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Infectious cDNA clones of porcine reproductive and respiratory syndrome virus and their potential as vaccine vectors

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Abstract

Full-length infectious cDNA clones have recently become available for both European and North American genotypes of porcine reproductive and respiratory syndrome virus (PRRSV), and it is now possible to alter the PRRSV genome and create genetically defined mutant viruses. Among many possible applications of the PRRSV infectious cDNA clones, development of genetically modified vaccines is of particular interest. Using infectious clones, the PRRSV genome has been manipulated by changing individual amino acids, deleting coding regions, inserting foreign sequences, and generating arterivirus chimeras. The limited available data suggest that all structural proteins of PRRSV are essential for replication of the virus, and that PRRSV infectivity is relatively intolerant of subtle changes within the structural proteins. The major tasks in PRRSV research are to identify virulence factors and pathogenic mechanisms, and to understand the structure–function relationships of individual viral proteins. Utilizing these infectious clones as tools, a new generation of safe and efficacious PRRS vaccines may be constructed.

Keywords: PRRSV; Infectious clone; Arterivirus; Reverse genetics; Vaccine vector

1. Introduction

The study of viral genetics largely relies on viral phenotypes and the isolation of mutant viruses. Stocks of RNA viruses usually contain a large proportion of mutants from which desired phenotypes are selected for genetic analysis by appropriate selection methods. Traditional phenotypes represent temperature-sensitive, drug-resistant, neutralization-resistant, or plaque-size variants. Host-range mutants or attenuated viruses may be selected via pathogenic studies in animal models. Mutation frequencies of RNA viruses are considered to be 1 mutation in 10^3 to 10^5 nucleotides per replication, while DNA viruses have rates of less than 1 in 10^8 (Leider et al., 1988; Steinhauer et al., 1992). This difference is attributed to a lack of proofreading ability in RNA replication enzymes. The low mutation rates of DNA viruses require the use of mutagens to induce random mutations in the genome, and the high mutation rates of RNA viruses make it difficult to dissect precise genetic determinants because of multiple mutations.

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Advances in recombinant DNA technology have made it possible to manipulate viral genomes and to introduce specific modifications to target sequences in order to create genetically modified mutant viruses. Homologous DNA recombination using a helper virus is a common approach to genetic manipulation of large DNA viruses and has been used for herpes viruses, adenoviruses, and poxviruses (for reviews, see Lai et al. (2002), Goins et al. (2004), Moss (1996) and Paoletti (1996)). For viruses with a smaller genome, a full-length double-stranded DNA copy of the viral genome is constructed in a bacterial plasmid and used for generation of virus. This technology, referred to as reverse genetics, is the most powerful genetic tool in modern virology and has the potential to be applied to a variety of uses, from the basic understanding of virus biology to the development of genetically engineered viral vaccines and specific gene delivery to cultured cells and animals.

2. Principles of viral reverse genetics

DNA viruses, with the exception of poxviruses, replicate in the nucleus of virus-infected cells and have access to cellular machinery for their genome replication and mRNA transcription. DNA polymerase, which can be of either viral or cellular origin, replicates the DNA genomes. For these viruses, assembly of progeny virus also occurs in the nucleus. The genetic methods to study DNA viruses are based on the infectivity of viral DNA. When viral DNA is deproteinized and introduced into permissive cells, an infection cycle begins and progeny viruses are produced. The complete genomes of adenoviruses, polyomaviruses, and papilloma viruses have been cloned into bacterial plasmids, and the cloned DNA has been shown to be infectious to cells.

For RNA viruses, with the exception of retroviruses, the genome does not enter a DNA phase during replication, and direct manipulation of RNA molecules is difficult. Therefore, genetic manipulation of RNA virus genomes relies on utilizing their cDNA intermediates. Single-stranded RNA viruses are grouped into two major classes: positive-strand and negative-strand RNA viruses. These two classes of RNA viruses are distinguished by the infectivity of RNA genomes. While positive-strand RNA is fully infectious in cells and can initiate a full range of infection cycles, the infectious unit associated with negative-strand RNA viruses is not a naked RNA genome but rather a ribonucleoprotein (RNP) complex. Reconstitution of infectious RNPs is technically difficult. Although the process of reconstitution is a major hurdle for reverse genetics in the area of negative-strand RNA viruses, it has been successfully overcome for some viruses. In the retroviruses, genomes are converted to double-stranded cDNA by reverse transcriptase and such proviral forms of DNA are infectious when introduced into cells. Therefore, retrovirus infectious clones resemble either DNA virus or positive-strand RNA virus.

