

## Rescue of Synthetic Minireplicons Establishes the Absence of the NS1 and NS2 Genes from Avian Pneumovirus

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**We have determined the nucleotide sequences of the regions 3' and 5' proximal to the avian pneumovirus (APV) N and L genes, respectively. These sequences were used in the construction of a synthetic minireplicon construct in which the chloramphenicol acetyltransferase (CAT) reporter gene was flanked at its 3' end with the APV leader together with the APV N gene start signal and at its 5' end with the APV L gene end signal and the genome trailer region. The ability of T7 RNA polymerase runoff transcripts to direct the replication and expression of the CAT reporter gene in APV-infected cells demonstrated the ability of the putative leader and trailer regions to direct genome replication and gene expression. Furthermore, this confirms the absence of the NS1 and NS2 gene analogs within the APV genome. We were able to detect the expression of CAT protein from cells that had been infected with supernatants from the initially infected and transfected cells. These results have identified the *cis*-acting sequences of APV responsible for viral replication, gene expression, and packaging into virus-like particles.**

Avian pneumovirus (APV) is currently classified along with respiratory syncytial virus (RSV) and pneumonia virus of mice (PVM) within the subfamily *Pneumovirinae* of the family *Paramyxoviridae*. This classification is based on the morphology of the APV virion (2, 6), the absence of hemagglutination, the electrophoretic mobility of virus-specific proteins, the number of mRNA species (4, 7, 19) and the homology of the APV genes with those from human RSV (HRSV) and PVM. Like other paramyxoviruses, APV consists of a negative-sense single-stranded RNA genome (genomic virion RNA [vRNA]) complexed to the nucleoprotein (N), phosphoprotein (P), and RNA-dependent RNA polymerase (L) proteins to form the helical ribonucleoprotein core. While the APV proteins show greater homology with those of the other members of the pneumovirus genus, nucleotide sequencing of eight genes (18, 20, 21, 26, 27, 31–33) has shown that the partial gene order of the APV genome (3' N-P-M-F-M2-SH-G-L 5') is more similar to that of the rubulaviruses than to that of the other pneumovirus members (3' NS1-NS2-N-P-M-SH-G-F-M2-L 5'). Like the other pneumoviruses, the APV genome also contains the M2 (or 22K) gene. The M2 genes of all pneumoviruses potentially code for two overlapping open reading frames (ORFs) (9, 20, 33; our unpublished observations). The first M2 ORF of HRSV has been shown to encode a transcription elongation factor, whereas the second M2 ORF encodes a polypeptide that is an inhibitor of RNA synthesis (11). To date no genes or candidate proteins equivalent to those of NS1 and NS2 in HRSV have been described for APV.

On the basis of the differences in genome organization APV has recently been assigned to a new, as-yet-unnamed genus within the subfamily *Pneumovirinae* (25). In view of the differences between the mammalian pneumoviruses and APV, it is of interest to investigate the molecular biology of this virus system to establish the limits of the differences and to deter-

mine the molecular details of replication and transcription of the APV genome.

