFGGSFVRDWSLDLPDACEHGAGLCCEVDGSTLCAECFRGCEGVEQCPGLFMGLLKLASPVPVGHKFLIGWYRAAKVTGRYNFLELLQHPAFA
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            101                                                150                                                200
VBS53 EAV   QLRVVDARLAIEEASVFISTDHASAKRFPGARFALTPVYASAWVASPAANSLIVTIDQEQDGFCWLKLLPPDRREAGLRLYYNHYREQRTGWLSKTGLRL
Hela-EAVP35 ..........................................V.....................................................
Hela-EAVP80 ..........................................V.....................................................

            201                                                250                                                300
VBS53 EAV   WLGDLGLGINASSGGLKFHIMRGSPQRAWHITTRSCKLKSYYVCDISEADWSCLPAGNYGGYNPPGDGACGYRCLAFMNGATVVSAGCSSDLWCDDELAY
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            301                                                350                                                400
VBS53 EAV   RVFQLSPTFTVTIPGGRVCPNAKYAMICDKQHWRVKRAKGVGLCLDESCFRGTCNCQRMSGPPPAPVSAAVLDHILEAATFGNVRVVTPEGQPRPVPAPR
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            401                                                450                                                500
VBS53 EAV   VRPSANSSGDVKDPAPVPPVPKPRTKLAKPNPTQAPIPAPRTRLQGASTQEPLASAGVASDSAPKWRVAKTVYSSAERFRTELVQRARSVGDVLVQALPL
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            501                                                550                                                600
VBS53 EAV   KTPAVQRYTMTLKMMRSRFSWHCDVWYPLAVIACLLPIWPSLALLLSFAIGLIPSVGNNVVLTALLVSSANYVASMDHQCEGAACLALLEEEHYYRAVRW
Hela-EAVP35 ............................................................G...........................
Hela-EAVP80 ............................................................G...........................

            601                                                650                                                700
VBS53 EAV   RPITGALSLVLNLLGQVGYVARSTFDAAYVPCTVFDLCSFAILYLCRNRCWRCFGRCVRVGPATHVLGSTGQRVSKLALIDLCDHFSKPTIDVVGMATGW
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................
```

```
            701                                                 750                                                 800
VBS53 EAV   SGCYTGTAAMERQCASTVDPHSFDQKKAGAIVYLTPPVNSGSALQCLNVMWKRPIGSTVLGEQTGAVVTAVKSISFSPPCCVSTTLPTRPGVTVVDHALY
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            801                                                 850                                                 900
VBS53 EAV   NRLTASGVDPALLRVGQGDFLKLNPGFRLIGGWIYGICYFVLVVVSTFTCLPIKCGIGTRDPFCRRVFSVPVTKTQEHCHAGMCASAEGISLDSLGLTQL
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            901                                                 950                                                1000
VBS53 EAV   QSYWIAAVTSGLVILLVCHRLAISALDLLTLASPLVLLVFPWASVGLLLACSLAGAAVKIQLLATLFVNLFFPQATLVTMGYWACVAALAVYSLMGLRVK
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            1001                                               1050                                                1100
VBS53 EAV   VNVPMCVTPAHFLLLARSAGQSREQMLRVSAAAPTNSLLGVARDCYVTGTTRLYIPKEGGMVFEGLFRSPKARGNVGFVAGSSYGTGSVWTRNNEVVVLT
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            1101                                               1150                                                1200
VBS53 EAV   ASHVVGRANMATLKIGDAMLTLTFKKNGDFAEAVTTQSELPGNWPQLHFAQPTTGPASWCTATGDEEGLLSGEVCLAWTTSGDSGSAVVQGDAVVGVHTG
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            1201                                               1250                                                1300
VBS53 EAV   SNTSGVAYVTTPSGKLLGADTVTLSSLSKHFTGPLTSIPKDIPDNIIADVDAVPRSLAMLIDGLSNRESSLSGPQLLLIACFMWSYLNQPAYLPYVLGFF
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            1301                                               1350                                                1400
VBS53 EAV   AANFFLPKSVGRPVVTGLLWLCCLFTPLSMRLCLFHLVCATVTGNVISLWFYITAAGTSYLSEMWFGGYPTMLFVPRFLVYQFPGWAIGTVLAVCSITML
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            1401                                               1450                                                1500
VBS53 EAV   AAALGHTLLLDVFSASGRFDRTFMMKYFLEGGVKESVTASVTRAYGKPITQESLTATLAALTDDDFQFLSDVLDCRAVRSAMNLRAALTSFQVAQYRNIL
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................
```

```
            1501                                                1550                                                1600
VBS53 EAV   NASLQVDRDAARSRRLMAKLADFAVEQEVTAGDRVVVIDGLDRMAHFKDDLVLVPLTTKVVGGSRCTICDVVKEEANDTPVKPMPSRRRRKGLPKGAQLE
Hela-EAVP35 ..........................................................R.....................................
Hela-EAVP80 ..........................................................R.....................................

