Deletion of the SH gene from avian metapneumovirus has a greater impact on virus production and immunogenicity in turkeys than deletion of the G gene or M2-2 open reading frame

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Subgroup A avian metapneumoviruses lacking either the SH or G gene or the M2-2 open reading frame were generated by using a reverse-genetics approach. The growth properties of these viruses were studied in vitro and in vivo in their natural host. Deletion of the SH gene alone resulted in the generation of a syncytial-plaque phenotype and this was reversed by the introduction of the SH gene from a subgroup B, but not a subgroup C, virus. Infected turkeys were assessed for antibody production and the presence of viral genomic RNA in tracheal swabs. The virus with a deleted SH gene also showed the greatest impairment of replication both in cell culture and in infected turkeys. This contrasts with the situation with other pneumoviruses in culture and in model animals, where deletion of the SH gene results in little effect upon viral yield and a good antibody response. Replication of the G- and M2-2-deleted viruses was impaired more severely in turkeys than in cell culture, with only some animals showing evidence of virus growth and antibody production. There was no correlation between virus replication and antibody response, suggesting that replication sites other than the trachea may be important for induction of antibody responses.

INTRODUCTION

Avian metapneumovirus (AMPV) is the type member of the genus Metapneumovirus, family Paramyxoviridae. It has been associated with significant morbidity and subsequent economic loss in the poultry industry (Cook, 2000). The only other member of the genus Metapneumovirus is human metapneumovirus (HMPV; van den Hoogen et al., 2001). AMPV strains have been divided into four subgroups on the basis of antigenicity and sequence diversity. Subgroups A and B are distinct, but closely related (Juhasz & Easton, 1994; Li et al., 1996; Randhawa et al., 1996), whereas subgroup C is more similar to HMPV (Toquin et al., 2003; Yunus et al., 2003). Subgroup B currently predominates in Europe, whereas subgroup C occurs in the USA and in ducks in France. Subgroup D is apparently restricted to a small number of historical isolates from France (Bàyon-Auboyer et al., 2000; Toquin et al., 2000).

The metapneumoviruses direct the synthesis of eight mRNA transcripts, encoding nine primary translation products. A reverse-genetics system, which permits rescue of infectious virus from cDNA clones, has been described previously for AMPV (strain LAH A) and used to show that infection with a recombinant virus unable to express both the SH and G genes resulted in the production of unusually large syncytia in Vero cells (Naylor et al., 2004). A deletion mutant of HMPV lacking the SH and G genes has also been described, but this did not display an altered fusion phenotype in the cells tested (Biacchesi et al., 2004). These data suggest that virus-directed fusion in AMPV is regulated by either the SH or the G gene and that this differs from HMPV.

The deletion of specific genes by using reverse genetics has been used to explore the function of virus proteins during infection in vitro and in vivo. Several pneumovirus genes have been shown to be dispensable for growth in cell culture, including those encoding the SH, G and M2-2 proteins. However, deletion of these genes has been reported to affect the growth of the viruses in animal models. HMPV lacking both the SH and G genes replicated less well in the upper and lower respiratory tracts of hamsters than wild-type virus. This effect was shown to be due to loss of the G gene, as virus lacking only the SH gene...
replicated more efficiently in hamster lungs than the wild type, whereas a G gene-deleted virus behaved in a similar way to the double mutant (Biacchesi et al., 2004). In the hamster model, the viruses lacking the G gene generated a protective response against challenge by wild-type virus (Biacchesi et al., 2004). Similar results were observed with recombinant HMPV in African green monkeys, where little effect was observed as a result of deleting the SH gene, whereas reductions in virus replication in both the upper and lower respiratory tracts were observed as a result of deletion of the G gene or the M2-2 open reading frame (ORF) (Biacchesi et al., 2005). However, in contrast to the observations with hamsters, replication efficiency in African green monkeys was more impaired in the lower than the upper respiratory tract, and all of the viruses were immunogenic and protective. Similar experiments have been performed with human respiratory syncytial virus (RSV) in rodents and chimpanzees. Deletion of the RSV G gene resulted in a marked reduction in replication of virus in the lungs of mice (Teng et al., 2001). Deletion of the RSV SH gene did not impair growth in tissue culture and, in some cell lines, actually improved yields. Deletion of the RSV SH gene resulted in impaired replication in the upper, but not the lower, respiratory tract of mice, and the virus was immunogenic and protective (Bukreyev et al., 1997). However SH gene-deleted RSV replicated less well in the lower than in the upper respiratory tract in chimpanzees and the symptoms of disease were milder (Whitehead et al., 1999). The effect of deletion of the RSV M2-2 ORF on virus growth in tissue culture varied from larger plaques and enhanced viral yield to smaller plaques and reduced virus yield. Yields of these deleted viruses in the lungs of infected mice and cotton rats were reduced, but the mice were protected from challenge (Jin et al., 2000). Replication was also reduced in chimpanzee lungs and the virus was highly immunogenic (Teng et al., 2000). These data indicate that deletion of the SH gene from pneumoviruses has a less pronounced effect on virus growth in vivo than deletion of the G gene or M2-2 ORFs. However, the effects on virus replication differ between animal models. It is therefore of interest to study the effects of deletion of these genes from AMPV on infection of the natural host. Here, we describe the generation of a series of deletion mutants of AMPV and the characterization of their growth characteristics in tissue culture and in vivo.