Among the RNA viruses, poliovirus was the first to have its genome converted to an infectious cDNA clone. It had been known for decades that the naked RNA genome of poliovirus is infectious (Alexander, 1958), but it was not until 1981, when a full-length genomic cDNA was constructed, that the infectivity of a cDNA cloned into a bacterial plasmid was demonstrated (Ricanello and Baltimore, 1981). The molecular mechanism by which cloned polivirus cDNA initiates infection is unknown but it has been suggested that the DNA enters the nucleus, where its transcription begins by DNA-dependent RNA polymerase using a cryptic promoter-like sequence present in the plasmid. The resulting full-length genome size RNA transcripts of positive polarity initiate an infection cycle and non-viral sequences at both termini are removed, thereby producing progeny virus. Analogous to poliovirus, infectious clones have been constructed for many positive-strand RNA viruses and their specific infectivity has been demonstrated for picornaviruses, caliciviruses, flaviviruses, togaviruses, as well as coronaviruses and arteriviruses (Sosnovtsev and Green, 1995; Boyer and Haenni, 1994; Yount et al., 2003; Almazan et al., 2000; van Dinten et al., 1997). With the advent of RT-PCR technology, an infectious cDNA clone may be generated faster than ever.

In contrast, the negative-strand RNA virus genome is not infectious per se. Negative-strand RNA cannot be translated into protein nor copied into positive-strand RNA in cells because host cells do not carry RNA-dependent RNA polymerases but only carry DNA-dependent RNA polymerases (polymerases I–III). Different approaches have been developed for

reverse genetics of negative-strand RNA viruses: orthomyxoviruses as segmented RNA virus (Hoffman et al., 2000) and rhabdoviruses and paramyxoviruses as non-segmented RNA viruses (Whelan et al., 1995; Schnell et al., 1994; Hoffman and Banerjee, 1997; for review, Conzelmann and Meyers, 1996). It is now possible to engineer their genomes.

3. Genome structure of arteriviruses and subgenomic mRNA production

Full-length genomic sequences have been determined for several arteriviruses. They include an equine arteritis virus (EAV, den Boon et al., 1991), more than 20 strains of porcine reproductive respiratory syndrome virus (PRRSV, Meulenberg et al., 1993b; Nelsen et al., 1999; Allende et al., 1999; Wootton et al., 2000; Ropp et al., 2004; Truong et al., 2004; and others in GenBank), two strains of lactate dehydrogenase elevating virus (LDV, Godeny et al., 1993; Palmer et al., 1995), and a simian hemorrhagic fever virus (SHFV) (Smith et al., 1997, GenBank accession AF180391). The arterivirus genome is a single-stranded, positive-sense RNA ranging in size from 12.7 kb for EAV to 15.7 kb for SHFV. The 5′ end of the SHFV genome contains a single type I cap in the form of m7G(5′)ppp(5′) (Sagripanti et al., 1986); from which it is anticipated that other arterivirus genomes are also methyl-capped. The 3′ end of the genome is polyadenylated. The arterivirus genome is polycistronic, containing two large open reading frames (ORF1a and b) and a set of six to nine ORFs downstream of the 1b gene. A small internal ORF is found within ORF2 of all arteriviruses (Snijder et al., 1999).

Both ORF1a and b are expressed from genomic RNA. While ORF1a alone is able to produce the 1a protein, ORF1b is always expressed in the form of 1a/1b fusion protein since 1b does not contain a translation initiation codon. Instead, the ORF1b expression is mediated by a −1 nucleotide ribosomal frame shift. Both 1a and 1a/1b fusion proteins are post-translationally cleaved into 12–13 cleavage products (van Dinten et al., 1999). These cleavage products are found in virus-infected cells but not virions, and therefore are considered to be non-structural components.

The downstream six to nine ORFs of the arterivirus genome, encoding the structural proteins, are translated from a set of 3′-coterminal subgenomic RNAs (sgRNAs) that are produced from the replicative intermediate complementary to the genomic RNA (Meulenberg et al., 1993a). Subgenomic RNAs share their 3′ end, but only the 5′ most gene is translated. Subgenomic RNAs of arteriviruses consist of a leader and a body, which are non-contiguous in the genome. The connection between the leader and body is formed by a consensus ‘‘transcriptional regulatory sequence’’ or TRS. The consensus sequence is unique for each arterivirus. For example, the consensus sequence for EAV is UCAACU (den Boon et al., 1996), whereas it is UUAACC for PRRSV (Meulenberg et al., 1993a). A sequence resembling UUAACC is found upstream of each structural gene in PRRSV, and all sgRNAs of PRRSV contain this sequence in the 5′ untranslated region preceding the ORF. Therefore, the TRS in arteriviruses functions analogously to a promoter in a conventional gene, and is the key control element for structural gene expression.