            1601                                                1650                                                1700
VBS53 EAV   WDRHQEEKRNAGDDDFAVSNDYVKRVPKYWDPSDTRGTTVKIAGTTYQKVVDYSGNVHYVEHQEDLLDYVLGKGSYEGLDQDKVLDLTNMLKVDPTELSS
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            1701                                                1750                                                1800
VBS53 EAV   KDKAKARQLAHLLLDLANPVEAVNQLNLRAPHIFPGDVGRRTFADSKDKGFVALHSRTMFLAARDFLFNIKFVCDEEFTKTPKDTLLGYVRACPGYWFIF
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            1801                                                1850                                                1900
VBS53 EAV   RRTHRSLIDAYWDSMECVYALPTISDFDVSPGDVAVTGERWDFESPGGGRAKRLTADLVHAFQGFHGASYSYDDKVAAAVSGDPYRSDGVLYNTRWGNIP
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            1901                                                1950                                                2000
VBS53 EAV   YSVPTNALEATACYRAGCEAVTDGTNVIATIGPFPEQQPIPDIPKSVLDNCADISCDAFIAPAAETALCDDLEKYNLSTQGFVLPSVFSMVRAYLKEEIG
Hela-EAVP35 ..............................P.....................................................................
Hela-EAVP80 ..............................S.....................................................................

            2001                                                2050                                                2100
VBS53 EAV   DAPPLYLPSTVPSKNSQAGINGAEFPTKSLQSYCLIDDMVSQSMKSNLQTATMATCKRQYCSKYKIRSILGTNNYIGLGLRACLSGVTAAFQKAGKDGSP
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            2101                                                2150                                                2200
VBS53 EAV   IYLGKSKFDPIPAPDKYCLETDLESCDRSTPALVRWFATNLIFELAGQPELVHSYVLNCCHDLVVAGSVAFTKRGGLSSGDPITSISNTIYSLVLYTQHM
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................

            2201                                                2250                                                2300
VBS53 EAV   LLCGLEGYFPEIAEKYLDGSLELRDMFKYVRVYIYSDDVVLTTPNQHYAASFDRWVPHLQALLGFKVDPKKTVNTSSPSFLGCRFKQVDGKCYLASLQDR
Hela-EAVP35 ....................................................................................................
Hela-EAVP80 ....................................................................................................
```

```
            2301                                                2350                                                2400
VBS53 EAV   VTRSLLYHIGAKNPSEYYEAAVSIFKDSIICCDEDWWTDLHRRISGAARTDGVEFPTIEMLTSFRTKQYESAVCTVCGAAPVAKSACGGWFCGNCVPYHV
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            2401                                                2450                                                2500
VBS53 EAV   GHCHTTSLFANCGHDIMYRSTYCTMCEGSPKQMVPKVPHPILDHLLCHIDYGSKEELTLVVADGRTTSPPGRYKVGHKVVAVVADVGGNIVFGCGPGSHI
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            2501                                                2550                                                2600
VBS53 EAV   AVPLQDTLKGVVVNKALKNAAASEYVEGPPGSGKTFHLVKDVLAVVGSATLVVPTHASMLDCINKLKQAGADPYFVVPKYTVLDFPRPGSGNITVRLPQV
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            2601                                                2650                                                2700
VBS53 EAV   GTSEGETFVDEVAYFSPVDLARILTQGRVKGYGDLNQLGCVGPASVPRNLWLRHFVSLEPLRVCHRFGAAVCDLIKGIYPYYEPAPHTTKVVFVPNPDFE
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            2701                                                2750                                                2800
VBS53 EAV   KGVVITAYHKDRGLGHRTIDSIQGCTFPVVTLRLPTPQSLTRPRAVVAVTRASQELYIYDPFDQLSGLLKFTKEAEAQDLIHGPPTACHLGQEIDLWSNE
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            2801                                                2850                                                2900
VBS53 EAV   GLEYYKEVNLLYTHVPIKDGVIHSYPNCGPACGWEKQSNKISCLPRVAQNLGYHYSPDLPGFCPIPKELAEHWPVVSNDRYPNCLQITLQQVCELSKPCS
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            2901                                                2950                                                3000
VBS53 EAV   AGYMVGQSVFVQTPGVTSYWLTEWVDGKARALPDSLFSSGRFETNSRAFLDEAEEKFAAAHPHACLGEINKSTVGGSHFIFSQYLPPLLPADAVALVGAS
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................