**Nucleotide sequence of the APV leader region.** vRNA of APV strain CVL14/1 was prepared from crude nucleocapsids isolated from infected BSC-1 cells as described previously (26). The sequences of both the leader and trailer regions were determined indirectly, as attempts to determine these sequences by direct RNA analysis proved unsuccessful due to the low yield of vRNA in APV-infected cells. A homopolymer A tail was added to the 3' end of the vRNA by using poly(A) polymerase (Gibco-BRL Life Technologies Ltd, Paisley, United Kingdom) in 50 mM Tris-HCl (pH 8.0)–250 mM NaCl–10 mM MgCl<sub>2</sub>–0.25 mM ATP–2.5 mM MnCl<sub>2</sub> for 15 min at 37°C. First-strand cDNA synthesis was primed by incubation of the polyadenylated vRNA at 42°C for 30 min in the presence of oligonucleotide Oli-T (GGCCCGGAAGCTTTTTTTTTT TTTTTT) and Superscript II reverse transcriptase (Gibco-BRL Life Technologies Ltd) and used in a PCR with Oli-T and an APV N-gene-specific primer, TN3a (TGCCTACCTCTGC TGCAGGCTCA), which anneals to the mRNA sense N gene at positions 189 to 212 (18). Amplification was achieved with *Taq* DNA polymerase (Gibco-BRL Life Technologies Ltd), with 40 cycles each of denaturation at 95°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 2 min. PCR-amplified products were sequenced directly. A PCR fragment approximately 300 bp in size was observed, suggesting that no extra gene exists between that encoding the N protein and 3' end of the genome. Direct sequence analysis of the PCR product gave the nucleotide sequence shown in Fig. 1. Attempts to determine the exact terminal residue of the leader region by ligation of an oligonucleotide linker to the 3' vRNA end proved unsuccessful, and we were unable to determine whether the terminal 3' nucleotide was a U or G residue. However, since all the published leader sequences of paramyxoviruses (except mumps virus, for which the terminal residue has not been determined) possess a terminal U residue, it is likely that the leader of APV also begins with a terminal U residue. A comparison of the APV leader sequence with those of other paramyxoviruses shows a higher degree of homology with the leader sequence of HRSV than with any

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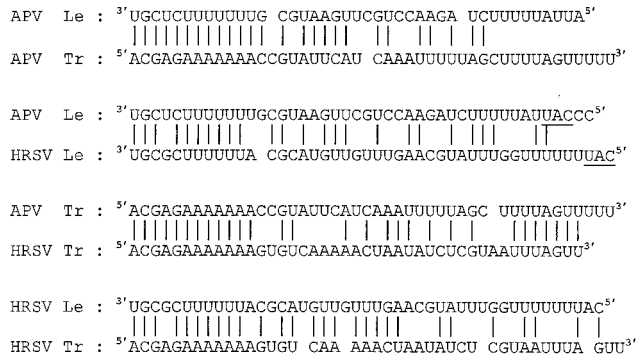


FIG. 1. Nucleotide sequence of the 3' leader (Le) and 5' trailer (Tr) regions of the APV genome. The APV sequences are aligned with the leader and trailer sequences of the A2 strain of HRSV (26). Underlined regions represent the conserved AUG motif located at the end of the leader sequences of both APV and HRSV. The G residue is part of the gene start signal of the first gene to be transcribed during virus infection.

other. The length (41 bases) of the APV leader sequence is similar to that of HRSV (44 bases), and the sequences have an overall nucleotide identity of 58%, with only one base difference between the terminal 11 bases of the leader sequences (Fig. 1). This one residue difference (position 4) is that for which mutational analysis of the HRSV leader sequence showed that replacing the G residue with either C or U (as in APV) produced a fourfold increase in expression from a chloramphenicol acetyltransferase (CAT) reporter gene contained in a minireplicon cDNA construct (12).

Interestingly, both the APV and HRSV leader sequences contain an AUG (anti-genome sense) (Fig. 1) motif in which the G residue is part of the gene start signal of the first gene to be transcribed during virus infection. For the other paramyxoviruses (measles virus, Sendai virus, parainfluenza virus type 3, bovine parainfluenza virus type 3, and parainfluenza virus type 2), the start signal of the first gene to be transcribed is preceded by a conserved UAG motif. It is not known whether these initiation or termination codons play a role in the replication and/or transcription of these viruses.