4. Construction of arterivirus infectious clones

As with other positive-strand RNA viruses, arterivirus genomes are infectious in cells (van der Zeijst and Horzinek, 1975; Meulenberg et al., 1998), and based on these findings, full-length infectious cDNA clones have been constructed for EAV and PRRSV (Fig. 1). The first infectious clone for PRRSV was developed for the European type PRRS Lelystad virus (Meulenberg et al., 1998). In this construction, a genome length cDNA copy was assembled and placed under the bacteriophage T7 RNA polymerase promoter in the low copy number plasmid pOK12 (Vierra and Messing, 1991). The plasmid was linearized by digestion immediately downstream of the polyA tail, and RNA transcripts were synthesized in vitro using T7 RNA polymerase in the presence of a cap analog. The synthetic transcripts would contain one non-viral ‘G’ at the 5′ terminus, a full-length genomic sequence, 109 polyA residues, plus 2 non-viral nucleotides derived from the vector plasmid. The in vitro synthesized RNA transcripts were transfected into baby hamster kidney (BHK-21) cells. BHK-21 cells are not permissive for PRRSV infection but provided

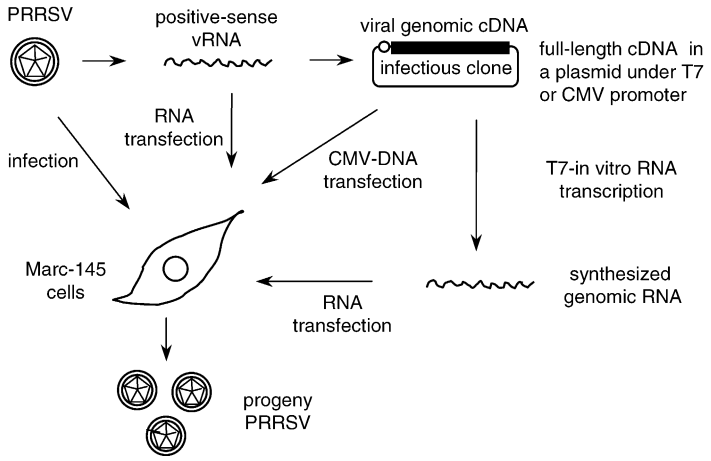

Fig. 1. Infectivity of PRRSV genomic RNA and generation of PRRSV from full-length infectious cDNA clones. The full-length genomic cDNA is typically placed under the bacteriophage T7 promoter or the eukaryotic cytomegalovirus (CMV) promoter. The full-length cDNA clone placed under the CMV promoter can directly be transfected into cells, where PRRSV genomic RNA is synthesized in vivo and initiates an infection cycle. If the full-length clone is placed under the T7 promoter, RNA needs to be synthesized in vitro which is then transfected into cells. Using the infectious cDNA clone, the PRRSV genome has been manipulated in its DNA form, and the respective mutant PRRSVs have been generated.

high transfection rates. When culture supernatant from transfected BHK-21 cells was transferred to PRRSV-permissive cells, cytopathic effects were observed, showing the infectivity of RNA transcripts derived from cDNA clones. A genetic marker was introduced into the cDNA clone to differentiate the reconstituted virus from parental virus. A new restriction enzyme site was introduced immediately downstream of the ORF7 gene, and the reconstituted virus contained this genetic marker. Coupling of a T7 promoter to a full-length cDNA clone originates from poliovirus reverse genetics (van der Werf et al., 1986). Specific infectivity of the poliovirus original promoterless cDNA clone was low, and only 10 plaque-forming units were produced from 1 μg of DNA. Cloning of the poliovirus infectious cDNA downstream of the bacteriophage T7 promoter facilitated in vitro RNA transcription with T7 RNA polymerase, and transfection of RNA instead of DNA into cells was more efficient at producing infectious progeny virus.

An infectious clone of VR-2332, the prototype North American PRRSV, has recently been developed (Nielsen et al., 2003). VR-2332 shares only 63% sequence identity with Lelystad virus. Approaches in constructing the VR-2332 clone were virtually identical to those for the Lelystad clone. The full-length genomic cDNA clone of VR-2332 was assembled and placed under the T7 promoter in pOK12. A new restriction site was created at nucleotide position 259 of the genome to serve as a genetic marker. The plasmid was linearized and viral RNA was synthesized in vitro by run-off transcription. The synthesized RNA would contain one methyl-capped non-viral 'G' at the 5′ end, a full-length genomic sequence, and 38 adenosines at the 3′ end, plus one non-viral 'A' nucleotide. The synthetic RNA was transfected into BHK-21 cells, and the culture supernatant recovered from BHK cells showed viral infectivity in Marc-145 cells. Infectivity of the transcripts was relatively low, and in order to observe CPE, the culture supernatant from BHK cells needed to be serially passaged four times in Marc-145 cells to amplify the infectivity. The reconstituted virus retained the genetic marker and was able to produce clinical symptoms in pigs when infected (Nielsen et al., 2003).

