

ABSTRACT

Title: REVERSE GENETICS OF AVIAN
METAPNEUMOVIRUS

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Avian metapneumovirus (AMPV) causes an acute respiratory disease in turkeys and is associated with “swollen head syndrome” in chickens, contributing to significant economic losses to the US turkey industry. With a long-term goal of developing a better vaccine for controlling AMPV in the US, a reverse genetics system to produce infectious AMPV entirely from cloned cDNA was established. To achieve this, the unpublished sequences of the G gene, the L gene, the leader and trailer region were first determined to complete the entire genome sequence of AMPV subgroup C strain Colorado (AMPV/CO). Our results showed that the full-length AMPV/CO genome was 14,150 nucleotides (nt) in length, denoting that AMPV/CO possessed the longest genome among known metapneumoviruses. Subsequently, a cDNA clone encoding the entire 14,150 nt genome was generated by assembling 5 cDNA fragments, representing the entire genome, between the T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme of a low-copy number transcription plasmid pBR 322. Transfection of this plasmid, along with the expression plasmids

encoding the N, P, M2-1 and L proteins of AMPV/CO, into cells stably expressing T7 RNA polymerase resulted in the recovery of infectious AMPV/CO. The recovered virus was observed to contain the genetic markers that were artificially introduced during cloning. Characterization of the recombinant AMPV/CO showed that its growth characteristics in tissue culture were similar to those of the parental virus. These results demonstrate that infectious AMPV can be generated entirely from cloned DNA using reverse genetics techniques. The potential of AMPV/CO to serve as a viral-vector was examined using green fluorescent protein (GFP) as a reporter. The recovered rAMPV/CO-GFP virus stably expressed GFP for at least five serial passages and showed characteristics similar to that of the parental virus, except that there was a one-log reduction in the virus titer. These results demonstrated that the established reverse genetics system can be utilized effectively for various studies involving AMPV molecular biology, pathogenesis and vaccine development.

REVERSE GENETICS OF AVIAN METAPNEUMOVIRUS

By

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Dedication

I wholeheartedly dedicate this work to my father, Mr. V. Dhanasekaran, and my mother, Mrs. D. Alamelu, who, through their unconditional love and many sacrifices have made me the person I am today.

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List of Abbreviations

aa	amino acid
AMPV	avian metapneumovirus
bp	base pair
BRSV	bovine respiratory syncytial virus
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CPE	cytopathic effect
dCTP	deoxycytidine triphosphate
Da	daltons
GE	gene-end
GFP	green fluorescent protein
GS	gene-start
HMPV	human metapneumovirus
HPIV-2	human parainfluenza type 2
HPIV-3	human parainfluenza type 3
HRSV	human respiratory syncytial virus
IG	intergenic
IRES	internal ribosomal entry site
kDa	kilodaltons
MEM	minimum essential medium
MOI	multiplicity of infection
mRNA	messenger RNA

MV	measles virus
nt	nucleotide
NV	Nipah virus
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RSV	respiratory syncytial virus
RT	reverse transcription
SV	Sendai virus

Chapter 1: General Introduction

1.1 Introduction

Avian metapneumovirus (AMPV) causes an acute respiratory disease in turkeys and is also associated with 'swollen head syndrome' in chickens (McDougall and Cook, 1986 ; Wilding et al., 1986; Wyeth et al., 1987; Cook et al., 1988 and Buys et al., 1989a and b). The virus was first isolated in South Africa in 1978 (Buys and Du Preez, 1980) and subsequently was isolated in Europe, Israel and Asia (Alexander, 1997; Cavanagh and Barret, 1988; Jones et al., 1986; McDougall and Cook, 1986 and Wilding et al., 1986). AMPV was first isolated in the US in 1996 from commercial turkeys in Colorado (Kleven, 1997 and Cook et al., 1999). Subsequently, AMPV outbreaks were reported in Minnesota, where the disease has emerged as a major economic problem for turkey farmers. The mortality due to AMPV infection and concomitant secondary bacterial infections ranges 0–30% in the US (Alvarez et al., 2003).

Based on nucleotide (nt) sequence divergence in the attachment glycoprotein (G) genes and their antigenic differences, AMPVs have been classified into four subgroups – AMPV-A, AMPV-B, AMPV-C and AMPV-D (Juhász and, 1994; Etteradossi et al., 1995 and Bâyon-Auboyer et al., 2000). The US isolates belong to subgroup C, and exhibit genetic and antigenic differences from isolates of other subgroups (Seal, 1998; Cook et al., 1999; Seal, 2000 and Toquin et al., 2000). The mammalian counterpart of AMPV, known as human metapneumovirus (HMPV), was recently isolated from children suffering from acute respiratory infections (van den Hoogen et al., 2001). Interestingly,

several reports have shown that AMPV-C has a closer resemblance to HMPV than to other AMPV subgroups (van den Hoogen et al., 2002; Yunus et al., 2003; Toquin et al., 2003; Govindarajan and Samal, 2004; Govindarajan et al., 2004 and Govindarajan and Samal, 2005).

AMPV belongs to the genus *Metapneumovirus* within the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (Pringle, 1998). The viral genome is a non-segmented, negative sense RNA which is 14,150 nucleotides long (Govindarajan and Samal, 2005). The genomic RNA is organized into eight genes, with a gene order of 3'-leader-N-P-M-F-M2-SH-G-L-trailer-5'. This differs from the gene order of the 10 genes of pneumoviruses such as respiratory syncytial virus (RSV), which is 3'-leader-NS1-NS2-N-P-M-SH-G-F-M2-L-trailer-5' (Collins et al., 1996; Ling et al., 1992 and Randhawa et al., 1997). The N gene encodes the nucleocapsid protein (N), which binds to the genomic RNA forming the nucleocapsid core. The P gene encodes the phosphoprotein (P) while the L gene encodes the large polymerase protein (L). Both P and L proteins associate with the nucleocapsid core and form the functional nucleocapsid within which reside the viral transcriptive and replicative activities. The M gene encodes the matrix (M) protein, which forms the inner layer of the envelope and is probably a driving force in viral assembly. The fusion protein (F) is a surface glycoprotein encoded by the F gene, and is involved in the fusion of the viral envelope to the cell membrane, thus enabling viral entry into the host cell. The M2 gene encodes two novel proteins (M2-1 and M2-2), which are made from two open reading frames on the M2 gene. M2-1 and M2-2 are believed to be involved in the regulation of transcription and replication of the

virus (Collins et al., 1996). Small hydrophobic (SH) gene encodes the SH protein, which is another surface glycoprotein whose function is not completely understood. The surface attachment glycoprotein (G) is encoded by the G gene and is believed to be involved in the attachment of the virus to the cellular receptors. In AMPV/CO, G protein is considered to be the major determinant of host immune response towards the virus (Seal et al., 2000).

Each gene on the AMPV genome is flanked by consensus gene-start (GS) and gene-end (GE) sequences and in between genes are short sequences known as the intergenic (IG) regions. The GS and GE regions play an important role in the process of transcription. Flanking the genes are the 3' extragenic region, known as the leader region (40 nt) and the 5' extragenic region known as the trailer region (39 nt). The leader and the trailer regions are believed to be the cis-acting regulatory elements involved in transcription, replication and packaging of the genomic as well as the antigenomic RNAs (Lamb and Kolakofsky, 1996).

AMPV follows the same general scheme of transcription and replication as that of other non-segmented negative-strand RNA viruses. The N protein is always associated with the nucleocapsid core during the processes of both transcription and replication. The polymerase complex, composed of the P and L proteins, enters the genome at the promoter in the 3' leader region and proceeds along the length of the genome, guided throughout by the short regulatory GS and GE signals. A free leader RNA and eight non-overlapping sub-genomic mRNAs are produced during this process. The IG regions are

not copied into the mRNAs. RNA replication occurs when the polymerase switches to a read-through mode, ignoring the transcription signals. This results in the production of a complete encapsidated positive-sense replicative intermediate, which in turn, serves as the template for production of progeny viral genomes.

Prior to this study, nucleotide sequences of six of the eight genes of AMPV strain Colorado (AMPV/CO) were determined (N and P genes – Dar et al., 2001; M gene – Seal, 1998; F gene – Seal et al., 2000; M2 gene – Dar et al., 2003; SH gene – Toquin et al., 2003; Yunus et al., 2003 and Lwamba et al., 2004). The sequences of G and L genes, and the complete genome sequence of AMPV/CO were not established. Knowledge of the entire genome sequence is a prerequisite for the genetic manipulation of the virus. The ability to introduce genetic changes directly into negative-stranded RNA viral genomes has proved to have important applications in the field of molecular biology of viruses and also in the development of attenuated vaccine strains.

Genetic manipulation of negative-sense RNA viruses has been hindered for a long time due to the lack of recombination in these viruses and the naked viral RNA not being infectious. Therefore, approaches different from those used for the positive-sense RNA viruses had to be developed to study genetically altered negative-sense RNA viruses. The most successful approach is the plasmid-complemented virus rescue system or the reverse genetics system. This approach is based on the cotransfection of different plasmids – one encoding the viral antigenome and others encoding the viral polymerase complex (N, P, M2 and L proteins), all under the control of T7 promoter. These

transfections are done in permissive cells that express the T7 RNA polymerase or cells infected with a recombinant vaccinia virus, which expresses the T7 RNA polymerase. Numerous negative-sense RNA viruses have been recovered through this plasmid-complemented rescue system (rabies virus, Schnell et al., 1994; vesicular stomatitis virus, Lawson et al., 1995; human respiratory syncytial virus, Collins et al., 1995; measles virus, Radecke et al., 1995; Sendai virus, Garcin et al., 1995; SV5, He et al., 1997; rinderpest virus, Baron and Barrett, 1997; parainfluenza virus, Hoffman and Banerjee, 1997; bovine respiratory syncytial virus, Buchholz et al., 1999 and Yunus et al., 2001; Newcastle disease virus, Peeters et al., 1999 and Krishnamurthy et al., 2000; human metapneumovirus, Biacchesi et al., 2004a and Herfst et al., 2004; and AMPV-A, Naylor et al., 2004).

Development of a reverse genetic system for AMPV/CO would lead to the possibility of not only studying the functions of each AMPV gene in an authentic virus system, but also other aspects of basic knowledge in metapneumovirus molecular biology. More importantly, establishment of a rescue system will create the ability to directly create mutations into the cDNA and hence help in engineering a recombinant live-attenuated vaccine candidate. This would be particularly important for AMPV infections in the US, since there are no effective vaccines currently available to control this emerging poultry pathogen.

This study is, thus, proposed to establish a rescue system for full-length AMPV strain Colorado, keeping in perspective the long term implications and advantages of

using such a system in studying the molecular biology of AMPV and generating a better vaccine for control of AMPV infections in the US.

1.2 Research objectives

The specific objectives in the present study on AMPV strain Colorado are:

1. To determine the entire genome sequence of AMPV subgroup C strain Colorado (AMPV/CO)
2. To construct AMPV/CO N, P, M2 and L gene expression plasmids and a full-length cDNA clone of the entire AMPV/CO genome
3. To rescue infectious AMPV/CO from full-length cDNA clone using reverse genetic system
4. To engineer AMPV/CO for the expression of a foreign gene, namely, enhanced green fluorescent protein

Chapter 2: Review of literature

2.1 Classification

AMPV is a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Pneumovirinae* and genus *Metapneumovirus* (Pringle, 1998). The genome of metapneumovirus is a non-segmented, single-stranded, negative-sense RNA. AMPV was assigned to a new genus because its genome contains eight genes arranged in a different order from the 10 genes of members of genus *Pneumovirus*, such as respiratory syncytial virus (RSV) (Collins et al., 1996; Ling et al., 1992 and Randhawa et al., 1997). The newly discovered human metapneumovirus (HMPV) is the only mammalian virus that has been included tentatively in the genus *Metapneumovirus* (van den Hoogen et al., 2001; van den Hoogen et al., 2002 and Njenga et al., 2003).

AMPV subgroups A and B (AMPV-A and -B, respectively) were originally defined based on nucleotide sequence divergence in the attachment glycoprotein (G) gene (Juhász and Easton, 1994) and antigenic differences (Toquin et al., 1992; Etteradossi et al., 1995 and Bâyon-Auboyer et al., 1999). In the US, AMPV was first isolated in 1996 from commercial turkeys in Colorado (Kleven, 1997 and Cook et al., 1999). The US isolates of AMPV were found to be genetically and antigenically different from AMPV-A and -B (Seal, 1998; Cook et al., 1999 and Toquin et al., 2000) and hence were designated AMPV-C. Subsequently, Bâyon-Auboyer et al. (2000) isolated a new subgroup of AMPV that was different from all three known subgroups and was tentatively designated AMPV-D.

2.2 Virion

AMPV are large pleomorphic enveloped virus particles ranging in size from 50 nm to more than 200nm in diameter (Cook and Cavanagh, 2002). Internal to the viral envelope is the long helical nucleocapsid structure 14 nm in diameter with a pitch of 7 nm and, externally the envelope is covered with spike glycoproteins about 15 nm long (Cook and Cavanagh, 2002). The nucleocapsid contains a single-stranded negative-sense genomic RNA of 14,150 nucleotides in the case of AMPV/CO (Govindarajan and Samal, 2005). As encountered in all paramyxoviruses, the genomic RNA is tightly encapsidated by nucleocapsid protein (N), forming the nucleocapsid core. This core is associated with phosphoprotein (P) and large polymerase (L) protein forming the transcriptive-replicative complex. The envelope of AMPV contains 3 surface glycoproteins: the attachment glycoprotein (G) responsible for attachment of the virus to the host cell, the fusion (F) protein required for the fusion of the viral envelope to the host cell membrane, and the small hydrophobic (SH) protein, the function of which is not completely understood. In AMPV/CO, the G protein is considered to be the major immunogenic protein and hence the main target of the host-cell immune response (Seal et al., 2000). The matrix (M) protein lies internal to the viral envelope and is believed to be important as an organizer during the assembly of the virus particle (Teng and Collins, 1998).

2.3 Genome organization

AMPV genome is organized into eight genes (3'-leader-N-P-M-F-M2-SH-G-L-trailer-5'), which are named according to the encoded protein (Fig. 1). Flanking the genes at the 3' end is the leader region, which is 40 nt long and at the 5' end is the trailer region

which is 39 nt long. Both leader and trailer regions are known to contain promoters and other regulatory sequence elements that control transcription, replication and packaging of the genome and antigenome (Lamb and Kolakofsky, 1996). Each gene is flanked by a short consensus gene-start and gene-end sequences which play a major role in transcription. In between two genes are intergenic regions, which range in length from 1 to 91 nucleotides for AMPV/CO.

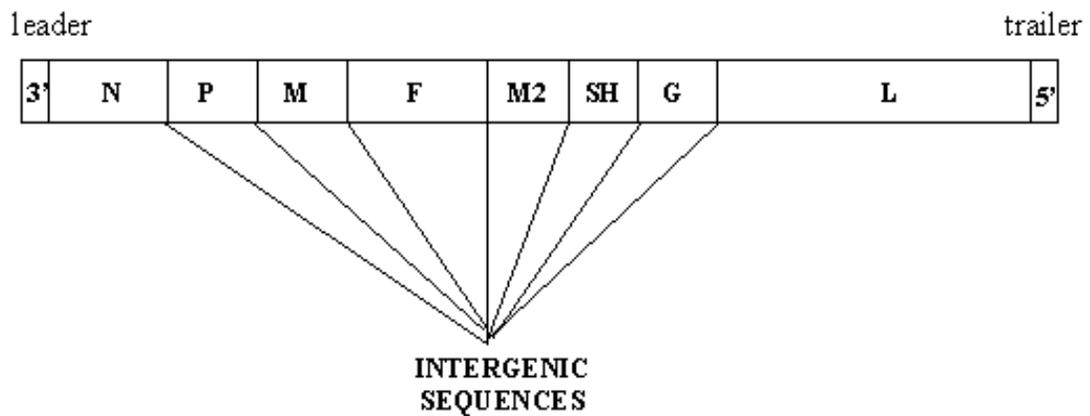


Figure 1. Genome organization of AMPV. N - nucleocapsid protein gene, P – phosphoprotein gene, M – matrix protein gene, F – fusion protein gene, M2 – second matrix gene, SH – small hydrophobic protein gene, G – envelope glycoprotein gene and L – large polymerase protein gene.

2.4 Viral proteins

The 8 genes of AMPV encode at least 8 proteins: N, P, M, F, M2, SH, G and L, unlike the pneumoviruses which encode at least 11 proteins. The M2 gene of AMPV encodes two proteins (M2-1 and M2-2) from two different open reading frames. These proteins are believed to be involved in the regulation of transcription and replication of the virus (Bermingham and Collins, 1999). Much of the knowledge regarding the functions of the AMPV proteins is derived from studies from other paramyxoviruses.

2.4.1 Nucleocapsid and nucleocapsid-associated proteins

The nucleocapsid protein (N) is always associated with the viral genomic RNA, thus forming the nucleocapsid core of AMPV. The nucleocapsid functions as the template for the viral replication and transcription. The phosphoprotein (P) and the large polymerase protein (L) associate with the nucleocapsid core to form the viral transcriptase-replicase complex.

N protein: The N protein is tightly bound around the viral genomic RNA, forming a RNase-resistant nucleocapsid. This nucleocapsid, in turn, associates with P and L proteins during transcription and replication, and most likely with the M protein during virus assembly. Studies based on the rhabdovirus vesicular stomatitis virus indicated that the intracellular concentration of the N protein may be a major factor that controls the relative rates of viral transcription and replication from genome templates (Blumberg and Kolakofsky, 1981; Blumberg et al., 1981). The complete nucleotide sequence of the N gene of AMPV strain Colorado (AMPV/CO) is 1206 nt long from the gene-start to the

gene end. The major ORF of 1188 nt contains a coding region of 394 amino acids (aa). The predicted molecular mass of the encoded polypeptide in the ORF is 43 kDa (Dar et al., 2001). Recently, an additional N polypeptide of 328 aa (molecular mass 36 kDa) was found to be encoded by a secondary ORF, in-frame with the major ORF, in Vero cells infected with AMPV/CO (Alvarez and Seal, 2005).

P protein: The P protein is highly phosphorylated and associates with the L protein to form the transcriptase-replicase complex. Though the large polymerase (L) protein is believed to possess all catalytic activities, the P protein is essential for viral RNA synthesis. It interacts with the N protein and keeps the latter in a soluble form and imparts specificity to the N protein to encapsidate viral but not cellular RNAs (Neumann et al., 2002). The P gene of AMPV/CO is 909 nt long and encodes a polypeptide of 294 aa, with a predicted molecular mass of 34 kDa (Dar et al., 2001).

L protein: The L protein is the largest viral protein and the least abundant of all structural proteins. It is the major component of the viral RNA dependant RNA polymerase in negative-stranded RNA viruses (Banerjee, 1987; Tordo et al., 1988). It is also thought to cap and polyadenylate the viral mRNAs, the latter activity is thought to be the result of polymerase stuttering on a short stretch of U residues. The L gene of AMPV/CO is 6173 nt in length from the gene-start to the gene end and encodes a polypeptide of 2005 aa in length. The predicted molecular mass of the polypeptide is 228,986 Da (Govindarajan and Samal, 2004).

