Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability

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Avian pneumovirus (APV) is a member of the genus Metapneumovirus of the subfamily Pneumovirinae. This study describes the development of a reverse-genetics system for APV. A minigenome system was used to optimize the expression of the nucleoprotein, phosphoprotein, M2 and large polymerase proteins when transfected into Vero cells under the control of the bacteriophage T7 promoter. Subsequently, cDNA was transcribed from the virion RNA to make a full-length antigenome, which was also cloned under the control of the T7 promoter. Transfection of the full-length genome plasmid, together with the plasmids expressing the functional proteins in the transcription and replication complex, generated APV in the transfected cells. The recombinant virus was passaged and was identified by cytopathic effect (CPE) that was typical of APV, the presence of a unique restriction-endonuclease site in the cDNA copy of the genome and immunofluorescence staining with anti-APV antibodies. Replacement of the full-length wild-type antigenome with one lacking the small hydrophobic (SH) protein and the attachment (G) genes generated a virus that grew more slowly and produced atypical CPE with syncytia much larger than those seen with wild-type virus.

INTRODUCTION

Avian rhinotracheitis is an important disease that is responsible for major economic losses in domestic poultry throughout most of the world (Jones, 1996). The most severe clinical effects are seen in turkeys, but there are significant economic losses in commercial chickens and it has been implicated in swollen head syndrome. In the 1980s, the combined efforts of several groups showed the cause to be a pneumovirus, named Avian pneumovirus (APV) (Jones et al., 1986; Wilding et al., 1986; Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988), which is now classified as a member of the family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus. It is distinguished from members of the genus Pneumovirus by its different gene order (Ling et al., 1992; Yu et al., 1992) and the absence of non-structural protein genes NS1 and NS2 (Randhawa et al., 1997). Recently, human metapneumovirus, a second member of the metapneumoviruses, was described, which is responsible for serious respiratory disease in children (van den Hoogen et al., 2001, 2002, 2003).

Manipulation of the genome and study of genome function in APV have been limited, due to the absence of a methodology whereby precise changes can be made. Reverse-genetics systems have been developed for other viruses of the family Paramyxoviridae, which have enabled specific mutations to be introduced into the virus genome and the subsequent phenotypic consequences determined. These have included Human parainfluenza virus 3, Human respiratory syncytial virus (HRSV), Bovine respiratory syncytial virus, Newcastle disease virus, Sendai virus, Rinderpest virus and Simian virus 5 (Marriott & Easton, 1999). These analyses have identified many important common features of the molecular biology of these viruses. However, analysis of HRSV by using reverse genetics has identified several aspects that differentiate it from other paramyxoviruses. Rescue studies with HRSV have shown that the minimal replicative unit is comprised of the nucleocapsid protein (N), phosphoprotein (P), M2 protein, RNA polymerase (L) and full-length viral genome in the antigenome sense (Collins et al., 1995). This paper describes the development of a reverse-genetics system for
APV. The system was used to produce APV that entirely lacked the SH and G genes, indicating that these genes are not essential for viability in tissue culture.

METHODS

Preparation of a full-length DNA copy of the APV genome. A full-length DNA copy of the APV genome was constructed in a series of PCR and ligation steps. The sequence of the APV genome is available from GenBank under accession no. AY640317. The cloning strategy is illustrated in Fig. 1 and all oligonucleotide primers used are shown in Table 1. RNA was extracted from Vero cells infected with APV (strain LAH A; Lohmann) by using an RNeasy kit (Qiagen). Two overlapping cDNAs were generated by reverse transcription (RT) for 1.5 h at 42 °C by using Superscript II (Invitrogen) (Fig. 1a). These RT reactions used the antigenomic sense primers APVLead and M2 start, corresponding to the leader and a sequence in the M2 gene. cDNA corresponding to the 3′ end of the genome was amplified by using primers APVLead ext and and a sequence in the M2 gene. cDNA corresponding to the 3′ end of the genome was amplified with T7L and APV Trail ext (Fig. 1b). These reactions were used as templates for eight further PCRs using Pfu polymerase (Stratagene) (Fig. 1c). These products, APV1, APV2, APV3, APV4+5, APV6, APV7, APV8 and APV9+10, were generated with primers listed in Table 1 that created Sall or XhoI sites at each end of the products. Digestion and ligation of these sites regenerating the viral sequence whilst eliminating the cloning sites, enabling insertion of the next fragment by using the same method.