A different isolate of North American PRRSV was used to construct an infectious cDNA clone in our laboratory (Calvert et al., 2002, 2003; Lee et al., 2004). P129 is a North American type PRRSV isolated in 1995 from an outbreak of highly virulent PRRS in the midwestern USA. The P129 virus produces severe and consistent respiratory disease in pigs and is considered more virulent than other field isolates of that time. P129 may be associated with what was to be termed the 'atypical' PRRS outbreak the following

year. The P129 genomic sequence differs from VR-2332 by 11%. The infectious clone of P129 was constructed by sequencing all regions of the genome with at least three-fold redundancy using cloned products from independent RT-PCR reactions, and using plaque-purified virus as template to reduce quasispecies-related variation. This allowed assembly of a consensus sequence for the virus. Nearly all of the individual PCR products appeared to deviate from the consensus by at least one base substitution, which may have resulted from viral mutation during plaque expansion and/or from PCR-induced mutations. To minimize possible introduction of lethal mutations into the full-length clone, individual cDNA fragments were repaired using subfragments from other reactions. Two specific mutations were retained as genetic markers for the infectious cDNA clone: one in nsp2 as a silent mutation and another in ORF2a as an amino acid substitution. The fully assembled cDNA clone in the high copy number plasmid pCR2.1 (Invitrogen), was placed under the T7 promoter for run-off in vitro transcription. The in vitro transcripts were predicted to consist of a capped single non-viral 'G', the full genomic sequence, a relatively short polyadenosine tail of 21-residues, plus 9 non-viral nucleotides derived from the plasmid. The RNA transcripts were transfected into Marc-145 cells and CPE was evident in these cells 3 days post-transfection, indicating the infectivity of the RNA transcripts. The cDNA-derived virus was virulent in pigs and produced clinical disease typical for PRRS with evidence of seroconversion (Calvert et al., 2002).

The P129 infectious cDNA clone was improved by replacing the bacteriophage T7 promoter with a eukaryotic promoter. Coupling of an infectious clone with a eukaryotic promoter was first described for poliovirus (Selmer et al., 1984). Coupling of the poliovirus clone with the simian virus 40 transcription and replication signals increased the original infectivity of the poliovirus clone by 100-fold. The P129 full-length clone was placed under control of the human cytomegalovirus (hCMV) immediate early promoter in the plasmid pCMVmc1, which was derived from pCMV-β (Clonetech). The circular plasmid DNA was directly transfected into Marc-145 cells. CPE appeared at 3 days post-transfection and became profound by 4 days post-transfection. The reconstituted virus retained the two genetic markers, indicating that the direct transfection of DNA plasmid was able to initiate a complete cycle of virus infection and produced progeny virus. The eukaryotic promoter system is advantageous since the in vitro transcription step is omitted. Furthermore, because plasmid DNA is directly introduced into cells, transfection efficiencies can be precisely controlled and optimized to maximize infectivity.

Another infectious North American PRRSV cDNA clone was recently described that produces progeny virus with the same virulence and transmission characteristics as the highly pathogenic parental virus (Truong et al., 2004).

For EAV, two infectious cDNA clones were constructed independently by two groups (van Dinten et al., 1997; de Vries et al., 2000). The construction approaches were almost identical to the Lelystad clone with only minor modifications. A high copy number plasmid pUC18 was used to carry the full-length cDNA copy of the EAV genome placed under the T7 promoter. The RNA transcripts synthesized off the initial construct appeared to be non-infectious in BHK-21 cells even though BHK cells are permissive for EAV. Further studies revealed that the genomic mRNA produced from this clone was replicative but defective in subgenomic RNA production. The entire EAV sequence was re-determined from two independent cDNA clones to identify any mutations that may have been introduced during cloning procedures, and mismatches were resolved by sequencing a third clone. Subsequently, a serine to proline mutation was identified in the nsp10 protein. When this mutation was reverted, the RNA transcripts became fully infectious. This finding was intriguing since a single mutation in nsp10 can determine switching between genome replication and mRNA synthesis. Such studies would not be possible without the use of an infectious clone, proving the usefulness of reverse genetics in understanding fundamental arterivirus biology.

5. Engineering of arterivirus infectious clones

Among many possible applications of infectious clones, the development of vaccine vectors is of particular interest. An ideal vaccine should be avirulent and safe to use without causing any clinical disease. The vaccine virus should be genetically stable

(no reversion to virulence) and broadly cross-protective upon virulent challenge. A genetic marker is a desirable additional feature, allowing the vaccine to be distinguished serologically from natural infection. For these purposes, various attempts were made using arterivirus infectious clones to substitute particular amino acids, to delete regions of the genome, to insert foreign sequences, or to substitute parts of viral genes. One of the challenges for arterivirus reverse genetics is to engineer the complex and extremely compact genome. Most arterivirus structural genes overlap one another at the ends, encoding proteins in two reading frames. The overlapping feature of arterivirus genes makes it difficult to introduce modifications to the terminus of one gene without affecting the overlapping gene. The arterivirus genomes appear to be intolerant to subtle changes, with minor alterations often leading to a failure to produce viable virus. Despite these difficulties, some progress has been made towards improving genetic manipulations of the EAV and PRRSV genomes (Fig. 2A).