**Nucleotide sequence of the APV trailer region.** The sequence of the trailer region of the APV genome was determined by homopolymer dG tailing of first-strand cDNA synthesized from vRNA by using oligonucleotide APVL27 (<sup>5719</sup>C ACTTAGTGAGCCTGTATGC<sup>5738</sup>), which anneals to the L gene, and Superscript II reverse transcriptase. The cDNA product was treated with RNase H for 20 min at 37°C and purified on a Glassmax column (Gibco-BRL Life Technologies Ltd). A homopolymer dG tail was added to the 3' end of the cDNA by using terminal deoxynucleotidyl transferase (Gibco-BRL Life Technologies Ltd) under standard conditions for 10 min at 37°C. Amplification of the sequence 5' of the APV L gene (vRNA sense) was achieved through the use of oligonucleotides APVL27 and C-Tail (AGATCTAGAAGCTTGGATCCCCCCCCC) and *Taq* DNA polymerase, with 35 cycles each of denaturation at 95°C for 15 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. PCR-amplified products were sequenced directly. A PCR product of 430 bp was observed, and sequence analysis showed that the region outside the previously determined L gene, equivalent to the APV trailer region, is 40 bases in length and shows a high degree of complementarity to the APV leader sequence (Fig. 1). This complementarity is commonly seen with other negative-strand viruses and was used in the determination of the sequence of

the APV L gene, where a leader-specific primer that annealed to the trailer region was used in amplification of the L gene (26). Although the leader regions of APV and HRSV are similar in length, the trailer region of APV is only 40 bases long whereas that of HRSV is 155 bases long. Furthermore, a comparison of the trailer sequences of APV and HRSV showed that the terminal 12 bases matched exactly (Fig. 1). These results, in conjunction with the finding that a CAT-based HRSV minireplicon in which only the terminal 40 bases of the 155-base-long HRSV trailer region were used was successfully rescued (10, 22), suggest that the *cis*-acting elements required for viral replication may be contained within this short terminal region of the trailer sequences of both viruses.

**Construction of a synthetic minireplicon containing the CAT reporter gene.** While the determination of the sequence of the region 3' of the APV N gene shows a high degree of homology with the leader sequence of HRSV, the apparent absence of equivalents of the mammalian pneumovirus NS1 and NS2 genes left open the possibility that we failed to amplify all of the RNA at this end of the genome and that the sequence was incomplete. In order to demonstrate that the sequence located 3' of the APV N gene was indeed the viral leader sequence and thus confirm the absence of the NS1 and NS2 genes, the putative leader sequence was incorporated into a minireplicon construct similar to the synthetic minireplicon of measles virus described by Sidhu et al. (29) together with the trailer sequence in such a way that expression of the CAT reporter gene would occur only if the defective interfering-like element could carry out replication and transcriptional events in the presence of *trans*-activating proteins provided by a helper virus.

A minireplicon in which the CAT reporter gene was flanked at its 5' end by the APV leader and N-gene start sequences and at its 3' end by the APV L gene end and trailer sequences was constructed. A summary of the process is shown in Fig. 2. Oligonucleotides Hga2leader (GGTACCGGATCCGACGCTATAAACGAGAAAAAACGCATTCA), which anneals to the APV leader sequence, and APVNCat (CCCGGAAGC TTCATATGGACTTGTCCTCATTATTTTCTAG) were used to amplify the APV leader sequence and the APV N-gene start sequence from Hga2leader-primed cDNA, using *Taq* DNA polymerase with 40 cycles each of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min. Hga2leader and APVNCat contain restriction endonuclease sites for *Hga*I and *Nde*I, respectively (underlined). The PCR product (Leader-GS) was digested with *Nde*I and purified. The CAT reporter gene was amplified with oligonucleotides 5'CAT CCCGGGAACGTTTCATATGGAGAAAAAA TCACTGGATA) and 3'CAT (GGCCTGCAGTCTAGAAA TTACTCTTACGCCCGCCTGCCACTCATCGC) by using *Taq* DNA polymerase. The amplified CAT reporter gene was digested with *Nde*I (site is underlined) and ligated to the *Nde*I-digested Leader-GS PCR product. An aliquot of the ligation product was used in PCR with oligonucleotides Hga2leader and 3'CAT as described above to generate a PCR product, Leader-Cat. The region of the L gene containing the termination codon, the 72-base noncoding region, and the gene end signal together with the APV trailer region was amplified in a separate PCR with oligonucleotides APVL-trailer (GTGCACTGCAGTAATAAAGCAATATCAACTC) and Trailer-T7 (CCCGGTACCAAGCTTAATACGACTC ACTATAGGACGAGAAAAAACCGTATTTCAT) with APVL27-primed cDNA as the template. APVL-trailer anneals to the genomic vRNA within the APV L gene at the termination codon and contains a restriction site for *Pst*I (underlined). Trailer-T7 binds to the genomic trailer sequence and contains