            3001                                                3050                                                3100
VBS53 EAV   LAGKAAKAACSVVDVYAPSFEPYLHPETLSRVYKIMIDFKPCRLMVWRNATFYVQEGVDAVTSALAAVSKLIKVPANEPVSFHVASGYRTNALVAPQAKI
Hela-EAVP35 ..................................................................................................
Hela-EAVP80 ..................................................................................................
```

```
            3101                                                3150                    3176
VBS53 EAV   SIGAYAAEWALSTEPPPAGYAIVRRYIVKRLLSSTEVFLCRRGVVSSTSVQTICALEGCKPLFNFLQIGSVIGPV-
Hela-EAVP35 ..............................................................................-
Hela-EAVP80 ..............................................................................-
```

Appendix 7. Alignment of amino acid sequences of E protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                    1                                                  50
E of VBS53 EAV      MGLVWSLISNSIQTIIADFAISVIDAALFFLMLLALAVVTVFLFWLIVAI
E of Hela-EAVP10    ..................................................
E of Hela-EAVP35    ..................................................
E of Hela-EAVP60    ..................................................
E of Hela-EAVP80    ..................................................

                    51              68
E of VBS53 EAV      GRSLVARCSRGARYRPV-
E of Hela-EAVP10    ..S.V............-
E of Hela-EAVP35    ..S.A............-
E of Hela-EAVP60    ..C.A............-
E of Hela-EAVP80    ..C.A............-
```

Appendix 8. Alignment of amino acid sequences of GP2b protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                    1                                                  50
GP2b of VBS53 EAV   MQRFSFSCYLHWLLLLCFFSGSLLPSAAAWWRGVHEVRVTDLFKDLQCDN
GP2b of Hela-EAVP10 ..............L.............W...................
GP2b of Hela-EAVP35 ..............S.............R...................
GP2b of Hela-EAVP60 ..............S.............R...................
GP2b of Hela-EAVP80 ..............S.............R...................

                    51                                                 100
GP2b of VBS53 EAV   LRAKDAFPSLGYALSIGQSRLSYMLQDWLLAAHRKEVMPSNIMPMPGLTP
GP2b of Hela-EAVP10 ..................................VM............
GP2b of Hela-EAVP35 ..................................VV............
GP2b of Hela-EAVP60 ..................................LM............
GP2b of Hela-EAVP80 ..................................LM............

                    101                                                150
GP2b of VBS53 EAV   DCFDHLESSSYAPFINAYRQAILSQYSQELLLEAINCKLLAVVAPALYHN
GP2b of Hela-EAVP10 ...........A......................................
GP2b of Hela-EAVP35 ...........T......................................
GP2b of Hela-EAVP60 ...........T......................................
GP2b of Hela-EAVP80 ...........T......................................

                    151                                                200
GP2b of VBS53 EAV   YHLANLTGPATWVVPTVGQLHYYASSSIFASSVEVLAAIILLFACIPLVT
GP2b of Hela-EAVP10 ..................................................
GP2b of Hela-EAVP35 ..................................................
GP2b of Hela-EAVP60 ..................................................
GP2b of Hela-EAVP80 ..................................................

                    201                       228
GP2b of VBS53 EAV   RVYISFTRLMSPSRRTSSGTLPRRKIL-
GP2b of Hela-EAVP10 ........................-
GP2b of Hela-EAVP35 ........................-
GP2b of Hela-EAVP60 ........................-
GP2b of Hela-EAVP80 ........................-
```

Appendix 9. Alignment of amino acid sequences of GP3 protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                    1                                                  50
GP3 of VBS53 EAV    MGRAYSGPVALLCFFLYFCFICGSVGSNNTTICMHTTSDTSVHLFYAANV
GP3 of Hela-EAVP10  ..................................................
GP3 of Hela-EAVP35  ..................................................
GP3 of Hela-EAVP60  ..................................................
GP3 of Hela-EAVP80  ..................................................

                    51                                                 100
GP3 of VBS53 EAV    TFPSHFQRHFAAAQDFVVHTGYEYAGVTMLVHLFANLVLTFPSLVNCSRP
GP3 of Hela-EAVP10  ..................................................
GP3 of Hela-EAVP35  ..................................................
GP3 of Hela-EAVP60  ..................................................
GP3 of Hela-EAVP80  ..................................................

                    101                                                150
GP3 of VBS53 EAV    VNVFANASCVQVVCSHTNSTTGLGQLSFSFVDEDLRLHIRPTLICWFALL
GP3 of Hela-EAVP10  ..............S...................L...............
GP3 of Hela-EAVP35  ..............G...................P...............
GP3 of Hela-EAVP60  ..............G...................P...............
GP3 of Hela-EAVP80  ..............G...................P...............

                    151       164
GP3 of VBS53 EAV    LVHFLPMPRCRGS-
GP3 of Hela-EAVP10  ..............-
GP3 of Hela-EAVP35  ..............-
GP3 of Hela-EAVP60  ..............-
GP3 of Hela-EAVP80  ..............-
```