A T7 promoter sequence was added to the leader end to generate APV1T7 and a Hepatitis delta virus ribozyme (HDVR) sequence was added to the trailer end to generate APV9+10HDVR. The HDVR was amplified from pOLT5 (Peeters et al., 1999) by using the HDVR start and HDVR back primers (Table 1). This product was ligated onto APV9+10 and the correct construct was amplified with APV11.9 XhoI+ and HDVR back.

APV1T7 and APV9+10HDVR were cloned into the low-copy plasmid pCTPE (a modified version of pOLT5 with the HDVR, T7 terminator and lacZ sequences removed to improve cloning efficiency; Fig. 1d). The remaining PCR products were cloned into a modified version of pUC18 with the Sall site changed to an EcoRI site by site-directed mutagenesis (Quickchange; Stratagene) (Fig. 1d). Site-directed mutagenesis was also used to correct coding changes that were identified by sequencing (Imperial College London Medical School service, London, UK) and to introduce an Sall restriction site into APV3. The latter introduced conservative (lysine to arginine) changes at aa 299 and 300 of the predicted F protein.

Sequential ligation of Sall/XhoI-cut fragments generated clones containing APV1T7+3 or APV6–10HDVR (Fig. 1e). Attempts to clone APV4+5 into these constructs were not successful. PCRs on APV1T7+3/APV4+5 and APV4+5/APV6–10 ligations carried out in the presence of Sall and XhoI were performed by using primers CTPE 240–111 and G11– or M2 mid and CTPE 190–, respectively (Fig. 1f). Exonuclease I (USB) was used to remove remaining oligonucleotides and an overlap PCR was used to join the two products, with T7 APVLead2 and CTPE 110– being added after two cycles (Fig. 1g). No primers were present in the first two cycles, in order to allow the fragments to anneal and copy each other from the overlap prior to the addition of the PCR primers. A 15–5 kb product was obtained, which was circularized by ligation and transformed into Escherichia coli (Fig. 1h). DNA from the resulting colonies was screened by multiplex PCR and restriction-enzyme digestion. One of two sequences of midiprep-purified DNA (Qiagen) lacked mutations in intergenic regions or coding changes.

After cloning, the full-length cDNA copy of the genome was modified between positions 3828 and 3831 (antigene sense) by site-directed mutagenesis (QuickChange; Stratagene) to introduce a convenient Sall restriction site. This also resulted in a conservative substitution at aa 299 and 300 of the F gene, with lysines being converted to arginine residues. Confirmation of the presence of the mutation after passage of the recombinant virus was obtained by RT-PCR amplification of the region of the genome between nt 3625 and 4063 using primers F6+ and F7–B (Table 1).

A defined deletion mutant of APV was generated by high-fidelity PCR (10 cycles) amplification of the full-length clone to introduce a deletion in the region of the SH gene. The amplicon was intended to terminate at genome position 5363, 1 nt prior to the SH gene transcription start signal, and to restart at position 5965, 2 nt prior to the G gene transcription start signal. Long-distance PCR (Thiel et al., 2003) was employed to amplify the full-length genome clone so as to remove the entire SH gene by using primers SH omit+ and SH omit–. The PCR mixture was run on an agarose gel to reveal solely the expected band (approx. 15 kb). This was self-ligated and used to transform competent cells. Resulting colonies were screened by multiplex PCR and restriction-enzyme analysis and a single clone from almost 1000 was identified as intact. This was sequenced across the M2–G gene junction region and it was found that several bases had been lost from the start of the G gene. The amplicon terminated at position 5363, as expected. However, the sequence unexpectedly continued from a point 12 nt into the ORF and hence the G gene lacked its transcription start, translation start and a small section of coding region. The entire sequence of the mutant genome was sequenced to confirm that this deletion was the only mutation present.