METHODS

Construction of full-length AMPV clones. A full-length clone of the CVL/14 strain of AMPV was constructed, with a T7 promoter adjacent to the leader sequence and a hepatitis delta virus ribozyme sequence positioned to cleave RNA transcripts at the end of the trailer region, followed by a bacteriophage T7 RNA polymerase terminator. The cDNA was constructed such that a full-length antigenome RNA was synthesized under the control of the T7 promoter, as described previously (Naylor et al., 2004). Two full-length recombinant genomes were generated. The first (APVA) represented the entire genome with only nine nucleotide changes. Five of these were synonymous changes in coding regions, and four were coding changes. The latter changes were asparagine to aspartate at aa 5 (NSD) in the L protein, introduced as a result of an error in the published L gene sequence, and three changes in the F protein (G68D, T123A and N170S), corresponding to the amino acids in strain UK/3B/85 (Yu et al., 1991) rather than the sequence of the F gene of strain CVL/14. In the second full-length genome, unique restriction sites were introduced into all of the intergenic regions to generate fragments that could be assembled to generate a ‘cassette’ clone (designated CASA). Details of the construction of the recombinant virus genomes are available from the authors on request. The sequences of the new intergenic regions present in these constructs are shown in Fig. 1. All of the deletion mutants described below were made by using the CASA clone as the genetic backbone. Mutants lacking the SH or G genes (dSH and dG) were generated by digestion at the restriction sites either side of the relevant gene (Agd and Eagl for the SH gene and Eagl and Bmg8l for the G gene; Fig. 1), followed by rendering the restriction sites blunt-ended by using T4 DNA polymerase, and subsequent religation. The mutant virus lacking the M2-2 ORF was generated by firstly excising the M2 gene and replacing it with a DNA fragment generated by PCR and containing only the M2-1 ORF flanked by FseI and AgeI restriction sites, which were present in the oligonucleotide primers and which allowed easy substitution. The SH gene in the CASA recombinant genome was replaced with the coding regions of the SH genes from subgroup B strain 98103 (D. Toquin & N. Eterradossi, unpublished) and subgroup C strain 99350 (Toquin et al., 2006), which were amplified by RT-PCR and inserted into the full-length clone by using the AgeI and Eagl restriction sites.