Appendix 10. Alignment of amino acid sequences of GP4 protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                    1                                                  50
GP4 of VBS53 EAV    MKIYGCIXGLLLFVGLPCCWCTFYPCHAAEARNFTYISHGLGHVHGHEGC
GP4 of Hela-EAVP10  ...Y...L..........................................
GP4 of Hela-EAVP35  ...H...L..........................................
GP4 of Hela-EAVP60  ...H...L..........................................
GP4 of Hela-EAVP80  ...H...L..........................................

                    51                                                 100
GP4 of VBS53 EAV    RNFINVTHSAFLYLNPTTLTAPAITHCLLLVLAAKMEHPNATIWLQLQPF
GP4 of Hela-EAVP10  ..................................................
GP4 of Hela-EAVP35  ..................................................
GP4 of Hela-EAVP60  ..................................................
GP4 of Hela-EAVP80  ..................................................

                    101                                                150
GP4 of VBS53 EAV    GYHVAGDVIVNLEENKRHPYFKLLRAPALPLGFVAIVYVLLRLVRWAQQC
GP4 of Hela-EAVP10  ........I.........................................
GP4 of Hela-EAVP35  ........I.........................................
GP4 of Hela-EAVP60  ........F.........................................
GP4 of Hela-EAVP80  ........F.........................................

                    151
GP4 of VBS53 EAV    YL-
GP4 of Hela-EAVP10  ..-
GP4 of Hela-EAVP35  ..-
GP4 of Hela-EAVP60  ..-
GP4 of Hela-EAVP80  ..-
```

Note: At position 8, X represents amino acid L (Leucine) or S (Serine).

Appendix 11. Alignment of amino acid sequences of GP5 protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                    1                                                  50
GP5 of VBS53 EAV    MLSMIVLLFLLWGAPSHAYFSYYTAQRFTDFTLCMLTDRGVIANLLRYDE
GP5 of Hela-EAVP10  ........F.....................................
GP5 of Hela-EAVP35  ........S.....................................
GP5 of Hela-EAVP60  ........S.....................................
GP5 of Hela-EAVP80  ........S.....................................

                    51                                                 100
GP5 of VBS53 EAV    HTALYNCSASKTCWYCTFLDEQIITFGTDCNDTYAVPVAEVLEQAHGPYS
GP5 of Hela-EAVP10  ..............................................P..
GP5 of Hela-EAVP35  ..............................................L..
GP5 of Hela-EAVP60  ..............................................L..
GP5 of Hela-EAVP80  ..............................................L..

                    101                                                150
GP5 of VBS53 EAV    VLFDDMPPFIYYGREFGIVVLDVFMFYPVLVLFFLSVLPYATLILEMCVS
GP5 of Hela-EAVP10  ................................................
GP5 of Hela-EAVP35  ................................................
GP5 of Hela-EAVP60  ................................................
GP5 of Hela-EAVP80  ................................................

                    151                                                200
GP5 of VBS53 EAV    ILFIIYGIYSGAYLAMGIFAATLAIHSIVVLRQLLWLCLAWRYRCTLHAS
GP5 of Hela-EAVP10  ................................................
GP5 of Hela-EAVP35  ................................................
GP5 of Hela-EAVP60  ................................................
GP5 of Hela-EAVP80  ................................................

                    201                                                250
GP5 of VBS53 EAV    FISAEGKVYPVDPGLPVAAAGNRLLVPGRPTIDYAVAYGSKVNLVRLGAA
GP5 of Hela-EAVP10  .I.....................................R....
GP5 of Hela-EAVP35  .I.....................................K....
GP5 of Hela-EAVP60  .V.....................................R....
GP5 of Hela-EAVP80  .I.....................................R....

                    251
GP5 of VBS53 EAV    EVWEP-
GP5 of Hela-EAVP10  .....-
GP5 of Hela-EAVP35  .....-
GP5 of Hela-EAVP60  .....-
GP5 of Hela-EAVP80  .....-
```