5.1. Point mutations

The full-length infectious cDNA clones have enabled molecular dissection of the determinants required for viral infections and attenuation. The GP5 and M proteins are two major envelope proteins in arteriviruses, which exist as a disulfide-linked heterodimer in the virion. The requirement for GP5-M heterodimerization was studied using an EAV infectious clone (Snijder et al., 2003). Individual cysteine residues in GP5 or M were precisely mutated and infectivity of the mutated cDNA clones was examined (Fig. 2A, a). The cysteine at position 8 in the M protein ectodomain was found to be responsible for heterodimerization and crucial for virus infectivity. For GP5, all five cysteines appeared to be essential for virus infectivity, and the cysteine at position 34 was found to be associated with the M protein. The GP5–M heterodimerization was shown to be essential for virus infectivity and a key step in the assembly of EAV envelope.

The importance of N protein for PRRSV infectivity was also studied using an infectious cDNA clone. The PRRSV N protein is present as a homodimer, and the North American type PRRSV N protein contains three cysteines with the cysteine at position 23 responsible for homodimerization (Wootton and Yoo, 2003). Cysteines in N proteins are not well conserved in arteriviruses. While LDV and the European type PRRSV contain two cysteines, the North American type PRRSV N protein contains three cysteines. Furthermore, EAV and SHFV are completely devoid of cysteines in their N proteins. Therefore, the significance of cysteine-linked homodimerization of N was of interest. This was studied in our laboratory using the P129 North American type infectious cDNA clone. Mutation of cysteine 23 in the N protein completely abolished virus infectivity, demonstrating that N protein dimerization is essential for viral infectivity in PRRSV (Lee et al., 2004).

The E protein is a newly identified unglycosylated small hydrophobic membrane protein believed to be present in all arteriviruses (Snijder et al., 1999). Using EAV reverse genetics, the E protein translation initiation codon was mutated to inactivate the expression of E. The resulting cDNA clone was defective in its infectivity, indicating the requirement of E protein for EAV infectivity. Similarly, the EAV GP2 protein was also shown to be essential for virus infectivity.

In EAV, the GP2–GP4 proteins are present as a heterotrimeric complex (Wieringa et al., 2003). The function of this complex was investigated for the role of four cysteine residues in the GP2 protein. Each cysteine residue of the GP2 protein was mutated in a full-length EAV cDNA clone. The results showed that the cysteine at position 102 forms an intermolecular cysteine bridge with one of the cysteines of GP4, while the cysteines at positions 48 and 137 of GP2 are linked by an intra-chain disulfide bond. Another cysteine residue in the GP4 protein is responsible for the covalent association of GP3 with the disulfide-linked GP2–GP4 heterodimer. The data demonstrate the importance for the correct association of the minor proteins for efficient incorporation into virions and virus infectivity.

5.2. Deletions

Attempts were made to identify a non-essential region in the viral genome so that it could be utilized as a potential site for foreign gene insertion. Initial attempts targeted ORF7 and the 3′ untranslated region (UTR) for deletion. ORF7 is situated at the 3′ end of