2.4.2 Matrix proteins

Matrix (M) protein: The M is the most abundant protein in the virion. The M protein is thought to be the central organizer in virus morphogenesis, due to its interactions with the cytoplasmic tails of the outer membrane proteins, the lipid bilayer and the nucleocapsids. The M protein of AMPV/CO is 868 nt long encoding a 254 aa long polypeptide from the longest ORF (Seal, 1998). Its predicted molecular mass is 27,651 Da.

M2 protein: The M2 protein is also known as the second matrix protein. The M2 gene of AMPV/CO has two ORFs encoding two proteins viz. M2-1 and M2-2. These proteins are believed to be involved in the regulation of transcription and replication of the virus (Bermingham and Collins, 1999). The M2 gene of AMPV/CO is 748 nt long encoding two polypeptides M2-1 (14 - 569 nt) and M2-2 (525 – 740 nt) of 184 and 71 aa length, respectively (Dar et. al., 2003).

2.4.3 Envelope proteins

AMPV possesses three surface envelope glycoproteins namely, the fusion (F) glycoprotein, the small hydrophobic (SH) glycoprotein and the attachment (G) glycoprotein.

F protein: The F protein is a type I integral membrane glycoprotein and mediates pH-independent fusion of the viral envelope with the cellular membranes, following which the viral nucleocapsid is delivered into the cytoplasm. The F protein is synthesized

as an inactive precursor (F_0) that is cleaved into F_1 and F_2 active domains by host-cell proteases, thus enabling the fusion process. The F gene of AMPV/CO is 1644 nt long and the major ORF codes for a 537 aa long polypeptide (Seal et al., 2000). The F protein of AMPV/CO possesses a multi-basic amino acid sequence (aa 99-KAR-aa 101) at its cleavage site (Seal et al., 2000). Hence, unlike HMPV, AMPV/CO is capable of growing in tissue culture without the addition of exogenous trypsin.

SH protein: The SH protein is a type II integral membrane glycoprotein whose precise function is not completely known. It was suggested that SH protein of human respiratory syncytial virus (RSV) possesses characteristics of a viroporin, which modifies membrane permeability (Gonzalez and Carrasco, 2003). The RSV SH protein has also been implicated in impairing the Th1-mediated host antiviral responses (Tripp et al., 2000). The AMPV/CO SH gene is 623 nt long and encodes a 175 aa long polypeptide (Toquin et al., 2003).

G protein: The G protein of metapneumoviruses is believed to be the putative cell attachment protein. It is also considered to be the major antigenic determinant of AMPV/CO (Seal et al., 2000). The AMPV G protein is a type II membrane glycoprotein, containing an N terminal intracellular domain, a short transmembrane domain and a C terminal extracellular domain. The G protein of AMPV/CO is 1798 nt long from gene start to gene end, encoding a predicted polypeptide of 585 aa. The predicted molecular mass of the G protein of AMPV/CO was 58754 Da with a significantly high content of G+C residues (Govindarajan et al., 2004).

2.5 Stages of AMPV replication

AMPV follows the same pattern of replication as other paramyxoviruses, which is described in the following sections.

2.5.1 Adsorption and entry

Initiation of infection starts with the adsorption of the virus to the cell surface receptors and subsequent fusion of the viral envelope to the cell membrane. The former is mediated by the G protein while the latter by the F protein. The precise nature of the cellular receptor that is involved in AMPV infection has not been identified. The viral nucleocapsid is then delivered into the cytoplasm and all stages of AMPV replication occur in the cytoplasm.

2.5.2 Transcription

The pattern of transcription of the AMPV genome is consistent with that proposed for all nonsegmented negative stranded RNA viruses. By analogy to other viruses, the 3' leader region on the AMPV genome acts as the polymerase entry site and is recognized by the viral polymerase complex. The viral RNA polymerase first transcribes the leader RNA (uncapped and untailed) and then makes capped and polyadenylated viral mRNAs, guided by the conserved transcriptional start and stop sequences of each gene.

Transcription continues in this start-stop fashion until the L mRNA is synthesized. The intergenic regions located between the individual genes are not transcribed during this process. Since the frequency with which the viral polymerase restarts mRNA synthesis at

each gene junction varies, the upstream genes are always transcribed more than the downstream genes leading to a gradient of mRNA abundance (Fig. 2).

2.5.3 RNA Replication

AMPV probably follows the same general mode of RNA replication as observed in other nonsegmented negative-sense RNA viruses. After primary transcription and translation of mRNAs, genome (-) replication starts with the synthesis of a full-length complementary copy known as the antigenome (+). The newly synthesized antigenome serves as the template for the synthesis of new progeny viral genomes (Fig. 2). During RNA replication, the viral polymerase follows a read-through process ignoring the transcription start and stop signals, thus leading to the formation of a faithful complement of the genome. Both the genome and antigenome are assembled into encapsidated nucleocapsids. The leader and trailer regions of the genome are assumed to contain specific sequences for encapsidation (Blumberg and Kolakofsky, 1981). The precise reason for the switching of the polymerase from transcriptive to replicative mode is not clearly known. The coupling of genome assembly/encapsidation and genome synthesis is believed to cause the polymerase to ignore the junctional and editing signals (Kolakofsky et al., 1991). In RSV, it was observed that though increased levels of N stimulated replication there was no evidence of a switch from transcription to replication (Fearn et al., 1997). However, M2-2 protein has also been observed to have a regulatory role in balancing viral replication and transcription (Bermingham and Collins, 1999).

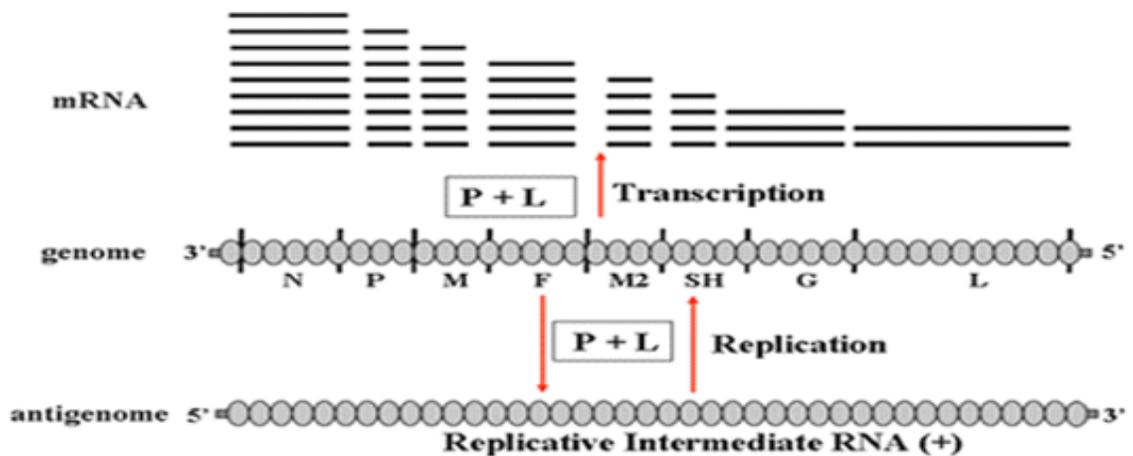


Figure 2. Schematic of AMPV transcription and replication. Genome and antigenome are shown as nucleocapsids with ovals representing the N protein subunits and vertical lines indicating the gene junctions. P-L complex transcribes the genome generating capped and poly A-tailed mRNAs. When sufficient viral protein levels are achieved, the viral polymerase switches to replicative mode resulting in the production of an antigenome, which in turn serves as the template for the synthesis of the progeny viral genome.

2.5.4 Packaging and release

The nucleocapsid assembly occurs in the cytoplasm of infected cells. The mechanism of viral assembly and release of AMPV is probably similar to that of other paramyxoviruses. Encapsidation of the viral genomic RNA into the nucleocapsid is the first step in viral assembly. The formation of the nucleocapsid occurs in two steps: first, the free N protein subunits associate to the genomic RNA to form the helical ribonucleoprotein, followed by the association of the P-L protein complex (Kingsbury et al., 1978). The accumulation of the M protein may also be crucial for initiating the assembly of virions (Teng and Collins, 1998). The viral envelope is assembled at the cell surface. The integral membrane glycoproteins (F, SH and G) are synthesized in the endoplasmic reticulum and then transported to the cell surface by the cellular secretory pathway. The nucleocapsid complex/M-protein structure, then associates with the cytoplasmic tails of the glycoproteins that are inserted into the cell membrane (Easton et al., 2004). Finally, release of the virus takes place by budding from the plasma membrane.

2.6 Avian metapneumovirus infections

2.6.1 Prevalence of AMPVs

AMPV virus was first isolated in South Africa in 1978 from turkeys exhibiting signs of sinusitis (Buys and Du Preez, 1980). Subsequently, presence of the virus was reported in United Kingdom (McDougall and Cook, 1986 and Wilding et al., 1986), France (Giraud et al., 1986 and Băyon-Auboyer et al., 2000), Germany (Naylor and

Jones, 1993), Netherlands (Cook et al., 1993), Japan (Tanaka et al., 1995 and Mase et al., 2003), Mexico (Decanini et al., 1991), Israel (Weisman et al., 1988 and Banet-Noach et al., 2005), Morocco (El Houadfi et al., 1991), Brazil (Arns and Hafez, 1992 and D'Arce et al., 2005) and Chile (Toro et al., 1998). AMPV was first isolated in the US in 1996 from commercial turkeys suffering from rhinotracheitis in Colorado (Kleven, 1997 and Cook et al., 1999). Currently, the subgroups A and B of AMPV are prevalent in Europe, United Kingdom, Japan, Brazil and Israel. Subgroup C is prevalent in many states of the US and Canada and the new subgroup, subgroup D is prevalent in France.

2.6.2 Disease in poultry

AMPV infections in poultry are essentially acute respiratory infections characterized by coughing, sneezing, and swelling of the infraorbital sinuses and other signs of respiratory distress often associated with nasal and ocular discharges. The disease has a high morbidity rate but the mortality rate ranges from 0 to 30% that may increase further with secondary bacterial infections. The clinical signs observed are more severe in birds infected with AMPV and other bacterial agents than with the virus alone (Alkhalaf et al., 2002; Jirjis et al., 2004 and Marien et al., 2005). Though turkeys and chicken are the main species of birds affected, the virus can also infect other species of birds (reviewed by Njenga et al., 2003).

2.6.3 Current control strategies

Currently, both live attenuated and killed vaccines are being used to control AMPV infections in Europe (Cook, 2000). However, in the US the killed vaccines have

not proven to be effective to control AMPV infections (Sheikh, 2001). A high-passage virus was observed to provide protection to turkey poults in response to immunization (Gulati et al., 2001). Studies have shown that cold-adapted AMPV can be used as a live attenuated vaccine and can prove to be safe and effective (Patnayak et al., 2002; Patnayak et al., 2003 and Patnayak and Goyal, 2004). A DNA vaccine expressing the F protein of AMPV was observed to reduce clinical symptoms in challenged birds better than one expressing the N protein (Kapczynski and Sellers, 2003). A virosome vaccine against AMPV was also observed to provide some level of protection following challenge (Kapczynski, 2004). However, the molecular basis of attenuation is unknown in the current vaccines being used in the US.

2.7 Minigenome and virus rescue systems for metapneumoviruses

Genetic manipulation of negative-stranded RNA viruses has been difficult, unlike the positive stranded ones. The major reasons for this are (1) lack of detectable genetic recombination in the negative sense RNA viruses and (2) the naked RNA is not infectious by itself. However, with the development of a reverse-genetic system, it is possible to introduce genetic changes directly into the negative-stranded RNA viral genome. This has significant applications in molecular biology studies as well as for the development of potential recombinant vaccines. The initial approach to genetically manipulate the genomes of negative-sense RNA viruses has been the “minigenome” system. A minigenome essentially consists of a synthetic construct made of genomic terminal sequences abutting a reporter gene. This is transcribed in vitro and then transfected into cells infected with the helper virus. A second approach involved the use of the support

plasmids (the nucleocapsid proteins) using the transient recombinant vaccinia virus/T7 RNA polymerase expression system, thus avoiding infection with the wild type helper virus. Since these minigenome systems do not represent the full-length genome, the main challenge was to rescue full-length genome RNAs.

The plasmid based approach has been utilized to recover infectious recombinant virus from full-length cDNA for several non-segmented negative-strand RNA viruses, namely rabies virus (Schnell et al., 1994), vesicular stomatitis virus (Lawson et al., 1995), measles virus (Radecke et al., 1995), rinderpest virus (Baron and Barrett, 1997), parainfluenza virus (Hoffmann and Banerjee, 1997), Newcastle disease virus (Peeters et al., 1999 and Krishnamurthy et al., 2000) and many other viruses. Among the members of subfamily *Pneumovirinae*, HRSV was the first to be recovered by reverse genetics system (Collins et al., 1995). Subsequently, a virus rescue system was also established for the bovine respiratory syncytial virus (Buchholz et al., 1999 and Yunus et al., 2001).

2.7.1 Rescue systems for avian metapneumoviruses

Among the avian metapneumoviruses, a minigenome system was first developed for AMPV-A strain CVL14/L (Randhawa et al., 1997). This minigenome system, containing the chloramphenicol acetyl transferase (CAT) gene as the reporter gene, utilized T7 RNA polymerase runoff transcripts transfected into AMPV-infected cells. Using this system it was established that NS1 and NS2 genes were absent from the genome of avian metapneumovirus. Recently, a reverse genetics system developed for AMPV-A strain LAH A demonstrated that the SH and the G genes were not essential for

virus survival in vitro (Naylor et al., 2004). Complete genome sequences of AMPV subgroups B and D are currently not available and hence development of a reverse genetics system for these viruses has not been possible.

2.7.2 Rescue systems for human metapneumoviruses

The first full-length HMPV to be recovered entirely from cloned cDNA was the Canadian isolate CAN97-83 (Biacchesi et al., 2004a). Subsequently, two isolates, NL/1/00 and NL/1/99, representing lineages A and B, respectively, were also recovered (Herfst et al., 2004). This recently established rescue system for HMPV has paved way for numerous studies related to HMPV molecular biology, pathogenesis and vaccine development. A recombinant HMPV expressing GFP as a foreign protein showed that the HMPV can also serve as a vector for foreign gene expression (Biacchesi et al., 2004a). In addition, GFP expression in living cells greatly helped in monitoring the initial recovery of these viruses by fluorescent microscopy. A recombinant HMPV that expressed an additional G gene and 2 additional copies of F gene indicated that the HMPV genome can be manipulated with relative ease and can accommodate up to a 30 % increase in length (Biacchesi et al., 2004a). Both the SH and G genes, alone or together, were observed to be dispensable for growth of HMPV in tissue culture as well as in respiratory tract of hamsters (Biacchesi et al., 2004b). Deletion mutants of HMPV lacking M2-1, M2-2, either individually or together, showed that though both of them are not required for in vitro growth, M2-1 is essential for virus growth in vivo (Buchholz et al., 2005).

Chapter 3: Determination of complete genome sequence

This chapter deals with the determination of the nucleotide sequences of attachment glycoprotein gene (Govindarajan et al., 2004), large polymerase protein gene (Govindarajan and Samal, 2004), and the leader and trailer regions of AMPV strain Colorado (AMPV/CO). Hence, with the previously determined sequences, this completes the entire genome sequence of AMPV/CO (Govindarajan and Samal, 2005).

3.1 Abstract

The nucleotide sequences of the G gene, L gene, and the genomic termini of AMPV/CO were determined. The G gene was 1798 nucleotides long and encoded a polypeptide of 585 amino acids. It was the longest G gene among all known metapneumoviruses. The L gene was 6173 nucleotides long and encoded a polypeptide of 2005 amino acids. Both the genes and their deduced proteins showed higher levels of identities to the corresponding genes and proteins of HMPV than to those of other AMPVs. The sequences of the 3' leader and the 5' trailer regions were determined to be 40 and 39 nucleotides long, respectively. Comparison of the genomic termini sequences indicated that there was high level of conservation among the metapneumoviruses and the genomic termini of AMPV/CO were more similar to those of the HMPVs than to the AMPVs. This study completes the sequence of the 14150 nucleotide genomic RNA of AMPV subgroup C strain Colorado.

3.2 Introduction

AMPV is a member of the genus *Metapneumovirus* in the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (Pringle, 1998). The virus contains a non-segmented, single-stranded, negative-sense RNA genome with eight genes encoding at least nine proteins (Ling and Pringle, 1988 and Yu et al., 1992): the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the second matrix proteins (M2-1 and M2-2), the small hydrophobic protein (SH), the attachment glycoprotein (G) and the large polymerase protein (P). Flanking the genes are the 3' and 5' extracistronic regions, known as the leader and trailer, respectively. These regions have been believed to be the cis-acting regulatory elements in replication, transcription and packaging of the genomic and antigenomic RNA (Lamb and Kolakofsky, 1996).

The nucleotide sequences of all eight genes of AMPV-A have been determined (Randhawa et al., 1997 and references therein). All of the genes except for the L gene of AMPV-B have also been sequenced (Jacobs et al., 2003 and references therein). Nucleotide sequences of six genes of AMPV/CO, namely N (Dar et al., 2001), P (Dar et al., 2001), M (Seal, 1998), F (Seal et al., 2000), M2 (Dar et al., 2003) and SH (Yunus et al., 2003; Toquin et al., 2003 and Lwamba et al., 2004) have been reported. However, there have been conflicting reports on the length of the G gene of AMPV/CO. The G gene of AMPV/CO was reported to be 1321 nt long with a predicted polypeptide of 435 aa (Alvarez et al., 2003; GenBank accession no. AY579780) or 783 nt long with a predicted polypeptide of 252 aa (Toquin et al., 2003; GenBank accession no. AJ457967). In both of these studies, G gene sequences were obtained from the genomic RNAs of

AMPV/CO. In our study, G gene sequences were obtained by sequencing the genomic RNAs and mRNAs of three different strains of AMPV-C.

The availability of a complete sequence of AMPV/CO genome is essential to rescue infectious virus entirely from cloned cDNA. And, the development of a rescue system for this virus should stimulate further research towards a complete understanding of this emerging turkey pathogen. Hence, with the ultimate goal of developing a rescue system, we report here the remaining undetermined sequences of AMPV strain Colorado and compare them to related viruses. These sequences include the complete G gene, L gene, and the entire leader and trailer regions.

3.3 Materials and methods

3.3.1 Cells, virus and RNA extraction

AMPV-C strain Colorado (AMPV/CO), obtained from the National Veterinary Services Laboratory (Ames, Iowa), was grown in Vero cells supplemented with 2% fetal bovine serum. AMPV-C strains Mn-1a and Mn-2a (used to determine the G gene sequence) were kindly provided by Sagar M. Goyal (University of Minnesota, St Paul, MN, USA). The virus propagation, purification and RNA extraction were performed in a manner similar to those described elsewhere (Govindarajan and Samal, 2004). Briefly, Vero cells were infected with AMPV/CO and the virus was harvested when maximum cytopathic effect was evident as extensive syncytia. The infected cells were scraped into the medium and lysed by three cycles of freezing and thawing to release the intracellular

virus. The cell lysate was clarified at 3000 g for 15 min, and the supernatant was made 10% with respect to PEG 8000 (Sigma) and incubated for 3 h at 4 °C. Subsequently, the virus was pelleted at 4000 g for 30 min at 4 °C. Viral genomic RNA was extracted from the viral pellet using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, except that an additional extraction with phenol plus chloroform was performed. Viral mRNAs were isolated from infected Vero cell lysates by using a polyA Spin mRNA Isolation kit (New England Biolabs).