Preparation of plasmids expressing the virus replication proteins. N, P and M2 gene sequences from strain APV-A were amplified by PCR and cloned. RNA was extracted (RNeasy; Qiagen) from infected cells, reverse-transcribed (Superscript II; Invitrogen) and amplified by PCR (Pfu polymerase; Stratagene) using primers that amplified the gene to include the start codon (N and P) or introduced the T7 promoter sequence (M2 and L) immediately prior to the start codon of each gene, as shown in Table 1. The downstream primer in all cases terminated beyond the gene’s stop codon. N and P genes were cloned into the Smal site of pCI (Promega) downstream of a bacteriophage T7 promoter, allowing gene expression under the control of the cytomegalovirus promoter. T7M2 was cloned into the Smal site of the modified pUC18 mentioned above.

The L gene was cloned in sections into the EcoRV site of pCTPE by using the sequential approach used for the complete viral genome. In order, APV9+10, APV8, APV7 and APV6 were ligated into pCTPE. It proved impossible to add the final section of the gene (APV7Lstart), representing approximately the first 400 bp of the gene, in a similar manner. However, the full L gene, together with the pCTPE plasmid, was prepared as a single PCR product (Pfu polymerase; Stratagene) following their ligation. The blunt-ended product was circularized by ligation and the mixture was treated with the restriction endonuclease DpnI to remove the original methylated plasmid and leave only the desired product for transformation into E. coli. Three resultant clones were sequenced fully and were found to be free of coding errors.

Testing of support proteins in a minigenome. The four support genes were tested functionally by using a cloned APV minigenome in which the virus genome had been modified so that all genes were replaced by a single copy of the chlorampenicol acetyltransferase (CAT) reporter gene, flanked by the virus leader and trailer regions. This adopted the method of Randhawa et al.
(1997) except that, in this instance, bacteriophage T7 polymerase was generated by a Fowlpox virus recombinant (FPT7; Britton et al., 1996) at an m.o.i. of 1 p.f.u. per cell and lipofectamine 2000 (Invitrogen) was used to transfect DNA into Vero cells. CAT reporter-gene expression was measured 48 h after transfection by ELISA, as described previously (Ahmadian et al., 2000).

Fig. 1. Schematic representation of cloning strategy for generation of a cDNA clone representing the APV genome. The primers used in specific steps are given in parentheses. The position of Sal site (s) and Xho sites (x) at the termini of fragments are shown. Details of the procedures are given in the text.
Virus rescue. The method adopted followed that used for the minigenome, except that full-length antigenome (wild-type or deletion) replaced the minigenome and incubation times were increased to allow time for any virus to produce detectable cytopathic effect (CPE). Fowlpox virus will destroy most avian cells before this time, but it is unable to package in mammalian Vero cells; hence, additional

<table>
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<td>APVLead</td>
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<td>APVLead ext</td>
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<td>T7 APVLead2</td>
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<td>APV 1.1 Sal−</td>
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<td>APV2</td>
<td></td>
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<tr>
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<td>APV 3.1 Sal−</td>
<td>CATTGCAAAGTGTGTTGTGACCATTTCC</td>
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<td>APV3</td>
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<tr>
<td>F6 +</td>
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</tr>
<tr>
<td>F7-B</td>
<td>CTACCATCAGCTACGGGTGTC</td>
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Table 1. Sequence of primers used in the generation of PCR fragments
means of limiting Fowlpox virus replication were unnecessary (Britton et al., 1996).

Control transfections of two types were also prepared whereby some cell sheets, containing 10^6 cells, were inoculated with Fowlpox virus (at an m.o.i. of 1 p.f.u. per cell) and transfection reagent, whereas others received solely the latter. The transfected cell sheet was viewed, freeze–thawed and filtered (0-2 µm) to remove cellular debris and to eliminate any possibility of effect from residual Fowlpox virus. The clarified material was used to infect new cells. Resultant cell sheets were examined daily for signs of CPE typical of APV.