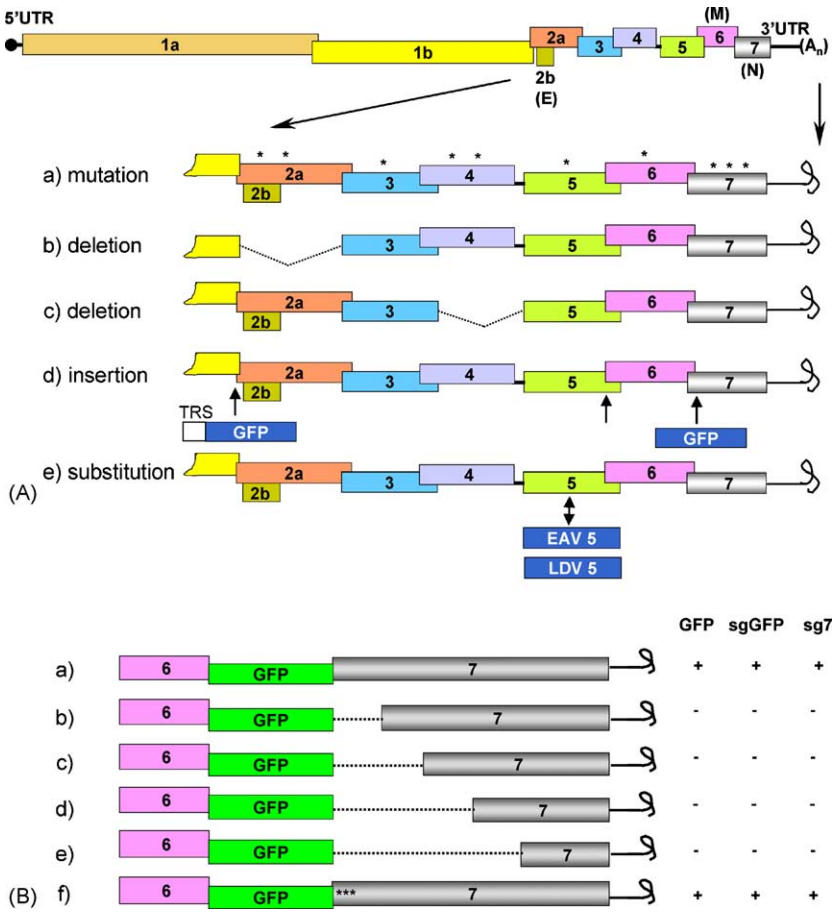

Fig. 2. Genetic engineering of PRRSV infectious cDNA clones. (A) PRRSV infectious cDNA clones have been engineered to mutate amino acids, delete open reading frames, insert foreign sequences, or substitute a gene with the corresponding sequence from other arteriviruses; GFP: green fluorescence protein gene; EAV: equine arteritis virus; LDV: lactate dehydrogenase elevating virus of mouse; TRS: transcription regulatory sequence. (B) The progressive deletions of ORF7 indicate that secondary RNA structures are important for subgenomic (sg) RNA synthesis; GFP: green fluorescent protein expression; sgGFP: subgenomic mRNA for green fluorescent protein gene; sg7: subgenomic mRNA for ORF7; ***: substitution of the translation initiation codon for ORF7 to a stop codon to suppress ORF7 expression.

the genome and codes for a nucleocapsid (N) protein. The N protein is the most immunogenic and abundant component of the virion. It is 123 and 128 amino acids in size for the North American and European genotypes, respectively (Meulenberg et al., 1993b; Nelsen et al., 1999). The 5′ end of ORF7 overlaps with the 3′ end of ORF6 by 11 nucleotides in both genotypes, and the downstream 3′ UTR is 159 and 122 nucleotides in size for VR2332 and Lelystad virus, respectively. When the N protein was truncated progressively from its carboxyl terminus, up to six amino acids could be deleted without abolishing the production of infectious virus (Verheije et al., 2001). Further deletion, for a total of seven amino acids, completely eliminated production of infectious virus. Small deletions in the middle portion of N were found to be intolerable as well. For the 3′ UTR, a deletion of seven nucleotides immediately downstream of ORF7 was possible, but additional deletion became lethal. It was suggested that the 3′ UTR was essential for viral RNA replication and the N protein truncation caused inhibition of virion assembly. The N protein is the major component of the viral capsid and exists as a dimer (Wootton and Yoo, 2003). The C-terminus of the N protein forms a strong beta-sheet and has been shown to be essential for the overall conformation of

the protein (Wootton et al., 2001; Doan and Dokland, 2003). The N protein has also been found to bind the viral genomic RNA (Yoo et al., 2003). This multifunctional nature of N may explain its intolerance to subtle genetic modifications.

In our laboratory, studies with the P129 infectious clone were conducted to determine the amount of coding region that could be removed from the 5′ end of ORF7 without eliminating the synthesis of subgenomic (sg) RNAs for the other structural protein genes. In order to facilitate these studies, ORF6 and ORF7 were functionally decoupled by duplicating the overlap shared by these genes, and unique restriction sites were inserted. Green fluorescent protein (GFP) was inserted between ORF6 and ORF7 under the control of the ORF7 TRS in order to visualize ''ORF7'' sgRNA activity in real time (Fig. 2A, d). This starting construct expressed GFP upon transfection of cells, and expected sgRNAs were detected by RT-PCR. A series of deletions were made in this background, all originating at the ATG start codon of ORF7 and extending for 21, 86, 145, 204, or 245 nucleotides downstream into the ORF7 coding region (Fig. 2B). All of these deletions completely abrogated synthesis of sgRNAs and GFP fluorescence. It appears that this region of the genome, including the extreme 5′ end of the ORF7 coding region, is involved in secondary RNA structures that are essential for sgRNA synthesis.

Central portions of other structural genes (ORFs 2a, 3–6) were individually deleted from the P129 infectious clone in such a way that the downstream TRS and coding sequences were not affected. All five genes were shown to be essential for replication of the virus in cell culture. Some of these deletion mutants may prove to be useful single-cycle vaccine candidates when propagated on complementary cell lines constitutively expressing the corresponding deleted genes (unpublished data).