3.3.2 Reverse transcription and PCR

G gene: The "genome walking" strategy was initially employed for obtaining the complete sequence of the G gene. AMPV genomic RNA was reverse transcribed at 42 °C using Superscript II reverse transcriptase (Invitrogen) and a G gene-specific positive-sense primer, G-513 (5'-CACAAGCAATAGCACAACACTGACAACACCAACAAC-3'). The cDNA synthesized from the above reverse transcription reaction was purified using a PCR purification kit (Qiagen) and then used for PCR amplification using G-513 and an L gene-specific negative-sense primer, L-713 (5'-GGCCCCTTGTAATTGCTTCTAA-3'); the primer sequences were obtained from the published G gene sequence (Toquin et al., 2003). Although a single, sharply defined amplification product was not obtained from this PCR, the obtained product was cloned and subsequently sequenced. Surprisingly, sequence analysis of numerous clones did not yield a single consensus sequence; instead, consensus sequences were obtained at the 5' and 3' ends of the cDNA clones, whereas the internal regions varied in length. As numerous attempts to determine the complete G gene

sequence from the viral genomic RNA proved unsuccessful, we decided to use the G mRNA as a template to determine the complete G gene sequence.

All RT reactions of mRNAs isolated from virus-infected cells were performed by using a ProtoScript First Strand cDNA Synthesis kit (New England Biolabs). Three separate RT reactions were performed for each virus, using an oligo(dT) primer and two G gene-specific reverse primers, G-1589 (5'-CAGTGCCGTCCCCAAAACAT-3') and G-1640 (5'-CATCATAGCAACCAGCCGGC-3'), which were designed based on the sequence obtained from viral genomic RNA. PCR was performed with TaKaRa LA *Taq* polymerase and GC buffer II (TaKaRa), using primers G-513 and G-1589. The following cycle parameters were used in the PCR: initial denaturation at 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and a final elongation step of 72 °C for 5 min. This PCR yielded a single 1.1 kb product. RT-PCR of mRNAs isolated from uninfected cells by using the same primers did not yield any product. The entire RT-PCR was performed three times, each time with a new viral mRNA preparation, and each time a single product of 1.1 kb was amplified. These products were either sequenced directly or were cloned and subsequently sequenced to yield the complete sequence of the G gene.

L gene: To determine the complete sequence of the L gene, the "genome walking" strategy was employed. AMPV genomic RNA was reverse transcribed at 42 °C using Superscript II reverse transcriptase (Invitrogen) and an L gene-specific positive-sense primer (5'-TAAAAATGGATCCACTAAATGAAGG-3'). The primer was

designed using the published partial L gene sequence of AMPV-C (Toquin et al., 2003). The c-DNA synthesized from the above reverse transcription reaction was purified using a PCR purification kit (Qiagen), then tailed with C nucleotides in a tailing reaction catalyzed by terminal deoxynucleotidyl transferase enzyme (Invitrogen). The dC-tailed cDNA was used for PCR amplification using L gene-specific forward primer, a poly dG-anchor reverse primer (Invitrogen) and Platinum Pfx polymerase (Invitrogen).

Leader region: As the leader and trailer regions of AMPV-A showed high resemblance to those of HMPV isolate 00-1 (van den Hoogen et al., 2002), we designed consensus primers to obtain the sequence of the AMPV/CO leader and trailer regions. To obtain the 3' leader sequence, the AMPV/CO genomic RNA was reverse-transcribed with a positive-sense consensus leader primer, le-For (5' GGAGGACGAGAAAAAACGC 3'). The resulting cDNA was subjected to a PCR with le-For primer and an N gene-specific negative-sense primer, N-540 (5' GATTGTTGATGCCAGCTTCGTGAA 3'). The PCR product thus obtained was cloned and the clones were hybridized with an N gene-specific radiolabelled probe. The 3' leader sequence was obtained from nt sequencing of numerous hybridization-positive clones. To further confirm the leader sequence, viral RNA was polyadenylated at its 3' end using poly A polymerase (Invitrogen) and reverse transcribed with an oligo dT primer. PCR was performed using a GeneRacer 3' forward primer (GeneRacer kit, Invitrogen) and the above-described N-540 primer. The amplified cDNA was either directly sequenced or cloned and several clones were sequenced to obtain the nt sequence of the 3' leader region of the AMPV/CO genome.

Trailer region: The sequence of the 5' trailer region was also obtained using the method described for obtaining the sequence at the 3' leader region. Briefly, the AMPV/CO genomic RNA was reverse-transcribed with an L gene-specific positive-sense primer, L-5787 (5' GTTGGAGGCAGCAGGGTCATAGAATC 3') and a PCR was performed with L-5787 and a negative-sense consensus trailer primer, tr-Rev (5' GGAG-GACGAGAAAAAACCGTAT 3'). The resulting RT-PCR product was either directly sequenced or cloned and sequenced to obtain the sequence of the 5' trailer region of AMPV/CO. The sequence of the 5' trailer region was further confirmed using the 5' RACE method (Krishnamurthy and Samal, 1998). The cDNA from the L-5787 RT reaction was tailed with dCTP using terminal deoxynucleotidyl transferase (Invitrogen), and PCR-amplified with L-5787 and an oligo dG anchor primer (Invitrogen). The PCR product was then cloned and several clones from three independent 5' RACE reactions were sequenced to obtain the sequence of the 5' trailer region of the AMPV/CO genome.

3.3.3 Cloning and sequencing

G gene: The PCR amplicons were either sequenced directly or were cloned and subsequently sequenced using an ABI 3100 DNA sequencer (Applied Biosystems). The PCR-amplified products were cloned into a TOPO-Zero blunt cloning vector (Invitrogen) and transformed into DH10B maximum efficiency competent cells (Invitrogen). Several colonies were sequenced and the correct sequence was obtained from sequences derived from at least three independent reactions. All sequencing procedures to determine the G gene sequence were performed in the presence of 5 % (v/v) DMSO or 1 M betaine (final concentration) in the sequencing reaction mixture. The sequences obtained from RT-PCR

products made from genomic and mRNAs were assembled to yield the complete sequence of the G gene.

L gene: The PCR-amplified products from the L gene were cloned as described above. The resulting colonies were hybridized with a radiolabelled L cDNA probe representing the first 713 nt of the L gene. Several hybridization-positive colonies having larger size inserts were selected and sequenced. The correct sequence was obtained from sequences derived from at least three independent clones. The entire sequence of the L gene was obtained by repeating the cloning and sequencing procedures. The L gene sequence obtained from each round of cloning was used to design the forward primer for the next round of RT-PCR. The RT-PCR products were also sequenced directly without cloning to derive at the consensus sequence.

3.3.4 Sequence analyses

All the sequence analyses were performed using the DNASTAR software program. Complete genome assembly was performed with the Seqman tool of the software and all the protein alignments were carried out using the ClustalW method (www.ebi.ac.uk/clustalw). Phylogenetic trees were constructed using the DNASTAR software program, while the lengths of distances between the sequences were obtained from the ClustalW program.

3.4 Results

3.4.1 G gene sequence

The complete G gene of AMPV/CO was 1798 nt (GenBank accession no. AY590691) from the start to the end of the gene, encoding a predicted polypeptide of 585 aa. The length of the G gene determined by us was 1015 nt longer than that reported previously for the same AMPV/CO strain (Toquin et al., 2003). To determine whether the lengths and sequences of the G genes were conserved among strains of AMPV-C, we cloned and sequenced the G genes of AMPV-C strains Mn-1a and Mn-2a. We observed that the gene length and the predicted protein length of G genes of Mn-1a and Mn-2a were exactly the same as those of AMPV/CO, but that considerable sequence variation existed between the two strains (Fig. 3).

The G gene of AMPV/CO, as observed for other metapneumoviruses, possessed the highly conserved gene-start (5'-GGGACAAGU-3', mRNA sense) and gene-end (5'-UAGUUAUUAAAAA-3') signals (Ling et al., 1992 and Biacchesi et al., 2003). Apart from the major ORF, four potential secondary ORFs (ORF2, 146–1771 nt, 541 aa; ORF3, 155–1771 nt, 538 aa; ORF4, 167–1771 nt, 534 aa; and ORF5, 1312–1608 nt, 98 aa) were also observed in the G gene of AMPV/CO. The G genes of Mn-1a and Mn-2a also contained similar secondary ORFs.

	Intra-cellular domain	Transmembrane domain	Extra-cellular domain	
AMPV/CO	MEVKVENVGKSQLKVKVKNFIKRSDCCKKLFALILGLVSFELTMNIMLSVMYVESNEALSLCRIQGTPAPRDNKNTENATKETTLHTT			90
Mn-1a	90
Mn-2a	90
AMPV/CO	TTTRDPEVRETKTTKQANEGATNPSRNLTTKGDKHQTRATTEAELEKQSKQTTEPGTSTQKHTPTRPSSKSPTTTQAIQQLTTPTPK			180
Mn-1a	180
Mn-2aA.....T..P...A..	180
AMPV/CO	ASTAPKNRQATTKKTETDTTTASRARNTNNPETATTTPKATTEETGKSKEGPTQHTTKEQPETTAGETTTTPQPRRTASRPAPTTKIEEEA			270
Mn-1aDS..G...A...AA.A.....GA.....A.....			270
Mn-2aG.....G.....R.....			270
AMPV/CO	ETTKTRTTKSTQSTGPPRPTGGAPSGAATEGSGRAAAAGGPSAASAGRRRTEAAAERDRRTRAGAGPTAGGARARTAAASERGADTAG			360
Mn-1a			360
Mn-2aN.....RST..KT... NNK.TTTTKR.NT..TDS.QQ.RIT..Q.QQ.QTR.K..TN..HPQ.TTTP.HNT..TN			360
AMPV/CO	SAGGGPGGDGATGGLSGGAPAEREDASGGTAAAGPGDGEADGRAPPAAALAGRTTESAAGAAGDSGRAGTAGWGSAADGRSTGGNAAAE			450
Mn-1aG.....			450
Mn-2a	.TK.S.KE.KT.RDP.SKT.T.Q....K....N..GSA...R....TTPT.....TT..DSG.E.TRRR....R....ST...			450
AMPV/CO	AGAAQSGRAAPRQPSGGTAPSTAPPNSGGSGRADAAPTEEVGVGSLWRGRYVCGPCGESVPEHPMNPFCFGDTAWICSDDGSLPAGC			540
Mn-1a			540
Mn-2a	..T.....T.K.....AGN.....NES.....A.....SI.....R...L.....G..N.....			540
AMPV/CO	YDGGTDGVVCCGVCNGNSCCCGRVECTCGGGAGLLSCCCGSYSWS	585		
Mn-1a	585		
Mn-2a	..S.A.....I.....N..	585		

Figure 3. Comparative alignment of the deduced amino acid sequences of the predicted G proteins of AMPV-CO, Mn-1a and Mn-2a. Proposed intracellular, transmembrane and extracellular domains of the G proteins are indicated above the sequences. Conserved amino acids relative to AMPV/CO are indicated by dots and potential *N*-linked glycosylation sites are underlined. The residues forming the hypervariable region (aa 300–450) on Mn-2a G protein have been highlighted in bold.

The predicted molecular mass of the G protein of AMPV/CO was 58 754 Da, having a net charge of 8.27 at neutral pH and an isoelectric point of 8.28. The G+C content of the G gene of AMPV/CO was 61 mol%, which was significantly higher than that of the G genes of other metapneumoviruses. The additional 1015 nt determined by us in the G gene of AMPV/CO had a much higher G+C content (73 mol%). The amino acid composition of the deduced G protein of AMPV/CO was relatively similar to those of other AMPV G proteins (Table 1). The G protein of AMPV/CO contained 7.2 % proline and 23.1 % serine/threonine residues, a consistent feature of mucin-like glycoproteins. The predicted G proteins of the three AMPV-C strains contained three conserved, potential *N*-linked glycosylation sites (Fig. 3). Seventy eight predicted potential *O*-linked glycosylation sites were observed on the G protein of AMPV/CO where as Mn-1a and Mn-2a G proteins possessed 74 and 103 predicted *O*-linked glycosylation sites. However, by analogy to HRSV and HMPV, it is unlikely that all of these sites are used. Majority of these sites were observed to lie between aa residues 150-300 (the predicted extracellular domain) in all the three viruses. The G ORFs of the three AMPV-C strains contained 18 conserved cysteine residues, 17 of which were present in the extracellular domains.

Hydropathy analysis of the putative G proteins of AMPV/CO, Mn-1a and Mn-2a showed characteristics of an anchored, type II membrane glycoprotein. The predicted hydrophobicity profile of AMPV/CO G protein included an amino-terminal, hydrophilic intracellular domain (aa 1–31), a hydrophobic transmembrane domain (aa 32–54) and a hydrophilic extracellular domain (aa 55–585) (Fig. 3). Although the G protein of

Table1. Features of G proteins of the metapneumoviruses

Virus	Length of ORF (aa)	Molecular Weight (kDa)	Percentage			No. of cysteine residues
			G+C content	Proline	Serine + Threonine	
AMPV/CO	585	58.8	61.7	7.2	23.1	18
AMPV-Mn1a	585	58.3	62.9	7.2	22.2	18
AMPV-Mn2a	585	61.6	54.0	7.7	27.4	18
AMPV-A	391	43.0	48.5	6.7	23.5	20
AMPV-B	414	44.6	49.5	8.5	24.6	20
AMPV-D	389	41.8	49.7	9.5	23.9	20
HMPV-001	236	25.8	47.0	8.5	33.9	1
CAN97-83	219	23.7	46.4	7.8	32.0	1
CAN98-75	236	25.5	46.6	5.5	33.9	2

AMPV/CO showed structural and biochemical features similar to those of the HMPVs, it showed only 23, 25 and 21 % amino acid identity to the corresponding proteins of HMPV-001, CAN97-83 and CAN98-75, respectively. However, the levels of amino acid identity to the G proteins of other AMPV subgroups were still lower: 14 % to those of AMPV-A and -B and 16 % to that of AMPV-D. Among the US strains, the G protein of AMPV/CO showed 98 and 81 % amino acid identity to those of Mn-1a and Mn-2a, respectively. The predicted G proteins of Mn-1a and Mn-2a exhibited 79 % amino acid identity between themselves. Sequence alignment of the G genes of the three AMPV-C strains revealed that Mn-1a and Mn-2a possessed 21 and 195 nt substitutions relative to AMPV/CO, resulting in 11 and 110 aa changes, respectively, in their deduced proteins. The majority of the changes in the predicted G protein of Mn-2a lay between aa 300 and 450, thus forming a hypervariable region with only 48 % amino acid identity (Fig. 3).

3.4.2 L gene sequence

The L gene of AMPV/CO was 6173 nt long from the gene-start to gene-end (GenBank accession no. AY513746). This size of the AMPV/CO L gene was comparable to the sizes of all other published paramyxovirus L genes (Table 2). The gene-start signal for the L gene of AMPV/CO was found to be 5'-GGACCAAGU-3' (mRNA-sense). It was different from the highly conserved gene-start signal (5'-GGGACAAGU-3') of all other genes of AMPV/CO (Yunus et al., 2003) and also from the L gene-start signal (5'-AGGACCAAU-3') of AMPV-A (Randhawa et al., 1996). The gene-end sequence of the L gene of AMPV/CO was 5'-UAGUUAUUUAAAA-3' (mRNA-sense), and showed resemblance to the gene-end signal of HMPV (5'-UAGUUAUUUAAAA-3') and AMPV-

A (5'-UAGUUAUAAAAA-3') L genes. However, it was different from the gene-end sequences of other genes of AMPV/CO (Yunus et al., 2003).

The longest ORF of the AMPV/CO L gene encoded a polypeptide of 2005 aa. The size of the L protein of AMPV/CO was exactly the same size as the L protein (2005 aa) of HMPV (van den Hoogen et al., 2002), but one amino acid longer than the L protein of AMPV-A (Randhawa et al., 1996). The M_r of the AMPV/CO L protein was 228,986 Da. The predicted AMPV/CO L protein was basic, with a net charge of +52 at neutral pH, and had a high leucine (11%) and isoleucine (6%) content.

Alignment of the L protein sequences of AMPV/CO, HMPV and AMPV-A revealed that the L protein of AMPV/CO showed an 80% amino acid identity with the L protein of HMPV; whereas, the amino acid identity between the L proteins of AMPV/CO and AMPV-A was only 64% (Table 2). The L protein of AMPV/CO showed a lower percentage (46–47%) of amino acid identity with other known pneumovirus L proteins. The resemblance of AMPV/CO L protein to those of subfamily *Paramyxovirinae* ranged only between 13 and 15%. We performed a phylogenetic analysis of the predicted amino acid sequences of all twelve L proteins to determine the relationship between AMPV/CO and other paramyxoviruses (Fig. 4). Our results showed that AMPV/CO was phylogenetically more closely related to HMPV than to AMPV-A.

Table 2. Comparison of the L gene and predicted L protein of AMPV/CO with those of other paramyxoviruses

	Gene size (nt)	Protein size (aa)	% nt similarity	% aa similarity
AMPV/CO	6173	2005	-	-
HMPV	6124	2005	73	80
AMPV-A	6099	2004	64	64
AMPV-D	- ^a	- ^a	66 ^b	- ^a
HRSV-A	6578	2165	39	46
HRSV-B	6580	2166	39	47
BRSV	6573	2162	55	47
Others ^c	6643-6955	2183-2262	1-3	13-15

a - Complete sequence not available

b - Alignment based on partially published sequence (1111-1416 nt)

c - Other viruses included human parainfluenza virus types 2 and 3, La Sota strain of Newcastle disease virus, Sendai virus, measles virus and Nipah virus.

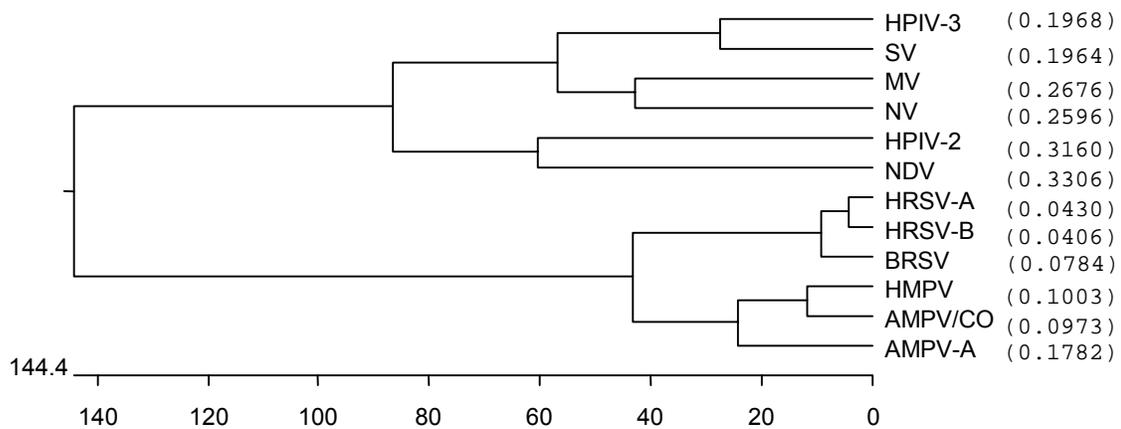


Figure 4. Phylogenetic analysis of the L ORFs of AMPV/CO and other paramyxoviruses.