If CPE was not evident, material was passaged further. When CPE was detected, RNA was extracted for RT-PCR by using an RNase kit (Qiagen) with the optional DNase digestion step. RNA was isolated 4 days post-infection. The region of the genome encoding the F gene containing the novel SstI mutation was amplified in the case of both full-length and deleted recombinant genome-derived material and a further PCR was used to amplify between the M2 and G genes in the case of the latter. All RT-PCR products were generated by using primers that were designed to anneal initially to genome RNA and were sequenced. Standard PCR conditions used were 10 s at 94°C, 20 s at 50°C and 60 s at 72°C for 30 cycles using Taq DNA polymerase (Promega). The F gene-derived products were incubated with the restriction endonuclease SstI to assess their cleavability.

Confluent Vero cells were inoculated with the passaged material. After 4 days, these were incubated with APV-specific polyclonal antiserum that was raised in turkeys. After washing, this was further incubated with fluorescein isothiocyanate (FITC)-linked anti-turkey antiserum that was raised in turkeys. After washing, this was further incubated with APV-specific polyclonal serum that was raised in turkeys. After washing, this was further incubated with fluorescein isothiocyanate (FITC)-linked anti-turkey conjugate to detect infected cells (Baxter-Jones et al., 1986) and then viewed by using fluorescence microscopy and photographed.

RESULTS AND DISCUSSION

The minigenome construct was used to identify functional copies of the genes encoding components of the virus ribonucleoprotein complex that are responsible for RNA synthesis. Plasmids expressing the APV N, P, M2-1 and L genes and the minigenome plasmid were transfected into cells that had previously been infected with FPT7. After 48 h, expression of the CAT reporter gene in the minigenome was assessed by ELISA. Fig. 2 shows that co-transfection of all four support-gene plasmids directed the synthesis of CAT protein. In the absence of the L gene plasmid, no reporter-gene expression was detected, as shown in Fig. 2. Optimum levels of the various plasmids to achieve maximal expression of the CAT gene were established by using this system and were determined to be 400 ng plasmid encoding the N protein, 100 ng plasmid encoding the P protein, 200 ng plasmid encoding the L protein and 10 ng plasmid encoding the M2-1 protein per well of a 12-well plate. Whilst expression of the CAT reporter gene was observed in the absence of the M2-1 protein, levels were low and expression was enhanced at least 100-fold in its presence. This is similar to the situation seen with HRSV (Collins et al., 1995).

A full-length copy of the APV genome was constructed in such a way that a unique restriction-enzyme site, not present in the sequence of the wild-type APV, was inserted into the genome. This generated an SstII site within the F gene coding region and altered the lysines at aa 299 and 300 to arginines. The plasmid carrying the modified full-length genome was under the control of the T7 promoter in such a way as to generate a positive-sense antigenome RNA. The plasmid was transfected into FPT7-infected cells together with plasmids expressing the APV N, P, M2-1 and L genes, as before. After the initial transfection, it was not possible to clearly identify areas of CPE, so at 6 days, cells were harvested and freeze–thawed and clarified supernatant was used to infect a fresh cell monolayer. Cellular changes typical of APV infection of Vero cells became apparent after 3 days, with patches of syncytial CPE visible across the entire cell sheet. After a further 2 days, this had led to generalized CPE that, 1 day later, led to multifocal destruction and detachment of the cell sheet. After harvesting this second passage in Vero cells, the titre was approximately 10^6 TCID_{50} ml^{-1}, which is typical of the yield obtained with Vero cell-adapted APV field isolates (Naylor & Jones, 1994).

RNA was extracted from the passaged virus in the presence of DNase I and a 438 bp region of the F gene containing the altered sequence was amplified by RT-PCR and sequenced to confirm the presence of the altered bases between positions 3828 and 3831. In addition, the product was digested with SstII and the two expected cleavage products were clear on an agarose gel, as shown in Fig. 3. A control PCR in which the reverse transcriptase was not included did not produce any DNA fragments (data not shown). These data indicate clearly that the passaged virus was derived from the cloned cDNA and that the amplified