5.3. Expression of foreign sequences

The potential of infectious cDNA clones as foreign gene expression vectors was explored for both PRRSV and EAV. A short sequence coding for the nine amino acid epitope of influenza virus hemagglutinin (HA) protein was inserted either at the 5′ or 3′ end of ORF7 of PRRSV, and a fusion protein of the HA tag and N protein was successfully expressed by the recombinant virus (Groot Bramel-Verheije et al., 2000). The growth of the virus was hindered however, and the HA tag began to be lost from the second passage, and was deleted completely by the fourth passage. Consistent with the Lelystad deletion data, modest changes to the N protein are not well tolerated. The genetic stability and viability of virus carrying the HA tag was improved with the insertion of a self-cleaving enzyme. A sequence derived from the foot-and-mouth disease virus (FMDV) autoprotease 2A was inserted in-frame between the HA tag and the N protein sequence (Groot Bramel-Verheije et al., 2000). The full-length PRRSV clone containing a foreign sequence coding for a total of 31 amino acids (9 aa for HA, 7 aa from FMDV 1D, and 17 aa from FMDV 2A) produced infectious progeny virus, and the HA–N fusion protein was proteolytically cleaved to release the HA epitope from the N protein. This virus retained its infectivity and genetic stability during four passages of examination. Small epitopes have also been expressed from EAV infectious clones. A nine amino acid peptide from the M protein of mouse hepatitis coronavirus was fused and expressed as an extension to the EAV M protein (de Vries et al., 2000).

An EAV clone has also been used as an expression vector for foreign genes. In one study, the chloramphenicol acetyltransferase (CAT) gene was inserted to replace part of ORF2 to achieve its expression from a modified sgRNA2 (van Dinten et al., 1997). This modification disrupted the ORF2 and E genes concomitantly and as a result, virus was not produced despite CAT gene expression in transfected cells. The CAT gene was then inserted immediately upstream of the ORF7 gene. This modification however, disrupted the ORF6 gene and resulted in abolished infectivity (van Dinten et al., 1997). de Vries et al. (2001) took an alternative approach where a foreign gene was expressed from an additional sgRNA without interruption of any EAV genes. In this approach, an expression cassette consisting of the GFP gene placed downstream of an EAV-specific TRS was inserted between the ORF5 and ORF6 genes of the EAV infectious cDNA clone. When RNA transcripts of the EAV clone were transfected into BHK-21 cells, the GFP gene was expressed and infectivity spread to adjacent cells, indicating the production of replication-competent virus. The virus-infected cells pro-

duced one additional sgRNA species and this additional RNA was GFP-specific. Serial passage of the virus, however, led to a gradual appearance of mutants with deletions in the GFP gene. These studies indicate that a genetic instability is a common feature of recombinant arterivirus vectors.

In our laboratory, the GFP gene was inserted into the P129 infectious clone at several locations. One of the most interesting of these is the insertion of GFP and an extra TRS element between ORF1b and ORF2a (Fig. 2A, d). This ''green'' virus is fully viable, replicates to titers approaching parental virus, and is phenotypically stable for at least 37 passages (Calvert et al., 2002; unpublished data). Thus, PRRSV and EAV can be used effectively as vectors for the expression of foreign genes, and have significant potential as vaccine vectors in swine and horses.

5.4. Viral chimeras

The development of infectious cDNA clones has made it possible to construct chimeric arteriviruses. Chimeric viruses were constructed to investigate the functional complementation of a protein between two different viruses and to examine their cell tropisms. An EAV infectious cDNA clone was engineered to substitute the ectodomains of two membrane proteins, GP5 and M proteins, with the corresponding sequences from PRRSV or LDV. GP5 and M are the two major membrane proteins in arteriviruses. The M protein is the most conserved structural protein of arteriviruses and its N-terminal half is believed to span the membrane three times, leaving only a short stretch of 10–18 amino acids exposed at the virion surface. The GP5 protein is predicted to be cleaved at the putative signal sequence at its N-terminus, leaving a 30 residue ectodomain for PRRSV, but about 95 residues in length for EAV. Similar to the M protein, the centrally located hydrophobic region of GP5 also spans the membrane three times followed by a cytoplasmic domain of 50–72 residues. The M and GP5 proteins exist as disulfide-linked heterodimers in arteriviruses, and the hetero-dimerization is essential in the assembly of the EAV envelope and subsequently for virus infectivity (Snijder et al., 2003). By analogy with other viruses and recognition by neutralizing antibodies, it was postulated that the ectodomain of GP5 might be involved in receptor binding. Using the EAV infectious clone, the EAV GP5 ectodomain was substituted with the ectodomain of three other arteriviruses (Dobbe et al., 2001); mouse arterivirus LDV, porcine arterivirus PRRSV, and monkey arterivirus SHFV. The EAV chimeric viruses containing the LDV and PRRSV GP5 ectodomain were viable but retained their infectivity for BHK-21 cells and rabbit kidney cells (RK-13). These cells can naturally be infected by EAV but not by either PRRSV or LDV.