The putative L ORF of AMPV/CO was aligned with those of other paramyxoviruses and a phylogenetic tree was generated using the DNASTAR software program. The lengths of each pair of branches (obtained from ClustalW alignment – www.ebi.ac.uk/clustalw) are proportional to the amount of inferred evolutionary change, while the units at the bottom of the tree denote the number of substitution events. HRSV-A and HRSV-B, human respiratory syncytial virus types A and B; BRSV, bovine respiratory syncytial virus; HPIV-2 and HPIV-3, human parainfluenza virus types 2 and 3; NDV, La Sota strain of Newcastle disease virus; SV, Sendai virus; MV, measles virus; NV, Nipah virus.

Sequence analysis of the polymerase proteins of various non-segmented negative-strand RNA viruses denoted that there are six conserved domains (I-VI) along the length of the protein, and the function of the L protein probably depends on these distinct domains (Poch et al., 1990 and Sidhu et al., 1993). All the six domains were also noticed in the AMPV/CO L protein as observed in the case of the AMPV-A L protein. The alignment of the four core polymerase motifs of domain III of 12 different paramyxovirus L proteins is shown in Fig. 5. Motifs A and C were perfectly conserved, while the motif B was nearly 100% conserved in the L proteins of the three metapneumoviruses. The D motif of the AMPV/CO L protein showed 70% and 90% identity with the corresponding motifs of AMPV-A and HMPV, respectively. The GDN tripeptide, which is similar to the catalytic GDD domain of positive-strand RNA virus RNA polymerases (Jablonski et al., 1991), was conserved in all twelve L proteins analyzed in this study.

The presence of an invariant tripeptide GHP in domain I was reported in the L proteins of AMPV-A (aa 365-367) and BRSV (aa 427-429) (Randhawa et al., 1996 and Yunus et al., 1998). A similar tripeptide was also observed at location 365-367 in the L protein of AMPV/CO. A stretch of hydrophobic residues (KERE), as observed in the BRSV L protein (aa 616-619), was also observed in the L protein of AMPV/CO (aa 554-557). A putative nucleotide-binding motif was reported to lie within domain VI of many negative-stranded non-segmented RNA viruses (Poch et al., 1990; Randhawa et al., 1996; Yunus et al., 1998; Harcourt et al., 2001 and van den Hoogen et al., 2002). An ATP-binding motif (aa 1673-1721) was also found in the L protein of AMPV/CO, but the number of the intermediate residues varied by one.

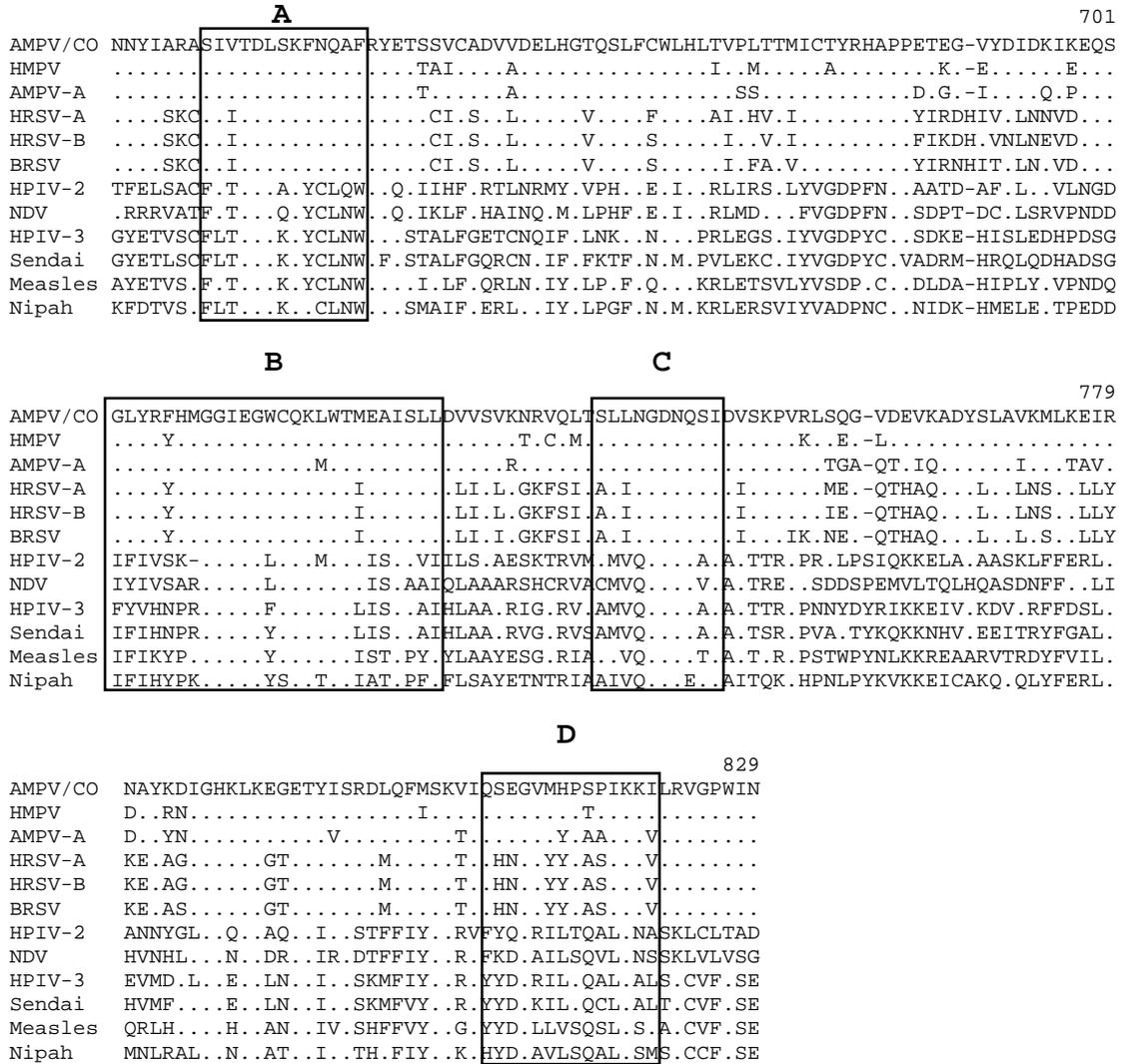


Figure 5. Amino acid sequence comparison of the putative polymerase core motifs (A, B, C and D) of domain III of the AMPV/CO L protein with those of other paramyxoviruses. All four motifs are boxed. Gaps are represented by dashes and perfectly-conserved amino acids relative to AMPV/CO are indicated by dots. HRSV-A and HRSV-B, human respiratory syncytial virus types A and B; BRSV, bovine respiratory syncytial virus; HPIV-2 and HPIV-3, human parainfluenza virus types 2 and 3; and NDV, La Sota strain of Newcastle disease virus.

3.4.3 Genomic termini sequences

The 3' leader of AMPV/CO was 40 nt long, and was comparable in length to those of AMPV-A (41 nt) and HMPV isolates CAN97-83 (41 nt), CAN98-75 (40 nt) and NL/1/99 (40 nt). The AMPV/CO leader region showed greatest similarity, both in length and nt identity, to those of CAN98-75 and NL/1/99. The 3' leaders of all three viruses were 40 nt in length and exhibited 85% nucleotide identity among them. The first 17 nt of the leader regions of AMPV/CO and AMPV-A were also identical (Fig. 6).

The 5' trailer region of AMPV/CO was 39 nt long and was very similar to that of AMPV-A (40 nt), but relatively shorter when compared to the HMPV trailers (96 nt in CAN97-83 and 68 nt in CAN98-75). However, the extreme 18 nt at the 5' end of both the Canadian isolates of HMPV were identical to that of AMPV/CO (Fig. 7). We observed that both the leader and trailer regions of AMPV/CO showed higher degrees of resemblance to those of HMPV than to those of AMPV-A, the only AMPV whose complete genomic sequence is available. This is in agreement with the observation that the other genes of AMPV/CO are also more similar to those of HMPV than to those of other AMPV subgroups. The leader and trailer regions of AMPV/CO were also highly complementary to each other; 11 of the first 13 nt and 18 of the terminal 30 nt were exact complements (Fig. 8). This high degree of conservation suggests that these residues might be part of the genomic and antigenomic promoters.

		10	20	30	40	N Gene
AMPV/CO	: 3'	UGCUCUUUUUU	UGCGUAUAUUC	GUGUUGAAGGUU	UGUUUUG-	<u>CCCUGUUCA</u>
AMPV-A	: 3'	AG...	GUCCA.GAUC..	U..A.UA	<u>CCCUGUUCA</u>
CAN97-83	: 3'	...G.....	...C.....	UAA..C..U...	U...GUA	<u>CCCUGUUCA</u>
CAN98-75	: 3'	...G.....	...C.....	UAA..U.....	<u>CCCUGUUUA</u>
NL/1/99	: 3'	...G.....	...C.....	UAA..U.....	<u>CCCUGUUUA</u>
HMPV 00-1	: 3'	...G.....	...C.....	UAA.CU.....	U...AU	<u>CCCUGUUCA</u>

Figure 6. Comparative alignment of the sequences (genomic-sense) of the 3' leader regions of metapneumoviruses (AMPV/CO, AMPV-A and HMPV isolates 00-1, CAN97-83, CAN98-75 and NL/1/99). Perfectly conserved nucleotides relative to AMPV/CO are indicated by dots.

```

                10         20         30
AMPV/CO   : 5' ACGGCAAAAAAACCGUAUUCAUCCAAUUUUAGUUCCUCA-
AMPV-A    : 5' ...AG.....A.A...UAGCUU.U.G
CAN97-83  : 5' .....A...U.....A..A..U..U.U
CAN98-75  : 5' .....A...U.....C.A..U..U.U
NL/1/99   : 5' .....A...U.....C.A..U..U.U
HMPV 00-1: 5' .....A...U.....A..A..U..U.U

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Figure 7. Comparative alignment of the sequences (genomic-sense) of the 5' trailer regions of metapneumoviruses (AMPV/CO, AMPV-A and HMPV isolates 00-1, CAN97-83, CAN98-75 and NL/1/99). Perfectly conserved nucleotides relative to AMPV/CO are indicated by dots.

```

                10         20         30         40
AMPV/CO le: 3' UGCUCUUUUUUUGCGUAUAUUCUGUUGAAGGUUUGUUUUG
                ***  *****      * *  ***  *      *  *
AMPV/CO tr: 5' ACGGCAAAAAAACCGUAUUCAUCCAAUUUUAGUUCCUCA-

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Figure 8. Complementarity between the genomic termini of AMPV/CO. The complementary nucleotides (in genomic RNA sense) are marked by asterisks.

3.5 Discussion

3.5.1 G gene

The complete G gene of AMPV/CO was 1798 nt long, encoding a predicted polypeptide of 585 aa (GenBank accession no. AY590691). The length of the G gene determined by us was 1015 nt longer than previously reported (Toquin et al., 2003). The G gene of AMPV/CO was significantly longer than those known for other MPVs. It was 538–613 nt longer than the G genes of other AMPV subgroups and 1066–1087 nt longer than those of HMPV. It is of interest to note that, although AMPV/CO showed genetic relatedness to HMPV with respect to most of its genes (van den Hoogen et al., 2002 and Govindarajan and Samal, 2004); it differed greatly from HMPV with respect to the length and sequence of the G gene. Determination of the G gene sequences of AMPV-C strains Mn-1a and Mn-2a revealed that the lengths and sequences of the G genes were conserved among strains of AMPV-C. Apart from the major ORF (ORF1), four potential secondary ORFs (ORF2, 146–1771 nt, 541 aa; ORF3, 155–1771 nt, 538 aa; ORF4, 167–1771 nt, 534 aa; and ORF5, 1312–1608 nt, 98 aa) were also observed in the G gene of AMPV/CO, Mn-1a and Mn-2a. The predicted polypeptides from ORFs 2, 3 and 4 were in frame in the predicted polypeptide from the major ORF1. They resemble secreted forms of G protein as observed by their hydropathy analysis. However, it would be interesting to determine whether these secondary ORFs yield additional protein products or secreted forms of the G protein following infection.

The G protein of AMPV/CO showed only 21-25 % amino acid identity to the G proteins of HMPVs. However, the levels of amino acid identity to the G proteins of other AMPV subgroups were still lower: ranging from 14-16 %. This is in agreement with the general observation that the glycoproteins show low levels of identities. Among the US strains, the G protein of AMPV/CO showed 98 and 81 % amino acid identity to those of Mn-1a and Mn-2a, respectively. The predicted G proteins of Mn-1a and Mn-2a exhibited 79 % amino acid identity between themselves. Our study also revealed that there is considerable variation in the G protein among the US strains of AMPV and a hypervariable region with only 48 % amino acid identity was observed between aa 300-450 of the Mn-2a G protein.

Careful examination of the nucleotide sequence of the G gene of AMPV/CO published by Alvarez et al. (2003) revealed that it contained partial sequences of the SH and G genes as a single G ORF. Our results also showed that all 783 nt of the G gene described by Toquin et al. (2003) were contained in the G gene sequence determined by us, but an additional 1015 nt insertion was identified between nt 752 and 753 of the G gene. These additional 1015 nt contained a higher G+C content (73 mol%) than the rest of the G gene (61 mol%). We believe that these 1015 nt probably contributed to the structural complexity in the genome that prevented successful and complete RT-PCR of the genomic RNA *in vitro*. In our study, the use of the smaller-sized G mRNA (compared with that of the genomic RNA), the direction of the RT reaction and the use of GC buffer II (TaKaRa) were probably responsible for our ability to amplify the secondary structure

region of the G gene successfully. This method will be useful in sequencing the G genes of other AMPV-C isolates.

3.5.2 L gene

The L gene of AMPV/CO was 6173 nt long from gene-start to gene-end (GenBank accession no. AY513746). The longest ORF encoded a polypeptide of 2005 aa. The size of the L protein of AMPV/CO was exactly the same size of the L protein (2005 aa) of HMPV (van den Hoogen et al., 2002), but one amino acid longer than the L protein of AMPV-A (Randhawa et al., 1996). Alignment of the predicted L protein sequence of AMPV/CO with that of HMPV revealed an 80% amino acid identity; whereas, the amino acid identity between the L proteins of AMPV/CO and AMPV-A was only 64%. A similar trend was also observed with the small hydrophobic protein (Yunus et al., 2003), the envelope glycoprotein (Govindarajan and Samal, 2004), and other proteins of AMPV/CO and HMPV (Govindarajan and Samal, 2005). The L protein of AMPV/CO showed a lower percentage (46–47%) of amino acid identity with other known pneumovirus L proteins and a much lower (13–15%) identity with the L proteins of the heterologous Paramyxovirinae subfamily. The L protein of HMPV also showed only 13–15% amino acid identity to those of *Paramyxovirinae* subfamily (van den Hoogen et al., 2002). To our knowledge, this is the highest observed amino acid identity between the polymerase proteins of two viruses (whose sequences are available) within the genus *Metapneumovirus* that affect different host species. A higher amino acid identity (83.8%) was also observed between the BRSV L protein and HRSV L protein (Yunus et al., 1998). It is very surprising that an avian metapneumovirus more resembles

a human metapneumovirus than its avian counterparts. This indicates that there could be a possible evolutionary significance linked to this resemblance. However, these speculations and hypotheses need further studies and stronger evidence.

3.5.3 Genomic termini

The 3' leader region of AMPV/CO was 40 nt in length, and was comparable to the leader regions of AMPV-A (41 nt) and the HMPVs (40-41 nt). The leader region of AMPV/CO also showed higher levels of sequence similarity to the isolates of HMPV. However, the first 17 nt of the leader regions of AMPV/CO and AMPV-A were also identical. The 5' trailer region of AMPV/CO was 39 nt long and was very similar to that of AMPV-A (40 nt), but relatively shorter when compared to the HMPV trailers (96 nt in CAN97-83 and 68 nt in CAN98-75). We also observed that trailer region of AMPV/CO showed higher degree of resemblance to that of HMPV than to the trailer region of AMPV-A. This is in agreement with the observation that the other genes of AMPV/CO are also more similar to those of HMPV than to those of other AMPV subgroups. The leader and trailer regions of AMPV/CO were also highly complementary to each other; 11 of the first 13 nt and 18 of the terminal 30 nt were exact complements, indicating that these residues might probably be part of the genomic and antigenomic promoters.

3.5.4 Complete genome

Determination of the nucleotide sequence of the G gene, L gene and the genomic termini, together with the published sequences of other genes and intergenic regions, enabled us to complete the entire nucleotide sequence of AMPV/CO genome. From our studies, we

found that AMPV/CO genome was 14,150 nt long (Fig. 9) and, thus possesses the longest genome among metapneumoviruses whose complete genomic data are currently available (Govindarajan and Samal, 2005). These results contradict the recent findings that the AMPV/CO full-length genome was 13,134 nt long and was the smallest of all pneumoviruses (Lwamba et al., 2004). Comparison of our sequence data with the recent findings revealed that the AMPV/CO genome was longer due to the longer G gene. The complete genome of AMPV/CO showed a structural organization similar to that of AMPV-A and HMPV, and all the genes except the G gene were comparable in size to the corresponding genes in AMPV-A or HMPV (Randhawa et al., 1997 and Biacchesi et al., 2003). As observed in other pneumoviruses, AMPV/CO did not seem to follow the “rule of six”, since the complete genome length was not a multiple of six (Randhawa et al., 1997; Biacchesi et al., 2003 and Samal and Collins, 1996).

The putative gene-start, gene-end and intergenic sequences of AMPV/CO are presented in Fig. 10. The gene-start signal 5' GGGACAAGU 3' (mRNA-sense) was highly-conserved in all the genes of AMPV/CO except the L gene, which had a slightly different gene-start signal, 5' GGACCAAGU 3'. The consensus gene-start signal for AMPV/CO genome was 5' GGGACAAGUnAAnAUG 3', the AUG being the first initiation codon for the gene. The consensus gene-end signal for AMPV/CO was 5' UAGUUnUnAAAAA 3' (Fig. 10), very similar to that of HMPV (Biacchesi et al., 2003), and showed resemblance to that of AMPV-A (Ling et al., 1992). The intergenic regions of AMPV/CO varied in length (1-91 nt) and did not show any discernible conserved features. The SH-G intergenic region of AMPV/CO was the longest,

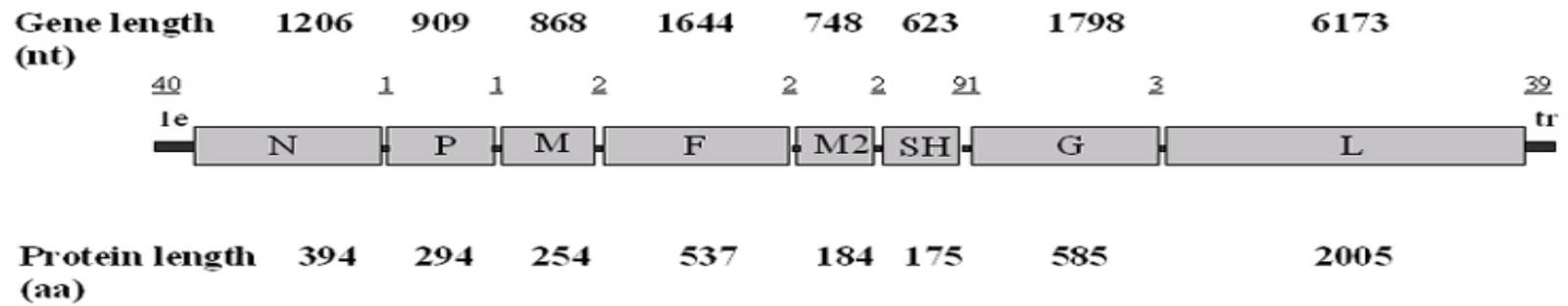


Figure 9. Structure of AMPV/CO genome. Individual genes are indicated by boxes. The gene length and protein length are indicated above and below each gene, respectively. The nucleotide lengths of the 3' leader, 5' trailer, and intergenic regions are underlined.