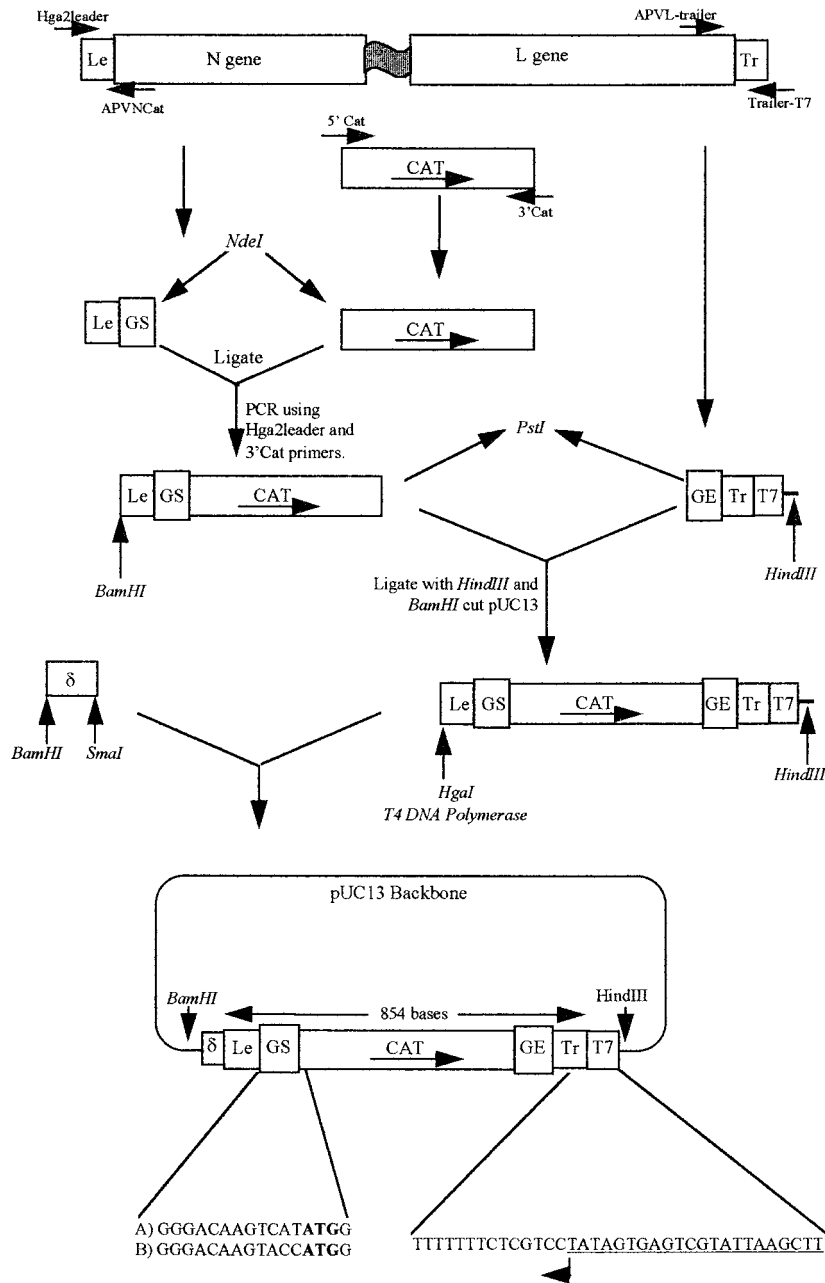


FIG. 2. Diagrammatic representation of the construction of the APV minireplicons containing the CAT reporter gene. The figure shows the strategy, as described in the text, used to generate a minireplicon containing the proposed leader (Le) and trailer (Tr) regions of the APV genome inserted into a plasmid containing the T7 RNA polymerase promoter (T7) and the hepatitis delta virus ribozyme ( $\delta$ ). GS and GE represent the start and end signals of the APV N and L genes, respectively. Sequence A is minireplicon 1, in which the AUG initiation codon of the CAT gene is in an unfavorable context for mammalian ribosomal translation. Sequence B is the optimized context of the CAT initiation codon for mammalian translation in minireplicon 2.

a restriction endonuclease site for *HindIII* (underlined) and a copy of the T7 DNA-dependent RNA polymerase promoter sequence. The PCR product was subsequently treated with *HindIII* and *PstI* and used in a three-way ligation reaction with *BamHI*- and *HindIII*-digested pUC13 and *PstI*- and *BamHI*-digested Leader-Cat PCR product. A clone containing the minireplicon was treated with *HgaI*, then with T4 DNA polymerase, and finally with *HindIII*. This clone was ligated in a three-way reaction with *HindIII*- and *BamHI*-digested pUC13 and *BamHI*- and *SmaI*-digested hepatitis delta virus ribozyme