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**Fig. 2.** CAT reporter-gene expression from an APV minigenome. A plasmid carrying a minigenome of APV containing the CAT reporter gene was transfected into 10^6 cells that had previously been infected with FPT7 at an m.o.i. of 1 p.f.u. per cell, expressing the bacteriophage T7 RNA polymerase. Plasmids carrying the N, P, L and M2 genes of APV under the control of the T7 promoter were also transfected into the cells by using the optimized conditions described in the text. CAT reporter-gene expression was measured by ELISA 48 h after transfection. 1, Cells infected with FPT7 alone; 2, cells transfected with plasmids encoding the N, P and M2 genes; 3, cells transfected with plasmids encoding the N, P, M2 and L genes; 4, cells transfected with plasmids encoding the N, P and L genes alone.
When unfixed cell monolayers that had been infected 3 days previously with the fully competent recombinant virus were incubated with APV hyperimmune serum and stained with anti-turkey–FITC conjugate, intense fluorescence that was absent from the uninfected controls was observed (Fig. 4).

In order to extend these observations and to investigate the requirement of specific genes for growth in vitro, a second APV genome construct was generated in which the entire transcribed region encoding the SH gene was deleted. The sequence of the entire insert in the plasmid bearing the recombinant virus genome was determined and showed that the cloning procedure had also generated an extension of the deletion to include the conserved G gene start, together with the first 11 nt of the G gene coding region. The deletion in the G gene included the translation initiation codon. No other unexpected sequence alterations were present. The plasmid was transfected into cells together with the support plasmids, as before. Obvious CPE was not detectable initially in the transfected cells but, following passage of clarified cell extracts, very small syncytial areas were seen after 6 days. When this was passaged again, the number of syncytial patches increased, but still required 6 days to be readily visible. In contrast to the wild-type rescue, neither generalized CPE nor monolayer detachment was observed and this was considered to be a clear indication of altered behaviour in cell culture. When passaged a third time, giant syncytia could be seen (Fig. 5). This CPE is strikingly different from that seen with conventional strains of APV. In addition, the deletion mutant was found consistently to generate yields of $10^{5.3}$ TCID$_{50}$/well per standard well of cells. This contrasts with the value of $10^{6.8}$ TCID$_{50}$/well that was seen with the

![Image](https://example.com/image1.png)

Fig. 3. RT-PCR amplification of cDNA from APV. A region of the F gene of APV was amplified between nt 3620 and 4063 of the APV genome RNA for both a wild-type non-recombinant and the recombinant virus derived from a full-length cDNA clone. Virus had been passaged twice in tissue culture following the original transfection and RNA was prepared 4 days after infection. Lane 1, 443 bp fragment amplified from non-recombinant wild-type virus; lane 2, DNA fragment from lane 1 digested with SstII; lane 3, size markers; lane 4, 443 bp fragment amplified from the recombinant virus recovered from a full-length cDNA clone; lane 5, DNA fragment from lane 4 digested with SstII, showing the generation of two novel fragments.

![Image](https://example.com/image2.png)

Fig. 4. Immunofluorescence staining of cells infected with rescued wild-type virus. Cells were stained 3 days post-infection. (a) Cells were infected with virus recovered from transfections with a full-length genome and virus antigen was detected by immunofluorescence. (b) Cells were infected with FPT7 alone and anti-APV antibodies were used to show lack of cross-reactivity with the control cells.
wild-type virus and suggests that the deletion mutant may be impaired slightly in its growth in vitro.

To confirm the presence of the deletion in the recombinant ΔSH/G-APV by RT-PCR, genomic RNA of the virus, isolated after three passages in tissue culture, was amplified between the M2 gene (antigenome position 5273) and the G gene (antigenome position 6605). Genomic RNA was prepared and treated with DNase I before amplification to ensure that no DNA from the original transfection had been carried forward during virus passage. This produced a product of 690 bp in size, in contrast to the 1330 bp product that would be expected from a virus with a full complement of genes (Fig. 6). The sequence of the shortened fragment was identical to that of the original plasmid in the junction region. No fragment was obtained in the absence of reverse transcriptase. This confirmed that the recombinant virus lacked the regions of the SH and G genes that had been deleted in the cDNA.