Appendix 12. Alignment of amino acid sequences of M protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                  1                                                  50
M of VBS53 EAV    MGAIDSFCGDGILGEYLDYFILSVPLLLLLTRYVASGLVYVLTALFYSFV
M of Hela-EAVP10  ..................................................
M of Hela-EAVP35  ..................................................
M of Hela-EAVP60  ..................................................
M of Hela-EAVP80  ..................................................

                  51                                                 100
M of VBS53 EAV    LAAYIWFVIVGRAFSTAYAFVLLAAFLLLVMRMIVGMMPRLRSIFNHRQL
M of Hela-EAVP10  ..................................................
M of Hela-EAVP35  ..................................................
M of Hela-EAVP60  ..................................................
M of Hela-EAVP80  ..................................................

                  101                                                150
M of VBS53 EAV    VVADFVDTPSGPVPIPRSTTQIVVRGNGYTAVGNKLVDGVKTITSAGRLF
M of Hela-EAVP10  ..................................................
M of Hela-EAVP35  ..................................................
M of Hela-EAVP60  ..................................................
M of Hela-EAVP80  ..................................................

                  151       163
M of VBS53 EAV    SKRAAATAYKLQ-
M of Hela-EAVP10  ............-
M of Hela-EAVP35  ............-
M of Hela-EAVP60  ............-
M of Hela-EAVP80  ............-
```

Appendix 13. Alignment of amino acid sequences of N protein of VBS53 EAV, Hela-EAVP10, Hela-EAVP35, Hela-EAVP60, and Hela-EAVP80

```
                  1                                                  50
N of VBS53 EAV    MASRRSRPQAASFRNGRRRQPTSYNDLLRMFGQMRVRKPPAQPTQAIIAE
N of Hela-EAVP10  ..................................................
N of Hela-EAVP35  ..................................................
N of Hela-EAVP60  ..................................................
N of Hela-EAVP80  ..................................................

                  51                                                 100
N of VBS53 EAV    PGDLRHDLNQQERATLSSNVQRFFMIGHGSLTADAGGLTYTVSWVPTKQI
N of Hela-EAVP10  ..................................................
N of Hela-EAVP35  ..................................................
N of Hela-EAVP60  ..................................................
N of Hela-EAVP80  ..................................................

                  101    111
N of VBS53 EAV    QRKVAPPAGP-
N of Hela-EAVP10  ..........-
N of Hela-EAVP35  ..........-
N of Hela-EAVP60  ..........-
N of Hela-EAVP80  ..........-
```

Appendix 14. Thermacycling conditions for site-directed mutagenesis PCR reactions

1st mutagenesis PCR reaction

Component	Amount per reaction
Nuclease free water	40.6 µl
10 × cloned Pfu reaction buffer	5.0 µl
dNTPs (25 mM each dNTP)	0.4 µl
pS45VBS53 DNA (1:20 dilution)	1.0 µl
Positive primer C211156P (20 µM)	1.0 µl
Negative mutagenic primer 11443N (20 µM)	1.0 µl
Pfu Turbo DNA polymerase (2.5 U/µl)	1.0 µl
Total reaction volume	50 µl

2nd mutagenesis PCR reaction

Component	Amount per reaction
Nuclease free water	36.6 µl
10 × cloned Pfu reaction buffer	5.0 µl
dNTPs (25 mM each dNTP)	0.4 µl
pS45VBS53 DNA (1:20 dilution)	1.0 µl
1st mutagenesis PCR mixture	5.0 µl
Reverse primer 11515N (20 µM)	1.0 µl
Pfu Turbo DNA polymerase (2.5 U/µl)	1.0 µl
Total reaction volume	50 µl

Thermacycling conditions for both 1st and 2nd mutagenesis PCR

Seg 1	95ºC 40ºC 72ºC	2 min 1.5 min 6 min	1 cycle
Seg 2	95ºC 40ºC 72ºC	45 sec 1.5 min 6 min	5 cycles
Seg 3	95ºC 50ºC 72ºC	45 sec 45 sec 6 min	34 cycles
Seg 4	72ºC	10 min	
Seg 5	4ºC	8	

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Abstracts and Presentations

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