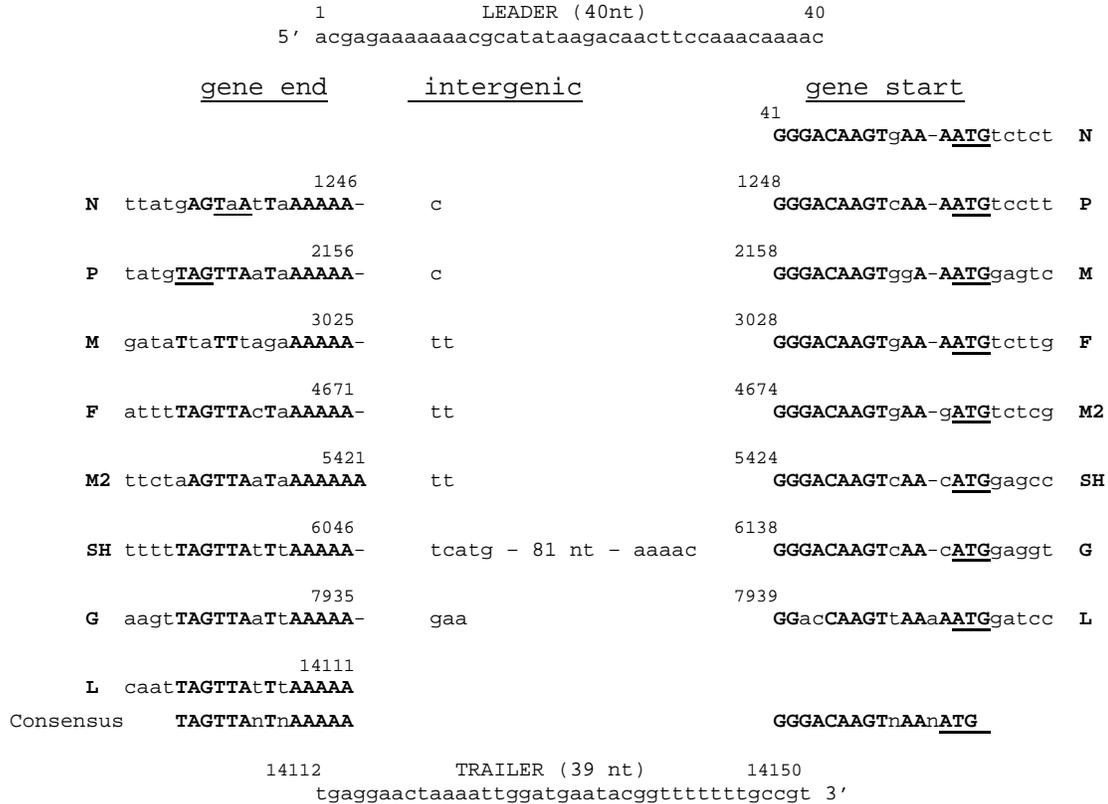


Figure 10. Complete gene map of AMPV/CO genome (in antigenome-sense). The last nucleotide of the gene-end, the first nucleotide of the gene-start, and the first and last nucleotides of the leader and trailer are numbered. Conserved sequence motifs at the gene-end and the gene-start of each gene are indicated in bold upper case, and a consensus is given below. Translational stop and start codons are underlined. Intergenic sequences are shown between the gene-end and gene-start sequences. In the SH-G intergenic region (91 nucleotides), only the first and last five nucleotides are shown, and the number of the remaining nucleotides is indicated.

comprising 91 nt, while the other intergenic regions ranged from 1 to 3 nt in length. The SH-G and the G-L intergenic regions were longer than the other intergenic regions in the Canadian isolates of HMPV (Biacchesi et al., 2003). The G-L intergenic region of AMPV-A was 69 nt long (Randhawa et al., 1996), while only 3 nt long in AMPV/CO. These observations denote that there is no discernible pattern in the nucleotide arrangement in the intergenic regions among the metapneumoviruses.

With the completion of the entire genomic sequence of AMPV/CO, we were able to compare the aa identity of each protein of AMPV/CO with the corresponding proteins of other MPVs. All the eight proteins of AMPV/CO invariably showed higher levels of aa identity with the corresponding proteins of HMPVs than with those of the AMPVs of subgroups A, B and D (Table 3). The N and M proteins of AMPV/CO showed the greatest identity (86-88%) with those of the HMPVs. The M2, F and L proteins of AMPV/CO also showed greater than 80 % aa identity with the corresponding proteins of the HMPVs. Among the membrane-associated glycoproteins, the F protein, rather than SH or G, of AMPV/CO showed greater aa identity with that of the HMPVs. These observations that AMPV/CO more closely resembles HMPV than other AMPVs are surprising and require further study with respect to virus evolution or cross-infectivity. Data on the complete genome is essential for the development of a reverse genetics system for AMPV/CO. Recovery of infectious AMPV-C from cDNA will be of great use in studying the molecular biology of this virus or other closely related human pathogens, such as HMPV.

Table 3. Percent amino acid identity between the putative ORFs of AMPV/CO and those of other metapneumoviruses

	N	P	M	F	M2	SH	G	L
AMPV-A	70	53	78	72	70	18	14	64
AMPV-B	70	52	77	71	65	13	14	- ^a
AMPV-D	- ^a	16	- ^a					
HMPV-001	88	66	86	81	85	24	23	80
CAN97-83	88	67	87	81	84	23	25	80
CAN98-75	88	67	87	80	85	20	21	80

a – sequence not available

Chapter 4: Construction of full-length cDNA clones and recovery of infectious AMPV/COs entirely from cDNA

4.1 Abstract

A full-length cDNA clone of AMPV/CO represented in 5 subgenomic cDNA fragments was assembled in a transcription plasmid based on pBR 322, between the T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme. Transfection of this plasmid into BSR-T7 cells that stably express the T7 RNA polymerase resulted in the synthesis of antigenomic AMPV/CO RNA. Simultaneous transfection of T7 promoter-driven plasmids expressing AMPV/CO N, P, M2-1 and L proteins resulted in replication and transcription of the generated antigenomic RNA. Recombinant virus was then amplified and recovered after further passage in susceptible Vero cells. The authenticity of the recovered recombinant virus was confirmed by the presence of the artificially-introduced restriction site markers. The recombinant AMPV/CO showed similar growth characteristics in tissue culture to that of the parental virus. These results demonstrate that it is possible to generate genetically engineered AMPV/CO by manipulating the viral genome at the DNA level through reverse genetic techniques. A recombinant virus rAMPV/CO-GFP expressing green fluorescent protein (GFP) as a foreign protein was also generated by similar techniques. The GFP-expressing recombinant virus grew to one-log lower titer than the wild-type virus but stably expressed GFP for at least five serial passages in Vero cells. These results indicate that using the newly established reverse genetic system AMPV/CO can be engineered to express a foreign protein and hence AMPV/CO can serve as a viral vector.

4.2 Introduction

Avian metapneumovirus (AMPV) causes an acute respiratory disease in turkeys and is associated with swollen head syndrome in chickens. The virus also affects many other species of birds. AMPV is a member of the genus *Metapneumovirus* in the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (Pringle, 1998). The genus *Metapneumovirus* contains the AMPV and its mammalian counterpart, the human metapneumovirus (HMPV). HMPV causes respiratory tract disease, especially in young children (van den Hoogen et al., 2001). Based on nucleotide sequence divergence in the attachment glycoprotein genes and their antigenic differences, AMPVs have been classified into four subgroups – AMPV-A, AMPV-B, AMPV-C and AMPV-D (Juhász and Easton, 1994; Eterradossi et al., 1995 and Băyon-Auboyer et al., 1999). The US isolates of AMPV belong to subgroup C, and exhibit genetic and antigenic differences from isolates of other subgroups (Seal, 1998; Cook et al., 1999 and Toquin et al., 2000). Interestingly, several reports have shown that AMPV-C shows a closer resemblance to HMPV than to other AMPV subgroups (van den Hoogen et al., 2002; Yunus et al., 2003; Toquin et al., 2003; Govindarajan and Samal, 2004, Govindarajan et al., 2004 and Govindarajan and Samal, 2005).

AMPV was first isolated in the US in 1996 in Colorado from commercial turkeys with rhinotracheitis (Kleven, 1997 and Cook et al., 1999). Subsequently, AMPV outbreaks were reported in Minnesota, from where it has spread to neighboring states. The disease has emerged as a major economic problem for turkey farmers, causing economic losses of approximately \$15 million annually. The mortality due to AMPV

infection and concomitant secondary bacterial infections ranges 0–30% in the US (Alvarez et al., 2003). Currently, killed or live-attenuated vaccines are being used to control this emerging pathogen (Goyal and Sheikh, 1999; Patnayak et al., 2002; Patnayak et al., 2003 and Patnayak and Goyal, 2004). However, the main disadvantage of the currently-available live attenuated AMPV vaccines is that they can themselves cause the disease leading to morbidity and mortality in vaccinated birds. Thus, development of a completely non-pathogenic AMPV vaccine would be highly beneficial to the turkey industry.

At present, there is no appropriate method to genetically engineer attenuated AMPV strains. Genetic manipulation of the virus would be needed to design an effective vaccine candidate, which is both safe and highly efficacious. Genetic manipulation of DNA viruses and positive-stranded RNA viruses has been established for quite some time. In recent years, it has been possible to genetically manipulate the genome of negative-sense RNA viruses due to the development of reverse genetic systems (reviewed in Collins and Murphy, 2002, Neumann et al., 2002 and Conzelmann, 1998).

AMPV contains a non-segmented negative-stranded RNA genome which is 14,150 nucleotides long (Govindarajan and Samal, 2005). The genome contains eight genes, which encode the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix proteins (M2-1 and M2-2), small hydrophobic protein (SH), attachment glycoprotein (G) and the large polymerase protein (L). As encountered in other non-segmented negative-sense RNA viruses, the genomic RNA of

AMPV is not infectious by itself. Other components of the ribonucleoprotein complex (N, P and L proteins) are essential to initiate virus-specific mRNA synthesis and thus the production of infectious virus. Due to recent technological advances, by co-transfecting plasmids expressing the full-length antigenomic viral RNA and the support plasmids (N, P, and L proteins), all under the control of T7 promoter, it is possible to recover infectious virus entirely from cloned cDNA. The source of the T7 RNA polymerase usually used for virus recovery is either a recombinant vaccinia virus expressing T7 RNA polymerase or a cell line that constitutively expresses the T7 RNA polymerase. Using this approach, numerous non-segmented negative-sense RNA viruses have been recovered (rabies virus, Schnell et al., 1994; vesicular stomatitis virus, Lawson et al., 1995; human respiratory syncytial virus, Collins et al., 1995; measles virus, Radecke et al., 1995; Sendai virus, Garcin et al., 1995; SV5, He et al., 1997; rinderpest virus, Baron and Barrett, 1997; parainfluenza virus, Hoffman and Banerjee, 1997; bovine respiratory syncytial virus, Buchholz et al., 1999 and Yunus et al., 2001; Newcastle disease virus, Peeters et al., 1999 and Krishnamurthy et al., 2000; human metapneumovirus, Biacchesi et al., 2004 and Herfst et al., 2004; and AMPV-A, Naylor et al., 2004).

In this chapter, we describe the recovery of AMPV-C strain Colorado (AMPV/CO) entirely from cloned cDNA using reverse genetic techniques. The recovered recombinant virus was biologically and antigenically similar to the parental wild type virus. This newly established virus rescue system will provide important applications for the development of safe and efficacious attenuated vaccine strains. In addition, the

manipulation of the AMPV genome at the DNA level will be highly useful for further studies on AMPV virulence, pathogenesis and molecular biology.

An important application of reverse genetics system is to engineer paramyxoviruses to express additional foreign proteins. Studies over the last many years have indicated the genome of paramyxoviruses to be very elastic and that they can be manipulated to stably express foreign proteins. The reverse genetics system developed by us was used to generate a recombinant AMPV/CO that stably expresses GFP as a foreign gene i.e. study the potential of AMPV/CO as a virus vector. The foreign gene selected for this study was enhanced green fluorescent protein (GFP) so that it will enable us to study both the rescue of the virus as well as its vector potential at the same time. The GFP-expressing recombinant AMPV/CO was similar to the wild type virus except that there was a slight reduction in virus titer. These results indicate that it is possible to engineer AMPV/CO to stably express foreign proteins through reverse genetic techniques.

4.3 Materials and methods

4.3.1 Cells and virus

Vero cells were maintained in MEM (Invitrogen GIBCO) supplemented with 10% fetal calf serum. Baby hamster kidney cells that constitutively express T7 RNA polymerase (BSR T7/5, Buchholz et al., 1999) were a generous gift from Dr. Karl-Klaus Conzelmann (Ludwig-Maximilians-University Munich, Munich, Germany). These cells were maintained in Glasgow MEM (Invitrogen GIBCO) supplemented with 10% fetal

calf serum, glutamine and amino acids under geneticin (1mg/ml) selection every second passage. AMPV-C strain Colorado (AMPV/CO) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA).

4.3.2 Viral RNA extraction

AMPV/CO and the recombinant AMPV described below were grown in confluent monolayers of Vero cells supplemented with 2% fetal calf serum. The virus propagation, purification and RNA extraction were performed in a manner similar to those described elsewhere (Govindarajan and Samal, 2004). Briefly, Vero cells were infected with the parental AMPV/CO or the recombinant virus and incubated at 37⁰ C for 72 h. Virus was harvested when maximum cytopathic effect was evident as extensive syncytia. The infected cells were scraped into the medium and lysed by three cycles of freezing and thawing to release the intracellular virus. The cell lysate was clarified at 3000 g for 15 min, and the supernatant was made 10% with respect to PEG 8000 (Sigma) and incubated for 3 h at 4 °C. Subsequently, the virus was pelleted at 4000 g for 30 min at 4 °C. Viral genomic RNA was extracted from the viral pellet using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, except an additional extraction with phenol plus chloroform.

4.3.3 Construction of expression plasmids

cDNA fragments bearing the ORFs of N, P, M2-1 and L genes were generated by RT-PCR. All RT reactions were performed with Superscript II reverse transcriptase (Invitrogen) and gene specific primers. The primers used in the RT-PCR reactions are

listed in Table 4. The expression plasmid pTM-1 that possesses the encephalomyocarditis virus internal ribosome entry site (IRES) downstream of the T7 RNA polymerase promoter and uses the translation initiation codon contained in the Nco I site of the IRES was used to clone the N, P, M2-1 and L ORFs. The N, P and M2-1 ORFs were cloned individually in pTM-1 between Nco I and BamH I sites. The L gene ORF was cloned between the Nco I and Xho I sites by a two-step cloning procedure using the Xma I site as the third restriction site. The Xma I site was introduced in the L gene ORF without any alteration of amino acid sequence by changing 2 nt (nt 9656 A→C and nt 9659 A→G). The N, P, M2-1 and L ORFs in pTM-1, designated as pN, pP, pM2-1 and pL respectively, were sequenced to their entirety using an ABI 3100 DNA sequencer (Applied Biosystems).

4.3.4 Construction of full-length plasmid

Restriction map analysis of the complete genome of AMPV/CO was performed to decide the strategy to be used to clone the complete genome into the plasmid pBR322/dr. Plasmid pBR322/dr was a modified form of plasmid pBR322 which contained a 72-nucleotide oligo linker between the EcoR I and Pst I sites and hepatitis delta viral 84-nucleotide antigenome ribozyme sequence and T7 RNA polymerase transcription termination signal between the Rsr II and Fse I sites (Krishnamurthy et al., 2000). Based on the restriction profile of the complete genome of AMPV/CO, we decided to clone the AMPV/CO genome into pBR322/dr using 5 cDNA fragments (Fig. 11).