sequence (24). Thus, in the final construct (minireplicon 1) a ribozyme sequence was introduced 3' of the leader sequence (vRNA sense). This would then result in the self-cleavage of the ribozyme sequence to generate the correct 3' terminus of the leader sequence.

While the 5' terminus of the synthetic vRNA contains an additional two non-virally encoded residues, GG (data not shown), the presence of these extra 5'-terminal bases has been shown not to interfere in rescue of synthetic viral genome analogs for other paramyxoviruses, though it may reduce the

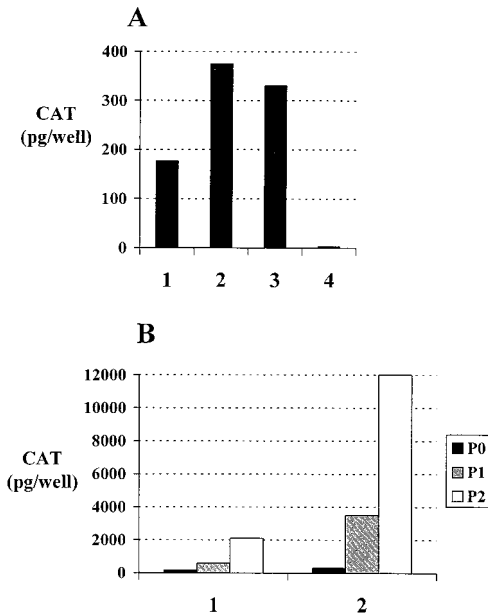


FIG. 3. Rescue of the APV minireplicon constructs in virus-infected cells. Each chart shows the level of expression of the CAT reporter gene determined in an ELISA system. (A) Lane 1, APV minireplicon 1 in APV-infected cells; lane 2, APV minireplicon 2 with optimized AUG context in APV-infected cells; lane 3, HRSV strain RSS-2 minireplicon in HRSV-infected cells; lane 4, APV minireplicon construct 2 in HRSV-infected cells. (B) Passage of APV minireplicons. Lane 1, minireplicon 1 in APV-infected cells; lane 2, minireplicon 2 with optimized AUG context in APV-infected cells. Values are shown for the primary rescue (P0), first passage (P1), and second passage (P2).