Verheije et al. (2002) revisited the above finding using a PRRSV infectious cDNA clone as the parental virus to generate chimeric viruses. The ectodomain of the PRRSV M protein was substituted with the corresponding sequence of EAV, LDV, and the North American PRRSV VR2332 (Fig. 2A, e). Viable viruses with a chimeric M protein were obtained in all three cases, but the latter two only became viable after the removal of the genomic overlap between the GP5 and M genes. The chimeric viruses were immunologically distinguishable from the wild-type PRRSV and genetically stable in vitro. Growth was impaired however in these chimeras compared to the parental virus. As with the EAV chimeric viruses, the PRRSV chimeric viruses also retained their infectivity in porcine cells and were not able to infect BHK-21 cells. These two studies clearly show that the surface structures composed of the arterivirus M and GP5 ectodomains do not fully account for viral tropism. Hence, the viral receptor binding proteins are presently unknown for arteriviruses and remain to be determined.

5.5. Animal studies

Little has been done in the way of animal studies using recombinant arteriviruses. The cDNA-derived EAV was tested in horses for pathogenicity and genetic stability. The reconstituted virus induced neutralizing antibodies without developing clinical signs in stallions, and the period for shedding in the horses was significantly reduced (Balasuriya et al., 1999). The parental virus used for construction of the EAV infectious clone was a cell-culture adapted laboratory variant of the Bucyrus strain, and the animal study indicates that the cell culture adpated EAV was attenuated.

Three PRRSV recombinants were recently tested in pigs for virulence and protective efficacies (Verheije et al., 2003). A recombinant PRRSV, in which the

ectodomain of M was replaced by that of LDV, was used to infect 8-week-old pigs. The infected pigs transmitted the virus to sentinel pigs although viremia was reduced. The pigs were protected against challenge with homologous (European) PRRSV but not protected from heterologous (North American) challenge. Another recombinant virus contained a deletion mutant that lacked the six C-terminal amino acids of the N protein (Verheije et al., 2001). This deletion mutant behaved similarly to the LDV-M substituted PRRSV mutant and was transmissible to sentinel pigs with a reduced viremia. The pigs were protected from homologous challenge but not from heterologous challenge. The third recombinant had two amino acid mutations in the GP2 protein, and grew to higher titers in cells. No significant phenotypic difference in the virus was observed in pigs. In this series of experiments however, only two pigs were used per group which places the significance of the findings in question. All three recombinant PRRSV mutants were genetically stable and their mutations were maintained throughout the viremic period.

Gene-deleted mutants of PRRSV also were engineered and tested in pigs as potential vaccine candidates (Calvert et al., 2003; Welch et al., 2004). The ORF2 or ORF4 gene of P129 was deleted individually from a full-length cDNA clone (Fig. 2A, b and c), and deletion mutants ΔORF2 or ΔORF4 were rescued from cells constitutively expressing GP2 or GP4 protein, respectively. Both gene-deleted mutants retained the same growth characteristics as the parent virus in the functionally complementing cells and were genetically stable over 40–50 passages in these cells. When these deletion mutants were tested in pigs in a vaccine/challenge study, the group given ΔORF2 intramuscularly with adjuvant induced high serum antibody titers prior to challenge. The same treatment led to a statistically significant reduction of virus load in serum and lung following challenge. However, lung lesions and rectal temperature spikes were more severe (Calvert et al., 2003). This paradoxical enhancement of pathology, coincident with reduced viral load, requires further investigation. Although somewhat discouraging as a vaccine candidate, we demonstrated the principle that essential PRRSV genes can be removed from the genome and complemented in trans to allow viral propagation. These gene-deleted mutants have potential to be used for studying the pathogenesis of PRRSV in host animals or to be used as replication-incompetent foreign gene delivery vectors.

6. Future prospects

Despite the tremendous efforts invested in controlling PRRSV infections, the virus continues to plague the swine industry and damage pig production worldwide. PRRSV infects immune cells and modifies the normal host immune function. The host response to infection is poor and the virus persists in infected pigs for prolonged periods of time. Genetic and antigenic variants rapidly arise, and an effective and safe vaccine is still unavailable. The recent development of reverse genetics systems for PRRSV allows us to engineer the viral genome and to create genetically defined mutant viruses. The next major task is to precisely identify virulence factors of the virus and to construct genetically attenuated viruses. The limited available information obtained from studies using infectious clones indicate that the arterivirus genome is organized in a complex manner, and that arteriviruses are relatively intolerant of subtle changes in protein conformation and RNA secondary structure. Future advances in our understanding of the biology of these viruses will provide insight into the structure–function relationships of individual PRRSV proteins. This information will allow us to specifically modify viral protein functions and generate useful genetically attenuated viruses using the available infectious cDNA clones. These new genetic tools will undoubtedly enable a second generation of safe and effective PRRS vaccines.

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