Table 4. Oligonucleotide primers used for cDNA synthesis and RT-PCR of
N, P, M2-1 and L ORFs

ORF	Primers
N	+ 5' CGCGCGTCATGAGCCTTCAGGGGATTCAGCTTAG3'
	- 5' GCGCGCGGATCCTTACTCATAATCATTCTGGCCTTCCTC3'
P	+ 5' GATCTCATGAGCTTTCCTGAGGGGAAAGATATATTG3'
	- 5' CGATCTCGAGCTACATAGTAAGGGAGTATAGGTC3'
M2-1	+ 5' CGCGCGTCATGAGCCGCAAGGCTCCCTGCAAATATG3'
	- 5' GCGCGCGGATCCTTAATCAGAGTTTGCACTATCTTGCATGGCAGC3'
L	+ 5' CGCGCGCCATGGATCCACTAAATGAAGGGTTGTG3'
	- 5' GCGCGCCTCGAGTCATTTCTTACTCATCAGCATGTAACC3'

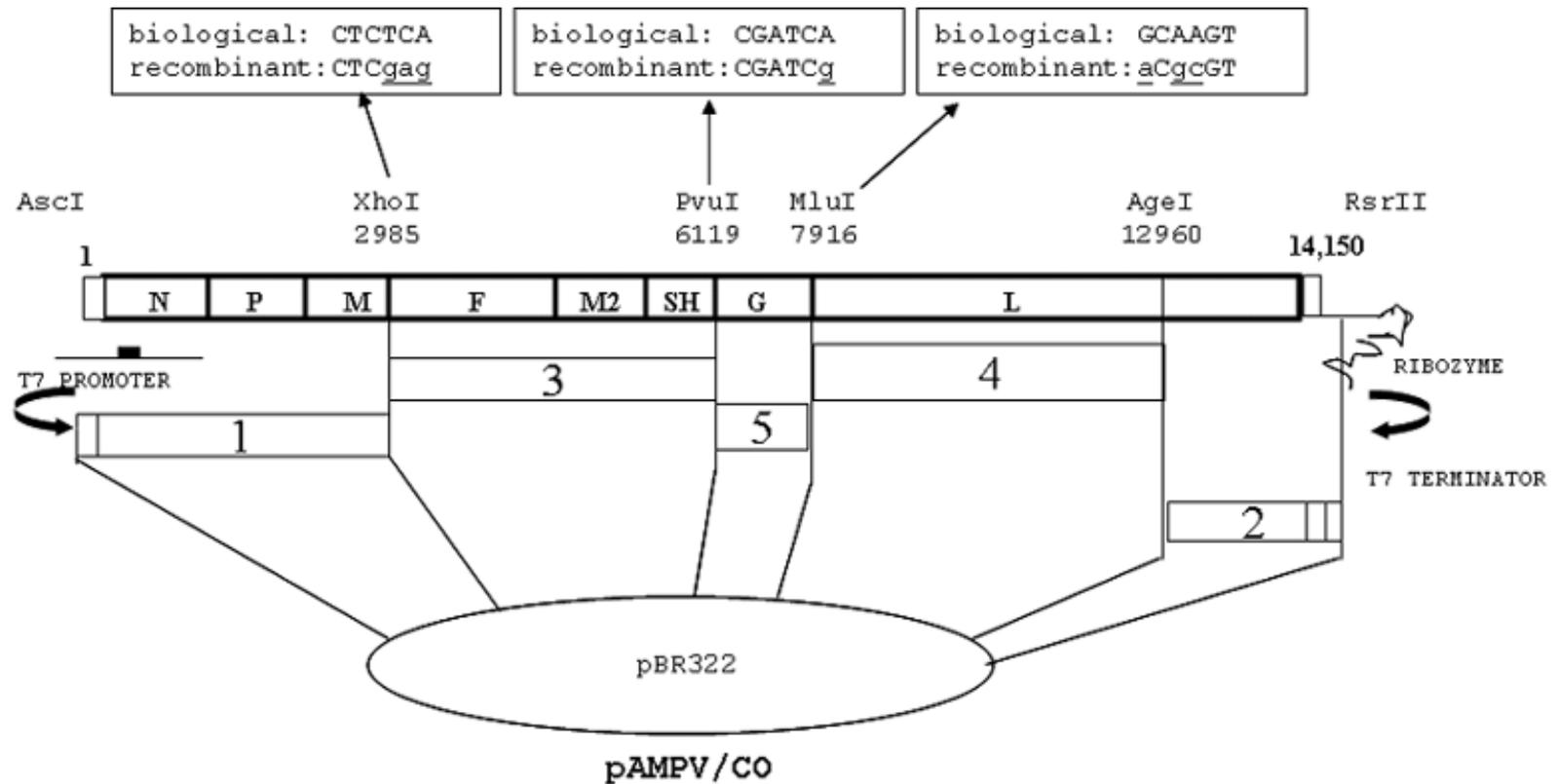


Figure 11. Generation of full-length AMPV/CO plasmid. Full-length AMPV/CO cDNA was assembled in pBR 322 from five subgenomic cDNA fragments that were generated by high-fidelity RT-PCR. The fragments were inserted in between the T7 RNA polymerase promoter sequence and the hepatitis delta ribozyme autocatalytic sequence which was followed by the T7 terminator sequence. The names of the restriction enzymes used for the assembly are shown at the top and the order in which the fragments were assembled is shown on each of them. The Xho I, Pvu I and Mlu I sites were introduced to facilitate construction and serve as markers (sequence changes shown in lower case and underlined).

All RT reactions, excepting the G gene fragments, were carried out using Superscript II reverse transcriptase (Invitrogen). The primers used for RT-PCR of each fragment are listed in Table 5. The G gene cDNA fragment bearing the complete G gene, from gene-start to gene-end, flanked by Pvu I and Mlu I sites was generated as described in section 3.3.2. Three restriction sites (Xho I, Pvu I and Mlu I) were artificially introduced during the cloning so as to help in cloning as well as to serve as markers to confirm the identity of the recovered recombinant virus. The Xho I site was introduced between the M and F genes, in the non-coding region after the M gene stop codon, by altering 3 nucleotides (nt 2988-2990). The Pvu I site was introduced in the SH-G intergenic region by altering one nucleotide (nt 6124). The Mlu I site was introduced between the G and L genes, in the non-coding region after the G gene termination codon, by altering 3 nucleotides (nt 7916, 7918 and 7919). None of the artificially introduced marker sites involved amino acid-coding sequences. The Age I restriction site (nt 12960) was a unique site already present on the genome, which was also included in the cloning process.

The five fragments were cloned in the order given in Table 5. After ligation into the plasmid, each fragment was sequenced completely using an ABI 3100 DNA sequencer (Applied Biosystems). The resulting AMPV/CO full length expression plasmid was termed pAMPV/CO. This plasmid contained three non-viral G residues adjacent to the T7 promoter, at the 5' end of the antigenome, to enhance promoter efficiency (Biacchesi et al., 2004a).

Table 5. Oligonucleotide primers used during full-length cDNA synthesis and RT-PCR

cDNA Fragments	Primers	Order of Cloning
	+ 5' <i>GTCAGGCGCGCCTAATACGACTCACTATAGGG</i> <u>ACGAGAAAAAACGCATATAAGAC</u> 3'	
I	- 5' <i>GTCAACGCGTGATCGCGATCGTAACTACTCGAGGGT</i> <u>TAAAAACGAAATTGTTACTGTG</u> 3'	1
	+ 5' <u>TAACCCTCGAGTAGTTATTACCTAGCTTGATATTAT</u> <u>TTAG</u> 3'	
II	- 5' <u>CTTCTCCGATCGTTTTTAATCATTGGATCACCTGTT</u> <u>CTCG</u> 3'	3
	+ 5' <u>AAAACGATCGGAGAAGGAAAAACGGGACAAGTCAAC</u> <u>ATGGAGGTCAAGGTAGAGAATGTTGGTAAG</u> 3'	
III	- 5' <i>ATTAACTAACGCGT</i> <u>TTCTAACTAACTCCAGCTGTA</u> <u>TG</u> 3'	5
	+ 5' <i>GCATACGCGT</i> <u>TAGTTAATTAATAAGGACCAAGT</u> <u>TAAAAATGGATCCAC</u> 3'	
IV	- 5' <u>TGCAACCGGTAGAGCTGAATACAAAATTG</u> 3'	4
	+ 5' <u>CTTACCGGTTGCAAAATAAGTGTC</u> 3'	
V	- 5' <i>AGCTCGGACCG</i> <i>cgaggaggtggagatgccatgccgA</i> <u>CCCACGGCAAAAAACCGTATTCATCCAA</u> 3'	2

The cDNA fragments correspond to the fragments in Fig. 11. T7 promoter sequences are marked in italic type, the virus-specific sequences are underlined, and restriction sites are marked in bold type. The partial HDV ribozyme sequence (24-nt) overhang is shown in lowercase. Orientation of the primer sequence is shown for sense (+) and antisense (-).

4.3.5 Construction of full-length plasmid encoding GFP

The antigenomic pAMPV/CO plasmid was modified by the insertion of a transcription cassette containing the ORF for enhanced GFP (Clontech, Inc.) (Fig. 12). The cassette was introduced as a single fragment at the Mlu I site created between the G and L genes, in the non-coding region after the G gene termination codon, by altering three nucleotides (nt 7916, 7918 and 7919). The transcription cassette contained the Mlu I recognition sequence followed by the 14-nt putative G gene-end sequence (TAGTTAATTAAAAA, positive sense, AMPV/CO nt 7922-7935), followed by a 2nt intergenic sequence (CC, positive sense), followed by the 16-nt putative N gene-start sequence (GGGACAAGTGAAA*ATG*, positive sense, AMPV/CO nt 41-56, N ORF initiation codon italicized and underlined), followed by the GFP ORF, followed by the Mlu I recognition sequence. The fragment was digested with Mlu I and cloned into pAMPV/CO resulting in the final construct pAMPV/CO-GFP (Fig. 12). The length of the encoded rAMPV/CO-GFP antigenome, excluding the non-viral sequences, would be 14,905 nt.

4.3.6 Transfection and recovery of infectious recombinant AMPV/COs

Transfection experiments were performed in BSR T7/5 cells grown to 90-95% confluency in 6-well plates (Fig. 13). The medium was changed with Glasgow MEM, without serum, at least 1 h before transfection. The cells were then transfected with 5 µg each of the full-length plasmids (pAMPV/CO and pAMPV/CO-GFP), 2 µg each of pN and pP, 1 µg each of pM2-1 and pL plasmids in a volume of 100 µl of Opti MEM per well. Transfection was carried out with Lipofectamine 2000 (Invitrogen), according to

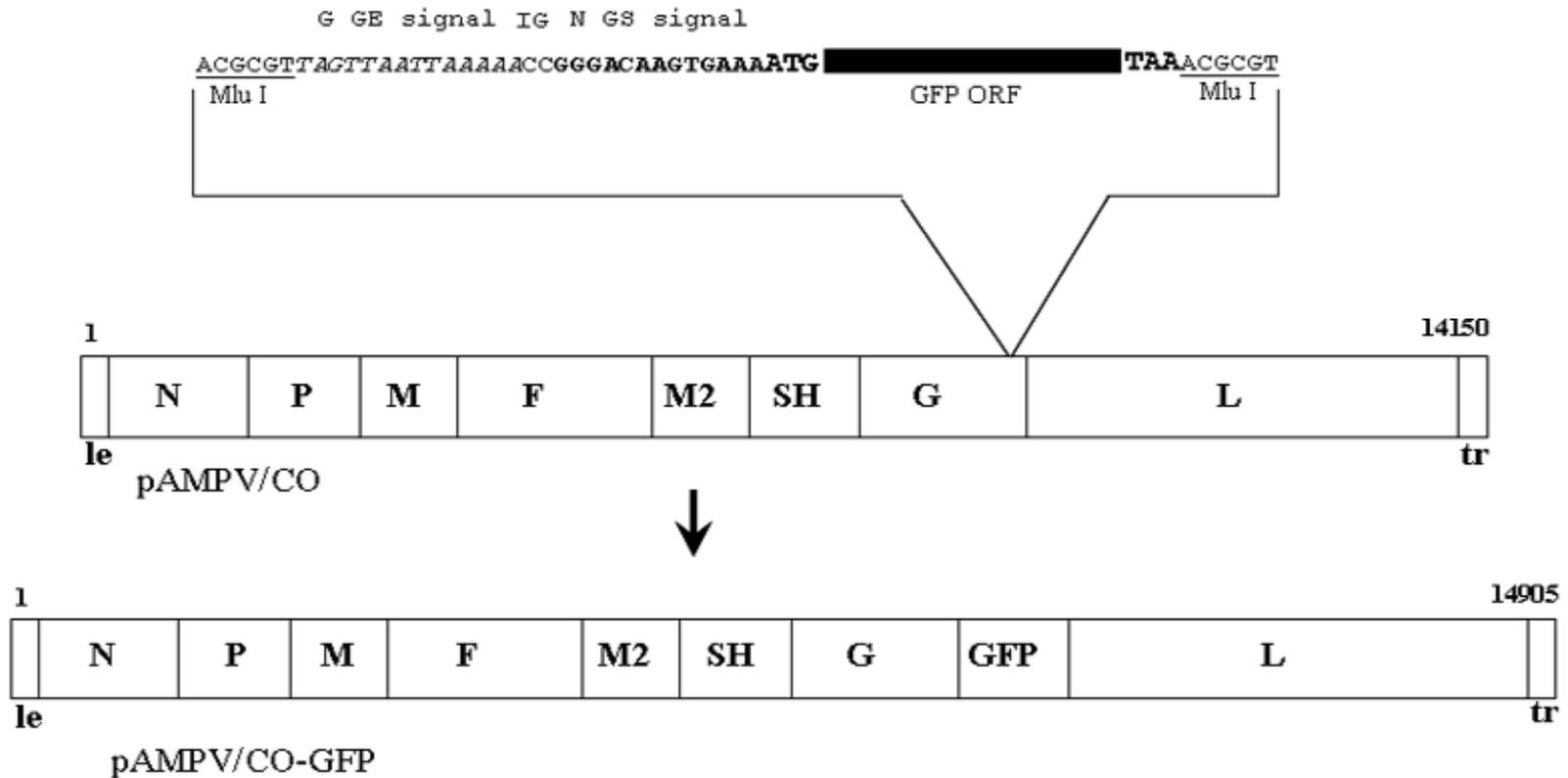


Figure 12. Construction of plasmid pAMPV/CO-GFP expressing the complete antigenomic RNA of AMPV/CO and GFP as a foreign gene. GFP was inserted in the putative 5' non-coding region of the G gene as a separate transcription cassette. The cassette contained the GFP ORF (black rectangle) that was flanked on the upstream side by a Mlu I site (underlined), the G GE motif (italicized), an intergenic region comprised of two C residues, and the N GS motif (bold). The GFP ORF was flanked on the downstream side by a Mlu I site (underlined).

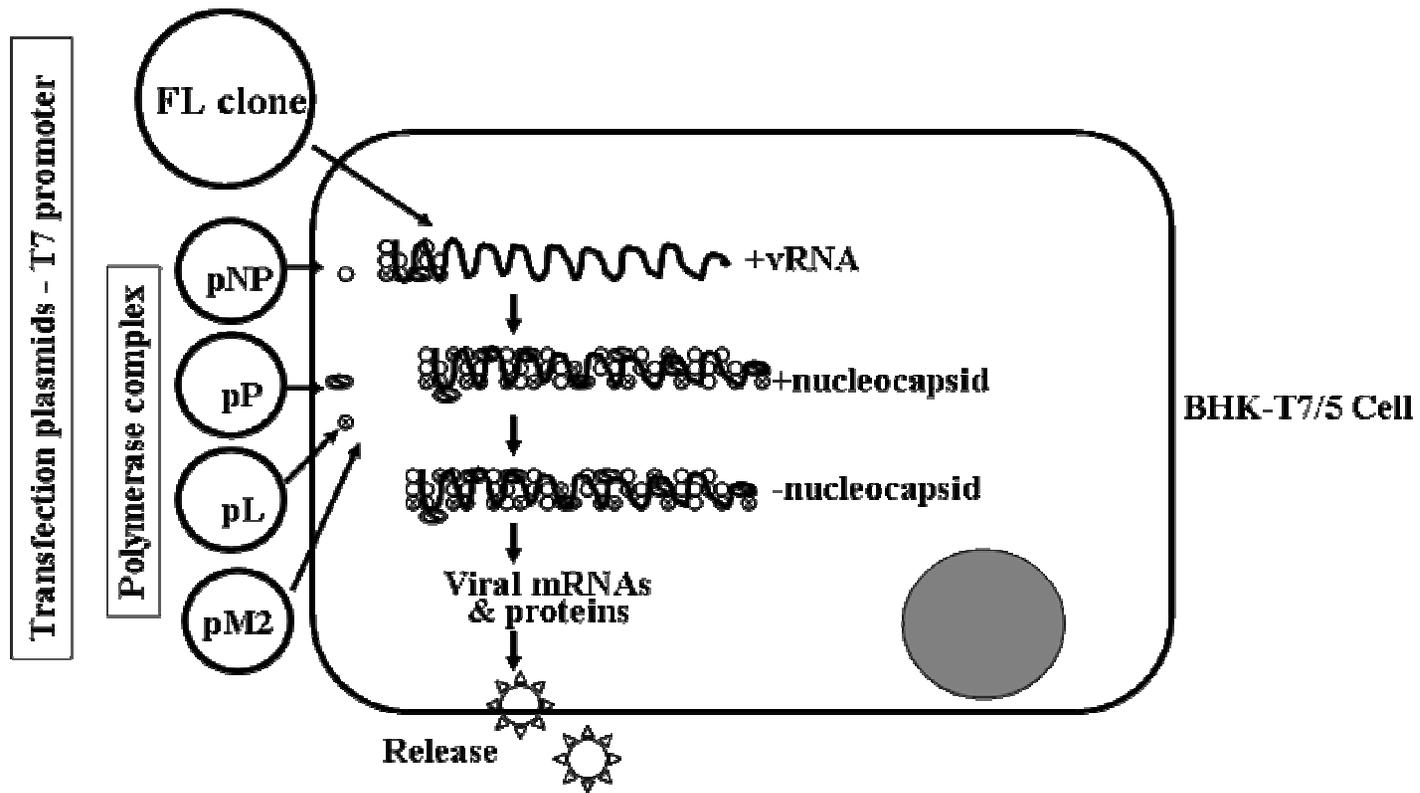


Figure 13. Plasmid-based recovery of recombinant AMPV/CO. BHK cells stably expressing T7 RNA polymerase were cotransfected with the antigenome plasmid and four expression plasmids encoding N, P, M2-1 and L proteins of AMPV/CO. This schematic represents the events that occur in the transfected cells leading to the recovery of recombinant viral particles.

the manufacturer's directions. The transfection mixture was removed after 6 h of incubation at 37⁰ C and the transfected cells were washed and maintained with Glasgow MEM containing 3% fetal bovine serum. Cells transfected with pAMPV/CO-GFP were monitored by fluorescent microscopy (Zeiss) for the expression of GFP. Three to four days after transfection, all the media was used for infecting a fresh batch of Vero cells in 6-well plates or T-25 flasks and observed for the development of virus-induced cytopathic effect (CPE) and also expression of GFP (cells transfected with the GFP plasmid). After one additional passage, the supernatant was harvested and clarified for further purification of the recombinant virus.

4.3.7 RT-PCR and demonstration of genetic markers

RT-PCR was performed on the RNA extracted from the recovered viruses to demonstrate the presence of the artificially introduced genetic markers. Briefly, the recombinant viruses were grown in Vero cells. Viral RNA was extracted using RNeasy Mini Kit (Qiagen) and RT-PCR was performed to demonstrate the presence of the Xho I, Pvu I and Mlu I markers that were artificially-introduced during the cloning process. We also performed RT-PCR to amplify the GFP gene from the recovered GFP-expressing recombinant AMPV/CO. The obtained RT-PCR products were then subjected to DNA sequencing to confirm the presence of the artificially-introduced markers. Control reactions without RT were included to show that the PCR products were derived from RNA and not the transfected plasmid DNA.

4.3.8 Virus growth

Multiple-step growth characteristics of the recombinant viruses were compared with those of the parental virus. Briefly, Vero cell monolayers grown in 6-well plates were infected with 0.01 MOI of the parental, rAMPV/CO and rAMPV/CO-GFP virus. Supernatants (0.2 ml) were collected at indicated time points post-infection and replaced by an equivalent volume of fresh medium. The collected samples were flash frozen, stored at -700 C, and titrated later in parallel by plaque assay. Each growth curve is based on the average of the virus titers from two infected monolayers.

4.3.9 Antibody staining of plaques

The recovered recombinant viruses were grown in Vero cells maintained in MEM supplemented with 2% fetal bovine serum and 0.8% methyl cellulose (Sigma). After incubation for 4 days at 37⁰ C, the overlay was removed and the monolayer was fixed with 4% paraformaldehyde solution. The cells were then permeabilized with 1:1 acetone in phosphate-buffered saline (PBS) at -20⁰ C for 2 min. The plaques were then incubated with rabbit polyclonal antisera (1:1000 dilution) raised against a polypeptide (KDNSGPIPQNQRPSS, aa 140 to 154 on N ORF) of the predicted N protein of AMPV/CO (Spring Valley Laboratories, MD, USA), followed by incubation with goat anti-rabbit IgG tagged with horseradish peroxidase (KPL, MD, USA). The plaques were visualized using light microscopy after staining with DAB substrate (Sigma).