The function of the SH protein in APV and HRSV is unknown at present. Its removal without apparent loss of viability is a finding similar to that of Bukreyev et al. (1997) for HRSV and shows that functional similarity extends across the subfamily Pneumovirinae. The timing and character of the CPE produced by this virus was indistinguishable from that seen with the rescued wild-type virus. The ΔSH-RSV recombinant was also attenuated when administered to chimpanzees (Whitehead et al., 1999) and it be will be interesting to explore whether ΔSH/G-APV is also attenuated. The similarity between the members of the genera Pneumovirus and Metapneumovirus may extend to being able to remove other genes that have been shown to be non-essential in HRSV, such as the second ORF of the M2 gene (Schmidt et al., 2002; Jin et al., 2003). Similarly, additional genes may be inserted into the APV genome.

By analogy with HRSV, the G gene of APV is anticipated to encode the attachment protein that mediates the initial interaction between the virus and host cell (Levine et al., 1987). Despite its central role in the virus life cycle, the HRSV G protein has been shown to be dispensable for virus growth in cell culture. Karron et al. (1997) reported the characterization of an HRSV vaccine candidate that was shown to contain a deletion that removed both the SH and G genes. Reverse-genetics approaches subsequently confirmed that loss of the G gene in HRSV, either singly or together with removal of the SH gene, did not result in loss of infectivity in vitro (Techaarpornkul et al., 2001; Teng et al., 2001). This is consistent with the observations

![Fig. 5. CPE of recombinant APV in Vero cells. Images of cells showing: (a) uninfected control cells; (b) cells 5 days post-infection with recombinant APV derived from a full-genome cDNA, showing standard CPE; (c) cells 5 days post-infection with the ΔSH/G-APV recombinant virus, showing abnormal syncytium formation.](http://vir.sgmjournals.org)

![Fig. 6. RT-PCR between the M2 and G genes of APV. The region between nt 5273 and 6605 in the APV genome was amplified by using standard conditions described in the text. Lane 1, non-recombinant wild-type APV; lane 2, recombinant APV derived from a full-genome cDNA; lane 3, ΔSH/G-APV recombinant virus; lane 4, RT-PCR using RNA from Vero cells infected with FPT7 alone; lane 5, size marker.](http://vir.sgmjournals.org)
reported here for ΔSH/G-APV and further strengthens the similarity of the two systems. However, deletion of the HRSV G gene was shown to affect the efficiency of replication in certain cell types (Teng et al., 2001). It was suggested that this effect was primarily at the level of attachment and entry, although the precise reasons for the host cell-dependent differences are not yet clear. As with the SH- and G-deleted vaccine candidate, recombinant virus analysis showed that the G protein is important for infection in vivo. It will be of interest to study the replication characteristics of ΔSH/G-APV in various cell types and in vivo.

The new ability to manipulate the APV genome will lead to fundamental questions being addressed concerning gene function. Several recombinant HRSV viruses have been shown to be attenuated (Marriott & Easton, 1999). Commercially derived, live APV vaccines for administration to poultry have been available for over 10 years in Europe and these have led to major improvements in disease control (Cook, 2000). However, doubts remain about their performance under certain conditions, especially where vaccine administration may not be optimal and significant numbers remain unvaccinated (Jones, 1996; Cook, 2000). APV vaccines are known to revert to a virulent state under experimental conditions (Naylor & Jones, 1994) and there is evidence to suggest that this also occurs in the field, especially where vaccine administration is poor (Naylor et al., 2003). It is widely believed that the current vaccines may represent the maximum stability that is achievable by empirical cell passage (Naylor et al., 2003) and attempts to protect by using recombinant and DNA vaccines have to date conferred protection markedly inferior to that conferred by the current products (Yu et al., 1994; Kapczynski & Sellers, 2003). Killed vaccines have been shown to be poorly effective unless preceded by an initial live vaccination (Cook, 2000). Current work in our laboratory, involving the study of previously derived empirical vaccines, together with associated progenitor strains and virulent revertants (Naylor & Jones, 1994), is starting to identify genome regions where specific mutations may significantly alter pathogenesis. It is anticipated that the reverse-genetics system described here will open new avenues for the development of live vaccines to improve on the empirical types that are currently in use. It is also probable that such developments may have directly useful applications in studies of the recently discovered human metapneumovirus (van den Hoogen et al., 2001).

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