efficiency of replication (15, 28). The APV minireplicon was linearized with *Bam*HI to ensure termination of transcription. An 854-nucleotide RNA runoff transcript was synthesized in vitro with T7 RNA polymerase (Gibco-BRL Life Technologies Ltd) for 1 h at 37°C, treated with RNase-free DNase I (RQ1; Promega) for 20 min at 37°C, and purified with the RNeasy total RNA kit (Qiagen). For the transfection procedure, a single well of a 12-well plate containing subconfluent 293 cells was infected with APV at a multiplicity of infection of 1 for 1.5 h at 37°C and then incubated for 4 h at 37°C with 2  $\mu$ g of RNA mixed with 4  $\mu$ l of LipofectACE (Gibco-BRL Life Technologies Ltd) in 0.5 ml of Optimum medium (Gibco-BRL). The transfected cells were supplemented with fetal bovine serum to a final concentration of 2.5% and incubated for 2 days at 37°C. Following cell lysis, CAT reporter protein was quantified by using a CAT enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim) by comparison with purified CAT protein standards (Fig. 3). For comparison, the rescue of a synthetic minireplicon based on the sequence of strain RSS-2 of HRSV in 293 cells is also shown.

After 48 h of incubation of the transfected cells, we observed mean CAT protein concentrations of 177 pg/well (Fig. 3) for the minireplicon 1 construct. The detection of CAT protein expressed from a cDNA construct containing viral leader, trailer, and gene start and end signals as well as a negative-sense copy of the CAT reporter gene from virus-infected cells has recently been documented for several paramyxoviruses (1, 10, 13, 14, 23, 29). The production of CAT protein indicates the ability of the constructs to undergo at least one cycle of replication and transcription, since primary transcripts of the cDNA constructs, synthesized in vitro, contain an antisense copy of the CAT gene and therefore cannot be directly translated. A consequence of the cloning procedure for the APV

minireplicon was that the initiation codon for the CAT reporter gene was in a less-than-favorable context for ribosomal initiation (16). In an effort to increase CAT gene expression from our minireplicon, the nucleotide sequence context around the CAT initiation codon was altered to a more optimal one that has been determined for eukaryotic ribosomal translational initiation (16). Oligonucleotides miniup-1 (GGG ACAAGTCACCATGGAGAAAA) and miniup-2 (TTTTT CTCCATGGTGACTTGTCCC) were used to create an *Nco*I site around the CAT reporter gene start codon with an A residue located at position -3. All PCRs were performed with *Taq* DNA polymerase, with 35 cycles each of denaturation at 95°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min. These oligonucleotides were used in separate PCRs with the minireplicon 1 plasmid construct (described above) paired with the reverse and universal cycle sequencing primers (which prime within the plasmid sequence). Aliquots of the products from these reactions were subsequently used in a single PCR with just the universal and reverse oligonucleotides. The product (minireplicon 2) from this reaction was cloned and sequenced in its entirety to confirm the alteration of the context for ribosomal translation as well as to check for any possible errors that might have arisen during the PCRs. This construct (minireplicon 2) showed a twofold increase (392 pg/well) in CAT protein over the original APV minireplicon (Fig. 3). Incubation of fresh 293 cells with cleared medium taken from cells transfected with either minireplicon 1 or minireplicon 2 resulted in the detection of CAT protein after incubation at 37°C for 2 to 3 days. Subsequent passage of cleared medium from these infected cells showed a further increase in CAT protein without the addition of helper virus, indicating the replication and subsequent transcription of the primary transcripts. Up to a 40-fold increase in CAT protein concentration was observed after the second passage over the initial transfections (Fig. 3). CAT protein expression was observed as late as the fourth passage of clarified supernatants. It has yet to be determined at which point the minireplicon ceases to be replicated. These results demonstrate that both minireplicon RNAs can be not only replicated and transcribed but also packaged into infectious virus-like particles. It is not known whether the minireplicon RNA is packaged into separate particles or whether it is enveloped along with the helper virus. Similarly, it is not known whether the extra GG dinucleotide is retained within the passed minireplicons.