4.3.10 Plaque assay

Plaque assays to measure the virus titers were performed on 24-well plates containing confluent Vero cells. The monolayer was incubated with 10-fold serial virus dilutions for 1h at 37⁰ C. After virus adsorption, the inoculum was removed and replaced with 1 ml of OptiMEM containing 2 % fetal bovine serum and 0.8 % methyl cellulose (Sigma) and the cells were incubated at 37⁰ C. After 4 days post infection, the methyl cellulose overlays were removed and the cells were fixed with 80 % methanol. The fixed monolayer was then blocked with 5 % (wt/vol) nonfat dry milk in PBS and the cells were incubated with rabbit polyclonal anti-peptide antibody (1:1000 dilution) raised against the AMPV/CO N protein (Spring Valley Laboratories, MD, USA). The cells were then incubated with horseradish peroxidase-labeled goat anti-rabbit antibodies (KPL, MD, USA). Viral plaques were counted following incubation with DAB substrate chromogen (Sigma) to determine virus titers. For the GFP-expressing rAMPV/CO-GFP, the number of viral foci was counted by fluorescent microscopy and was also confirmed with the number obtained following an immunostaining reaction.

4.4 Results

4.4.1 Construction of a plasmid encoding the full-length AMPV/CO genome

A cDNA clone encoding the complete 14,150-nt antigenome of AMPV-C strain Colorado was constructed by sequential cloning of individual cDNA fragments, as depicted in Fig. 11. Five overlapping cDNA fragments were generated: fragment 1 contained the putative N, P, and M genes, flanked by T7 RNA polymerase promoter at

the upstream end and an Xho I site at the downstream end. The Xho I site was created by three nucleotide substitutions in the putative non-coding region following the termination codon of the M gene and served as a genetic marker to distinguish between the recombinant and the wild-type parental virus. Fragment 2 contained the putative F, M2 and SH genes and was flanked by the Xho I site at the upstream end and a Pvu I site at the downstream end. The Pvu I site was created by one nucleotide substitution in the putative SH-G intergenic region and also served as a genetic marker to distinguish between the recombinant and the wild-type parental virus. Fragment 3 contained the putative G gene and was bordered on the upstream end by the Pvu I site and on the downstream end by a Mlu I site that was created by three nucleotide substitutions in the putative non-coding region following the termination codon of the G gene. Fragment 4 contained the upstream sequences of the putative L gene and was bordered on the upstream end by the Mlu I site and on the downstream end by a naturally occurring Age I site. Fragment 5 contained the remaining sequences of the putative L gene and the trailer sequence, flanked by the Age I site at the upstream end and part of the hepatitis delta virus ribozyme sequence ending in an Rsr II site at the downstream end. The Rsr II site is a naturally occurring restriction site in the hepatitis delta virus ribozyme. The low-copy number plasmid vector pBR322 supplied the remaining part of the hepatitis delta virus ribozyme followed by a T7 RNA polymerase terminator sequence. The full-length plasmid was named as pAMPV/CO. The resulting AMPV/CO cDNA in the plasmid pAMPV/CO was sequenced to its entirety. Sequence analysis revealed that pAMPV/CO was a faithful copy of the 14150-nt AMPV/CO genome except for the artificially introduced genetic markers at the Xho I, Pvu I and Mlu I sites.

4.4.2 Construction of full-length plasmid encoding GFP

In order to assist in the recovery and identification of the cDNA-derived virus, the full-length plasmid pAMPV/CO was modified such that it contained a transcription cassette encoding GFP at the Mlu I site created between the G and L genes in the putative 5' untranslated region. In addition to providing a means to monitor the recovery of rAMPV, this also helped in studying the potential of AMPV to serve as a viral vector. The procedure of cloning the GFP transcription cassette into the full-length plasmid pAMPV/CO is shown in Fig. 12. The foreign gene cassette consisted of appropriate viral GS and GE signals to enable proper transcription. The resulting plasmid, pAMPV/CO-GFP, was sequenced at the flanking sites of the Mlu I cloning site to confirm the insertion of the foreign cassette.

4.4.3 Recovery of infectious recombinant viruses

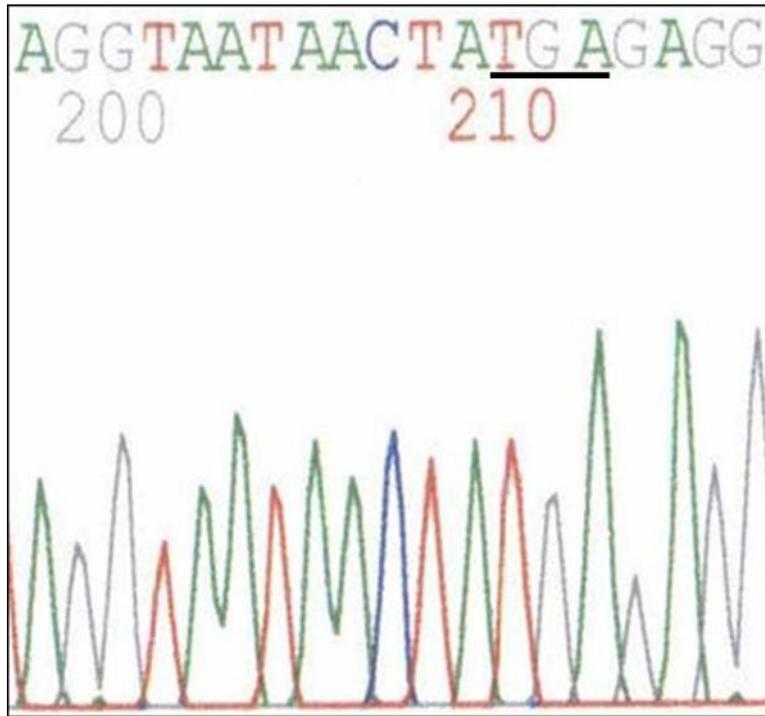
The antigenome plasmids pAMPV/CO and pAMPV/CO-GFP, along with a panel of the support plasmids encoding the N, P, M2-1 and L proteins, were transfected into BSR T7/5 cells that stably express the T7 RNA polymerase. In a parallel transfection, plasmid encoding the L protein was excluded to serve as a negative control. In the case of pAMPV/CO-GFP, the transfected cells were examined by fluorescent microscopy on successive days after transfection. Green cells were visualized by the day after transfection. Initially, they appeared as scattered isolated cells and subsequently they formed foci of more cells that later on developed into well-formed syncytia, similar to the cytopathic effect induced by the parental AMPV/CO virus. Recovery of infectious

rAMPV/CO-GFP was also possible without the inclusion of M2-1 protein in the transfection mixture.

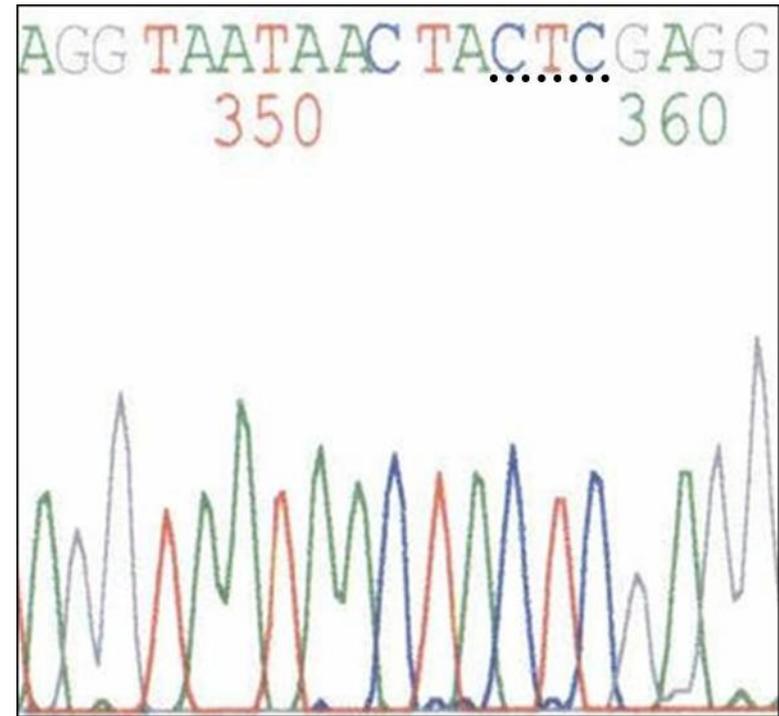
Three days after transfection, the cells were scraped into the medium, and the total suspension was transferred onto fresh Vero cells and observed for the appearance of CPE characteristic of AMPV/CO. Further passages were performed onto new Vero cells to amplify the recovered recombinant viruses (rAMPV/CO and rAMPV/CO-GFP). RT-PCR was performed on the RNA extracted from the recovered virus and the obtained products were then subjected to DNA sequencing to confirm the presence of the artificially introduced genetic tags (Figs. 14 and 15).

4.4.4 Characteristics of the recovered rAMPV/COs

The recovered rAMPV/CO and rAMPV/CO-GFP viruses were compared to its parental wild-type AMPV/CO for their growth characteristics. Multiple-step growth cycle replication of rAMPV/CO, rAMPV/CO-GFP and wild-type AMPV/CO was evaluated following inoculation of Vero cells at an MOI of 0.01 (Fig. 16). Both the kinetics and magnitude of replication of rAMPV/CO were similar to those of the parental virus. Virus titers of wild-type AMPV/CO and rAMPV/CO in the supernatants collected at 72 h post-inoculation were around $10^{6.0}$ pfu/ml (Fig.16). On the other hand, the kinetics and magnitude of replication of the GFP-expressing rAMPV/CO-GFP were slightly lower than those of the wild-type AMPV/CO as well as rAMPV/CO. Virus titer of rAMPV/CO-GFP at 72 h post-inoculation was around $10^{5.3}$ pfu/ml (Fig.16).



wt AMPV/CO



rAMPV/CO

Figure 14. Identification of genetic marker in the genome of rAMPV/CO. Primers spanning the M-F gene junction were utilized for RT-PCR and the obtained product was subjected to DNA sequence analysis. Wild type AMPV/CO was used as control. The 3 nucleotides (TGA, underlined in wt AMPV/CO) were modified during cloning to accommodate an Xho I restriction site (CTC, underlined with broken lines in rAMPV/CO) that served a genetic marker.

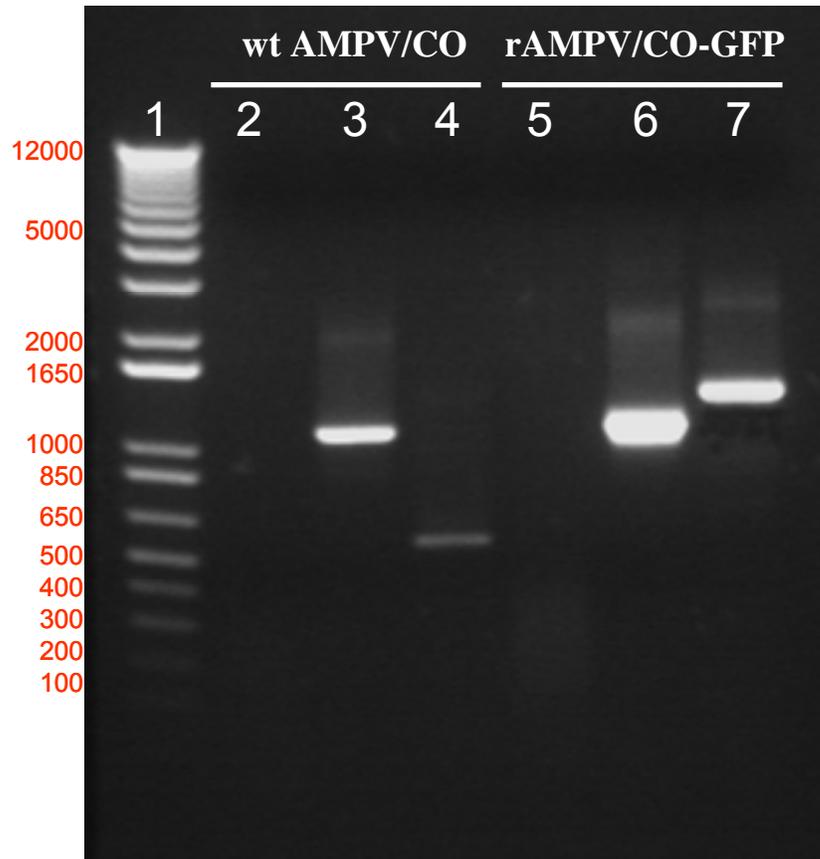


Figure 15. Confirmation of the presence of GFP gene in the genome of the recovered rAMPV/CO-GFP. Specific primers spanning the M-F gene junction (lanes 3 and 6) and the G-L gene junction (lanes 4 and 7) were utilized for RT-PCR. The larger product (1311 bp, lane 7) from rAMPV/CO-GFP confirmed the presence of GFP gene compared to the smaller product (556 bp, lane 4) from wt AMPV/CO. Lane 1 – 1 Kb Plus marker; lanes 2 and 5 – reactions without RT enzyme.

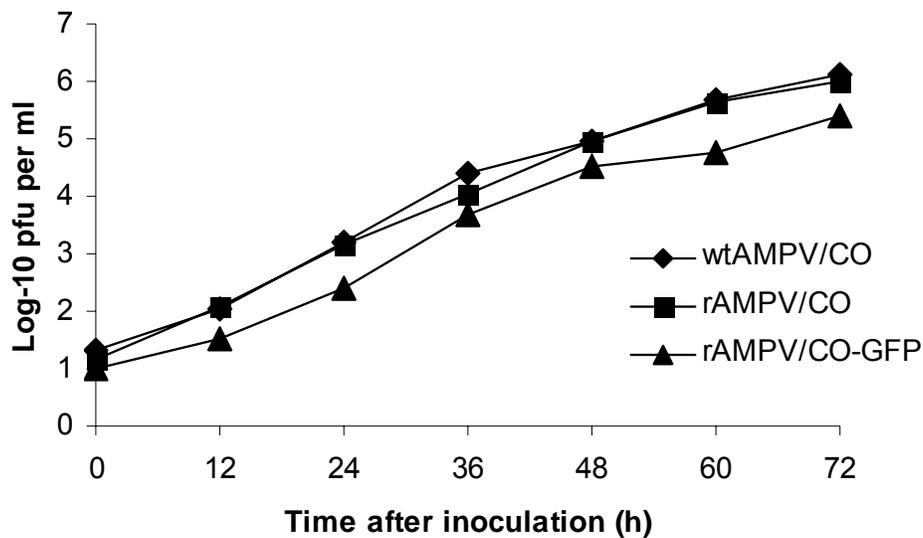


Figure 16. Replication kinetics of wild-type AMPV/CO, rAMPV/CO and rAMPV/CO-GFP. Vero cells were infected at a multiplicity of infection of 0.01 with wtAMPV/CO (◆), rAMPV/CO (■), or rAMPV/CO-GFP (▲). Supernatants (0.2 ml out of a total volume of 3 ml per well) were taken at the indicated time points post-inoculation and replaced by an equivalent amount of fresh medium. The samples were flash frozen and analyzed later for virus titers by plaque assay and immunostaining. Each time point was represented by two wells, and each titration was performed in duplicate. The mean virus titers are shown.

Both rAMPV/CO and rAMPV/CO-GFP induced CPE, consisting of large syncytia, similar to that of the wild type virus in cultured Vero cells (Fig. 17). The syncytia induced by both viruses were similar and were indistinguishable. The maximal CPE in Vero cells infected with either virus was observed after 72 h of infection. Furthermore, the recovered viruses were compared with the wild-type AMPV/CO for their antigenic characteristics in an immunoperoxidase plaque-staining reaction (Fig. 18). We observed that the plaques induced by either virus were similar in size and morphology and thus indistinguishable.

4.5 Discussion

In this study, we report the recovery of infectious AMPV-C strain Colorado (rAMPV/CO and rAMPV/CO-GFP) entirely from cloned cDNA. The complete genome data available for AMPV/CO (Govindarajan and Samal, 2005) was used to generate a reverse genetics system for this emerging turkey pathogen. A vaccinia virus-free recovery system comprising a T7-expressing cell line was used for virus recovery (Buchholz et al., 1999). In this system BSR/T7 cells that stably express T7 RNA polymerase were cotransfected with the full-length plasmid encoding the complete antigenomic RNA of AMPV/CO and plasmids expressing N, P, M2-1 and L proteins. A recombinant virus representing the full-length AMPV/CO (rAMPV/CO) was recovered using this system. This vaccinia-virus-free recovery system has also been used to recover several other non-segmented negative-stranded RNA viruses (Buchholz et al., 1999; Biacchesi et al., 2004 and Herfst et al., 2004). Alternatively, a vaccinia-based virus recovery system has also been utilized for the rescue of several viruses (rabies virus, Schnell et al., 1994; vesicular

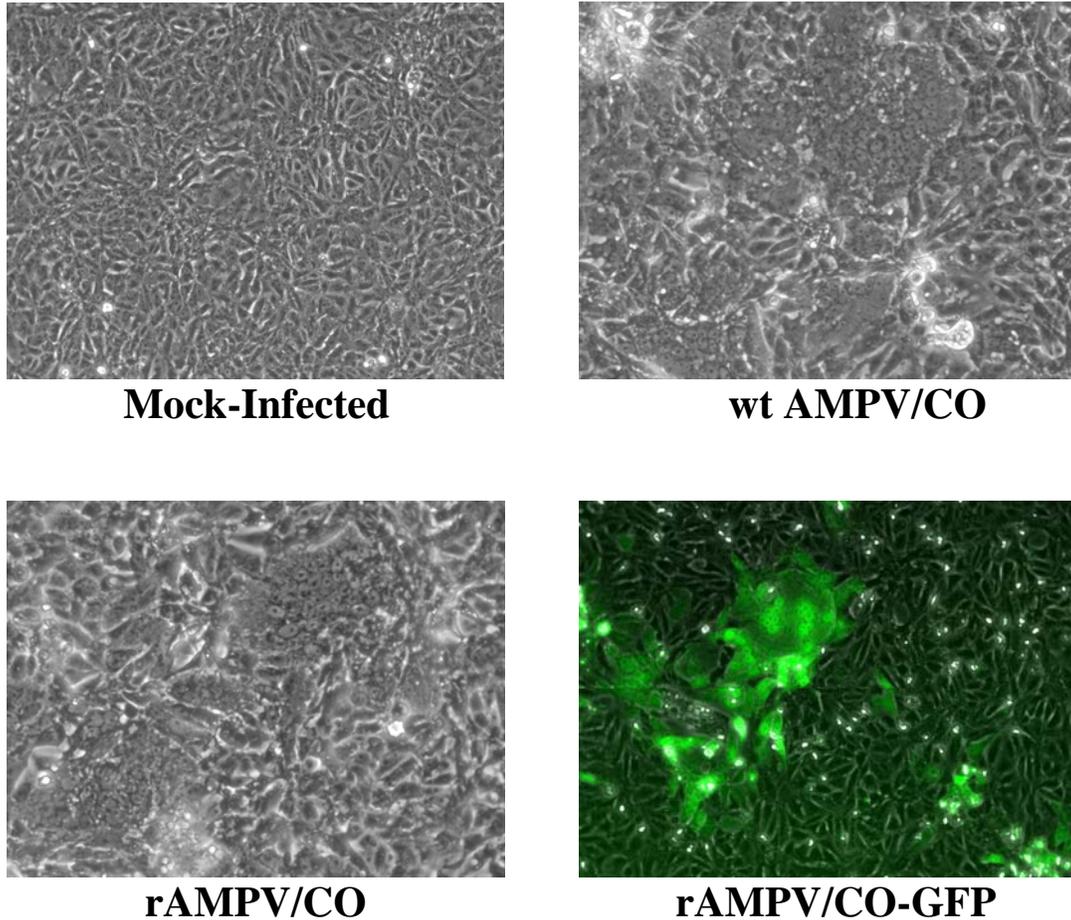


Figure 17. Cytopathic effect of recombinant AMPV/CO-GFP in Vero cells. Vero cells were either infected with wtAMPV/CO, rAMPV/CO, and rAMPV/CO-GFP or mock-infected with PBS and observed for appearance of CPE characteristic of AMPV. The rAMPV/CO and rAMPV/CO-GFP viruses induced syncytia indistinguishable from those of wild type AMPV/CO, at 72 h post infection, at an MOI of 0.1. The rAMPV/CO-GFP virus stably expressed GFP for at least five serial passages in Vero cells.

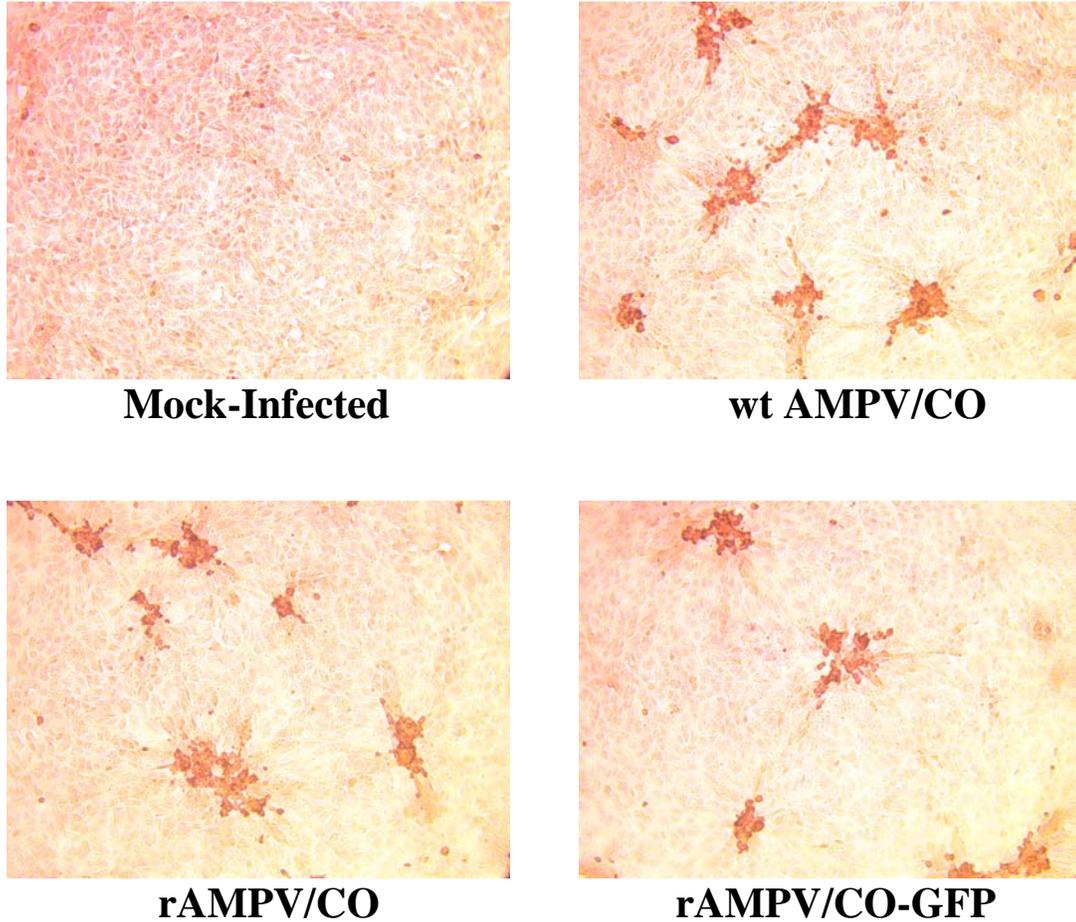


Figure 18. Immunostaining of Vero cells infected with rAMPV/COs. Vero cells were either infected with 0.1 MOI of wtAMPV/CO, rAMPV/CO, and rAMPV/CO-GFP or mock-infected with PBS and the infected monolayer was grown in 2 % MEM and 0.8 % methyl cellulose overlay. After 72 h of infection, the overlays were removed and the monolayers were subjected to immunoperoxidase staining. An antipeptide antibody raised against the N protein of AMPV/CO was utilized for the staining. The rAMPV/CO and rAMPV/CO-GFP viruses induced plaques indistinguishable, in morphology and staining characteristics, from those induced by wild type AMPV/CO.

stomatitis virus, Lawson et al., 1995; human respiratory syncytial virus, Collins et al., 1995; measles virus, Radecke et al., 1995; Sendai virus, Garcin et al., 1995; SV5, He et al., 1997; rinderpest virus, Baron and Barrett, 1997; parainfluenza virus, Hoffman and Banerjee, 1997; bovine respiratory syncytial virus, Yunus et al., 2001; Newcastle disease virus, Peeters et al., 1999 and Krishnamurthy et al., 2000; AMPV-A, Naylor et al., 2004). However, the former system is being utilized more increasingly due to its ease. Another advantage of the T7 expressing cell line is that the recombinant virus can be easily amplified without the vaccinia-virus associated problems. AMPV/CO is relatively slow growing virus compared to the vaccinia virus. Hence, in a vaccinia-virus recovery system, the vaccinia virus will outgrow the recombinant AMPV/CO. Hence, the T7 expressing cell line-based virus-rescue system is more efficient to recover AMPV/CO entirely from cloned cDNA.

The wild-type like recombinant virus rAMPV/CO, developed in this study, showed similar growth characteristics in tissue culture as those of its wild-type parental virus. The CPE induced by both wild-type AMPV/CO and rAMPV/CO in cultured Vero cells were very similar and the plaques formed by rAMPV/CO in Vero cells were also indistinguishable in size and shape from those of the parental virus. Furthermore, rAMPV/CO was also found to be antigenically similar to its wild-type parental virus, as revealed by an immunoperoxidase staining reaction of the plaques. These findings indicated that it is possible to recover a wild-type-virus-like recombinant virus entirely from cloned cDNA.

An important application of reverse genetics system is to engineer viruses to express additional foreign proteins. Studies over the last many years have indicated the genome of paramyxoviruses to be very elastic and that they can be manipulated to stably express foreign proteins to very high levels. There are several reasons to use paramyxoviruses as potential virus vectors. First, the inserted foreign genes in most cases are expressed stably even after many passages in vitro (Bukreyev et al., 1996, Mebatsion et al., 1996; He et al., 1997 and Biacchesi et al., 2004a). Second, paramyxoviruses are safe and stable vectors since they do not show any measurable rate of homologous RNA recombination (Lamb and Kolakofsky, 1996 and Palese et al., 1996). However, Spann et al. (2003) experimentally demonstrated a low rate of intermolecular recombination in RSV. But the authors suggested that such low recombination events are not a concern for vaccine stability and safety. Third, these viruses can accommodate large foreign proteins and still do not show drastic reduction in growth (Sakai et al., 1999; Haglund et al., 2000 and Huang et al., 2001 and Biacchesi et al., 2004). Finally, attenuated strains of many paramyxoviruses are available for use as safe vectors.

In the present study, the reverse genetics system developed by us was used to generate a recombinant AMPV/CO that expresses GFP (rAMPV/CO-GFP) as a foreign gene. GFP was inserted as a transcription cassette in putative 5' non-coding region of the G gene of full-length AMPV/CO cDNA. The recovered rAMPV/CO-GFP virus was similar to the parental virus in terms of its growth characteristics but yielded viral titers one-log lower than the parental virus. We also observed that rAMPV/CO stably expressed GFP for at least five serial passages in Vero cells. These results indicated that

the AMPV/CO can be genetically manipulated through reverse genetics to stably express foreign proteins to relatively higher levels and thus has a great potential to serve as a viral vector.

Attenuation following expression of foreign genes has been reported among numerous paramyxoviruses (Krishnamurthy et al., 2000 and Biacchesi et al., 2004a). However, our findings showed that the insertion of GFP as a foreign gene at the G-L gene junction of AMPV/CO genome, did not drastically affect the replication of the recombinant virus. We chose GFP as the foreign gene because of the ability to directly visualize virus recovery and spread in the transfected cells. And at the same time, it will also give an indication about the vector potential of AMPV/CO. Furthermore, the ability to express foreign proteins from artificially inserted transcription cassette also confirmed the identification and functionality of the transcription signal sequences of AMPV/CO. This rAMPV/CO-GFP virus will also be helpful in studies involving virus tropism and pathogenesis. In the future, this virus can also be effectively used for the diagnosis of AMPV through tests such as neutralization assays, as done for HMPV (Biacchesi et al., 2005). The recovered virus can also be used in various studies involving vaccine production. This recovered virus can be engineered to carry multiple foreign genes or immunogenic epitopes of other poultry pathogens and hence can be effectively used as a multivalent vaccine in poultry. In addition, through reverse genetics techniques, AMPV/CO can also be engineered to carry foreign proteins of viruses causing diseases in human and other non-avian species and their potential as a vaccine vector can further be investigated.

Although numerous paramyxoviruses have been recovered entirely from cloned cDNA over the past many years, the establishment of a reverse genetics system for the US subgroup of AMPV has considerable significance. AMPV is a recently recognized pathogen that has become a major problem for turkey farmers in the US. Ever since the initial identification of AMPV/CO in Colorado, the virus has spread to many adjoining states causing huge economic losses to the turkey industry. Since there are no effective vaccines currently available to control AMPV infections in affected birds, the development of a reverse genetics system is an important step for developing live-attenuated vaccines against AMPV. Moreover, recovery of AMPV subgroup C entirely from cloned cDNA indicates that the viral genome can be manipulated with relative ease and hence this system would prove to be an effective tool in further studying AMPV pathogenesis and molecular biology. And, given the close resemblance between AMPV/CO and HMPV, our newly developed reverse genetics system will also be helpful in various studies involving HMPV.

The ability to recover wild-type-like recombinant virus entirely from cloned cDNA denotes that the currently available nucleotide sequences of AMPV/CO are functional, at least in vitro. The recovery of a GFP-expressing recombinant AMPV/CO also indicates that the identified transcription signals of AMPV/CO are functional, even in a foreign gene context. However, the authenticity of these sequences needs to be evaluated in the natural host-system, the turkeys and chickens. Our future studies focus on evaluating these recombinant viruses in parallel with their wild-type parental virus to characterize their infectivity, replication, safety, immunogenicity, and protective efficacy.

Chapter 5: Conclusions and prospects

5.1 Conclusions

With the ultimate goal of producing a recombinant live-attenuated vaccine to control AMPV infections in the US, we have established a reverse-genetics system for AMPV subgroup C strain Colorado (AMPV/CO). Information about complete genomic sequence of AMPV/CO is however essential for genetic manipulation. Hence, in this study, prior to the development of the virus rescue system, we determined the unknown sequences (G gene, L gene, and genomic termini) of AMPV/CO. We observed that the G gene of AMPV/CO is the longest known glycoprotein gene sequence among the metapneumoviruses. In addition, we observed considerable sequence variation in the G gene sequence of US isolates of AMPV-C. The L gene and the genomic termini, however, were very similar to the other metapneumoviruses. Our findings showed that the AMPV/CO genome is 14,150 nt long and possesses the longest genome among the metapneumoviruses.

The complete genomic sequence of AMPV/CO determined by us was utilized to establish a reverse genetics system for AMPV/CO. A recombinant virus, rAMPV/CO, representing the wild-type virus and another recombinant virus, rAMPV/CO-GFP, expressing GFP as a foreign protein were recovered through standard reverse genetic techniques. Both the recovered recombinant viruses showed growth properties similar to that of the wild-type virus in tissue culture, but the titer of the GFP-expressing

rAMPV/CO was slightly reduced when compared to the other two viruses. The rAMPV/CO-GFP virus expressed GFP stably for at least five serial passages in Vero cells. These results demonstrate that it is possible to generate recombinant AMPV/CO entirely from cloned cDNA through reverse genetic techniques.

Ever since its identification in the US, AMPV-C has been a major problem for the turkey farmers. Currently, no effective vaccine is available to control AMPV infections in affected birds, thus leading to huge economic losses to the US turkey industry. Both live attenuated and killed vaccines are being used in the US to control AMPV infections in affected birds. However, as encountered in any live attenuated vaccine, AMPV vaccine may still cause disease in vaccinated birds. Moreover, the currently available live attenuated vaccines are empirically made and the molecular basis of attenuation is not known. Hence, a highly stable and efficacious vaccine becomes the ultimate objective to control AMPV infections in the US.

Genetic engineering of AMPV/CO is needed to make a safe and effective live attenuated vaccine to control AMPV infections in the US. Unfortunately, the recently developed reverse genetic system for the European subgroup of AMPV (AMPV-A, Naylor et al., 2004) cannot be used to generate a live recombinant vaccine to control AMPV infections in the US for two main reasons: (i) AMPV-A is distinct both genetically and antigenically from subgroup C and hence would not be effective against the latter and (ii) an AMPV-A based vaccine in the US would mean introduction of a new subgroup of AMPV into the US. Hence, our newly developed reverse genetics system for

AMPV subgroup C would be the only tool available to engineer recombinant live attenuated AMPV vaccine. Furthermore, the availability of the reverse genetics system will make it possible to understand the molecular biology and pathogenesis of AMPV-C and also the closely-related HMPV.

5.2 Future prospects

The newly established reverse genetics system for AMPV/CO will have numerous applications. The ability to genetically manipulate the AMPV/CO genome opens many aspects of studying AMPV biology and its pathogenesis. Some of the potential applications of this infectious virus-recovery system have been discussed in the following sections.

5.2.1 Insights into AMPV molecular biology and pathogenesis

An important application of this reverse genetics system for AMPV/CO will be to study the basic molecular biology of the virus. Though the metapneumoviruses are similar to other paramyxoviruses in many aspects, there are few important differences in them that have warranted the creation of a new genus. Moreover, they are relatively a new group of viruses that have not been characterized well in terms of their basic molecular biology. Most of their characteristics are believed to be similar to other paramyxoviruses. However, there is not enough evidence on these aspects. Hence, we believe that our newly established virus-recovery system will be helpful in studying the molecular biology of AMPVs in further detail. The role of individual viral gene can be easily studied in the context of infectious virus, as opposed to studying the gene function

in isolation. Desired mutations or deletions can be introduced in the AMPV genome with relative ease and the role of viral promoters, transcription signals, non-coding regions and the intergenic sequences can be investigated in detail. For instance, the attachment glycoprotein (G) gene of AMPV/CO has been observed to be a novel one and hence the exact role of this gene in AMPV/CO survival and pathogenesis can be studied easily with this newly developed reverse genetics system.

The G protein of AMPV/CO is believed to be the major viral attachment protein and also the major viral immunogenic protein towards which the host immune-responses are targeted (Seal et al., 2000). However, in HMPV it was observed that G protein is not essential for virus growth in tissue culture (Biacchesi et al., 2004b). Furthermore, the G-deletion recombinant virus was also able to induce neutralizing antibodies in hamsters denoting that G is dispensable for protection. These results indicate that denoting that F protein alone is sufficient for viral replication *in vivo*. We have also recovered a viable deltaG recombinant AMPV/CO using reverse genetic techniques. We have future plans of studying the growth characteristics and the pathogenicity of this deltaG recombinant virus in turkeys. We believe that the results from these studies will provide more information about the functional role of the G protein of AMPV/CO. It was recently observed in HMPV that the F protein is the major immunogenic protein and that the attachment G protein is not a major protective antigen (Skiadopoulus et al., 2005). We strongly believe that our reverse genetics system for AMPV/CO will be helpful in determining the major viral protective antigen among the three surface glycoproteins F, SH and G.

We believe that the recombinant AMPV/CO that expresses GFP as a reporter gene will be very helpful in further studies involving AMPV pathogenesis. Our future plans also include utilizing this GFP-expressing virus as a trackable virus to study virus tropism in the natural host system – the turkeys. Furthermore, it will also have important diagnostic applications. The GFP-expressing AMPV/CO can be used in assays such as virus neutralization tests for detecting AMPV infections from field samples.

5.2.2 Potential for vaccine development

There is an urgent need for a safe and effective vaccine to control AMPV infections in the US. This newly developed reverse genetics system will be a powerful tool for the generation of live-recombinant vaccine candidates. It will be possible to introduce attenuating mutations in the AMPV genome and the effect of such mutations on the pathogenicity of the virus can be easily evaluated. A promising candidate for AMPV vaccine would be a virus mutant with an altered fusion protein cleavage site. Recently, a cold-adapted AMPV has been shown to provide some protection against AMPV infections in turkeys. Another approach to generate a live-recombinant vaccine would be to import the mutations from this cold-adapted vaccine virus into the AMPV/CO genome through reverse genetic techniques and investigating the protective efficacy of the recovered recombinant virus. Similarly, various gene-deletion viral mutants can be also be generated and their potential as vaccine candidates can also be evaluated. A potential for creating chimeric viruses between AMPV and HMPV for use as vaccines in human and/or poultry can also be envisioned.

5.2.3 Studying the vector potential of AMPV

Numerous paramyxoviruses have been engineered by reverse genetic system to express foreign genes and thus serve as viral vectors. With the newly developed virus-rescue system, it will be possible to design AMPV/CO to express foreign genes. The generation of a recombinant AMPV/CO expressing GFP was a first step towards studying the vector potential of AMPV/CO. The results from our present study indicate that though there was a slight degree of attenuation in the GFP-expressing virus, it stably expressed GFP for at least five serial passages in tissue culture. We believe that the one-log reduction in the virus titer of rAMPV/CO-GFP was probably attenuation following insertion of GFP. We also expect that if the foreign genes are inserted at promoter proximal positions, as opposed to the G-L region in this case, the expression of the foreign gene will be higher and the resulting virus will be less attenuated. We have future plans to study the optimal position on the AMPV/CO genome to insert foreign genes for high-level expression of foreign proteins without much attenuation of the virus. In future, immunogenic proteins from other poultry, non-avian or human viruses can be inserted in the AMPV/CO genome and study the ability of AMPV to serve as a vaccine vector.

5.2.4 Insights into HMPV molecular biology and pathogenesis

Given the close resemblance between AMPV/CO and HMPV, it can be expected that this newly established reverse genetic system for AMPV/CO can also be used for studying the molecular biology and pathogenesis of HMPV. AMPV/CO and HMPV are very similar in their genome organization and exhibit similar growth properties in tissue culture. They also produce similar disease symptoms in the affected subjects. Hence, we

believe that both these viruses will also possess similar in vivo growth properties. Though various animal model systems have been evaluated for studying HMPV pathogenesis, an ideal animal model has not yet been found. Moreover, such systems do not mimic the natural host-system of HMPV. Non-human primates have been used in some HMPV pathogenesis trials. However, such experiments cannot be expected to be cost effective in a long run. Hence, AMPV/CO will prove to be an excellent and appropriate model system for studying HMPV molecular biology and pathogenesis, mainly due to the ease of studying the former in its natural host system. And, the newly developed reverse genetics system will be very helpful to achieve this.

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