Since the leader and trailer sequences of APV and HRSV show a high degree of conservation we attempted to rescue the minireplicon 2 construct from cells infected with heterologous virus. For each heterologous transfection, the amount of CAT protein never exceeded 3% of the amount observed with homologous transfections (Fig. 3). Presumably, even though the leader and trailer regions of APV and HRSV are similar, differences in the gene start and end signals may prove crucial during viral transcription. Mutation of either the gene start or end signal from an HRSV-based CAT-containing minireplicon led to a dramatic decrease in the expression of the reporter gene. In a dicistronic construct, altering the gene end signal of the first gene or the gene start signal of the second gene resulted in a decrease or abolition of expression from the second gene (17). Stillman and Whitt (29a) reported the first three bases of the conserved vesicular stomatitis virus (VSV) gene start sequences to be critical in the synthesis of transcripts from a dicistronic VSV minigenome analog.

Pneumoviruses are distinguished from other paramyxoviruses by a number of criteria. Morphologically, the virion particles exhibit several differences in the nature of the nucleocapsid (2). In addition, there are several differences at the

molecular level in terms of genome organization: pneumovirus genes are smaller than their functional counterparts in other viruses, and the genomes of RSV and PVM contain three genes which do not have equivalents in other viruses. The "extra" pneumovirus genes include the M2 gene and those encoding the NS1 and NS2 proteins, though there is no sequence conservation between the RSV and PVM NS1 and NS2 genes or the predicted protein products (5).

The data presented here describes the sequence of the elements present 3' and 5' of the APV N and L genes, respectively. Their similarity to the leader and trailer regions of HRSV suggested that genes equivalent to those encoding the NS1 and NS2 proteins that are present in other pneumoviruses were absent from the 3' and 5' ends of the APV genome. Together with the previously published sequences for eight contiguous APV genes, this strongly suggests that such genes are absent entirely from the APV genome. The ability of synthetic constructs containing these potential leader and trailer sequences to direct replication and transcription of this genome in the presence of helper virus confirms the role of these elements in viral replication and transcription. Since such elements are always located at the immediate termini of the virus genome this also confirms the absence of the NS1 and NS2 genes. This further opens the question as to the function of the mammalian pneumovirus NS1 and NS2 gene products. While the function of the NS1 and NS2 gene products has not been established, they may be analogous with the nonstructural V and C proteins of other paramyxoviruses and thus play a role in the regulation of viral gene expression. The absence of these genes in APV and the lack of conservation of the NS1 and NS2 genes of RSVs and PVM may represent differences in the replication of a pneumovirus in their respective hosts.

This report of the leader and trailer sequences, together with the sequences of the virus genes (18, 20, 21, 26, 27, 31–33) and intergenic regions (unpublished data), completes the nucleotide sequence of APV strain CVL14/1. Like the intergenic regions of HRSV, those of APV are of variable length and have no discernible conserved features (8). This means that the APV strain CVL14/1 genome is 13,373 nucleotides in length and is 1,849 nucleotides shorter than that of HRSV strain A2 (22) and 1,817 nucleotides shorter than that of HRSV strain RSS-2 (30), mainly due to the absence of the NS1 and NS2 genes in APV and the difference in size of the L genes (26). This indicates that, like HRSV (28, 30), APV does not obey the rule of six, which states that the genome or minireplicons must contain a number of residues divisible by six, possibly indicating a binding requirement of the replicative assembly (3, 29).

This work confirms that the gene order of APV is 3' leader-N-P-M-F-M2-SH-G-L-trailer 5', which is different from that of HRSV and PVM. Except for the presence of the APV M2 gene, which is common to pneumoviruses, the gene order is identical to that of rubulaviruses. This reinforces the designation of APV as a representative of a new genus in the subfamily *Pneumovirinae* (25). The determination of the complete APV nucleotide sequence and identification of *cis*-acting elements responsible for viral transcription and replication and packaging of synthetic minireplicons should allow us to further define these *cis*-acting elements and enable us to develop virus-free rescue systems in order to identify the role of *trans*-acting proteins in viral replication, gene expression, and maturation of virus particles.

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