Production and assay of recombinant AMPV. Recombinant viruses were generated by transfection into BSRT7/5 cells, using Lipofectamine 2000 (Invitrogen), of plasmids containing the full-length or deleted virus genomes (Buchholz et al., 1999). Plasmids containing the genes encoding the AMPV N, P, M2-1 and L proteins, inserted so as to give expression from the AUG codon adjacent to the internal ribosome entry site in pCITE4 (Novagen) under the control of the bacteriophage T7 promoter, were also transfected simultaneously into the BSRT7/5 cells. The amounts of plasmids used were those described previously for human RSV (Collins et al., 1995) or HMPV (Herfst et al., 2004). The medium from the transfected cell cultures was collected 5 days after transfection and used to infect BSC-1 cells. Transfections and virus growth were carried out at 33 °C. RT-PCR of RNA extracted from infected cells verified the presence of the deletions. Virus from the third passage after transfection was used to generate growth curves and to assess the ability of the recombinant viruses to generate syncytia. Growth curves were generated in BSC-1 cells, using an m.o.i. of 0.045 p.f.u. per cell. Infected cells were scraped into the culture medium, pelleted for 1 min at 13 000 g and resuspended in Glasgow’s modified Eagle’s medium containing 1% fetal bovine serum (Biosera). The supernatants and resuspended cell pellets were stored at −70 °C prior to microplate assay. Briefly, serial dilutions of samples were incubated for 2 days with monolayers of BSC-1 cells then fixed for 10 min with cold acetone:methanol (1:1). The monolayers were then washed three times in PBS containing 0.1% Tween 20, in which they were then stored at 4 °C. The plaques were visualized by immunostaining with anti-AMPV P protein mAbs (supplied by P. Rueda, Ingenasa, Madrid), horseradish peroxidase (HRP)-conjugated anti-mouse IgG and aminoethylcarbazole as the substrate, with three washes with PBS/Tween being carried out between each reagent addition.

Infection of turkeys. Six groups of ten 6-week-old, specific-pathogen-free turkeys (AFSSA-Ploufragan) were housed in separate filtered-air isolation units. Blood samples were taken from all birds for serological testing prior to intranasal inoculation. One group was kept as a non-infected control in a positive-pressure isolation unit, and received only Eagle’s minimal essential medium (Eurobio) with HEPES (Sigma-Aldrich) (0.1 ml per bird). The five other groups were
housed in negative-pressure isolation units and received the APVA, CASA, dSH, dG or dM2-2 recombinant viruses (under a permit from the French Commission on Genetically Modified Organisms). All viruses were inoculated at a dose of 10^{3.5} TCID_{50} (0.1 ml) per bird, except for dSH, which was inoculated at 10^{3.2} TCID_{50} (0.3 ml) per bird. Clinical symptoms were observed and tracheal swabs were prepared individually from all birds on days 3, 6, 10 and 14 post-infection. Final blood samples for serology were collected at 20 days post-inoculation.

**Assay of AMPV antibodies.** Antibodies to AMPV were quantified by ELISA, as described previously. An antigen derived from AMPV strain 85051 (Toquin et al., 2000), belonging to AMPV subgroup A (the same subgroup as the inoculated viruses), was used to improve sensitivity of the ELISA (Toquin et al., 1996). Antigens derived from AMPV strains 86004 and 85035, belonging to subgroups B and D, respectively (Toquin et al., 2000; Bâyón-Auboyer et al., 2000), were run in parallel to check whether the deletion of the highly subgroup-specific SH or G genes from the inoculated recombinant viruses resulted in an altered inter-subgroup cross-reactivity of the antibodies elicited by these genetically modified viruses.

**Quantitative PCR of AMPV N gene.** A specific, N gene-based Taqman real-time RT-PCR (RRT-PCR) was developed and validated for quantitative use according to a previously reported methodology (Guionie et al., 2007). Briefly, suitable primers and a Taqman FAM-labelled probe were defined from the sequence of the N gene of AMPV strain CVL14.1 by using Primer Express version 2.0 software (Applied Biosystems). The Taqman RRT-PCR assay was run in a 96-well format, using the ABI Prism 7000 sequence detection system (Applied Biosystems) and a QuantiTect probe RT-PCR kit (Qiagen). Reaction mixes and thermocycling parameters were as described previously (Guionie et al., 2007), except that 40 cycles were used. Data were analysed with the Sequence Detection version 1.2.3 software (Applied Biosystems). The baseline and threshold values were determined automatically (Auto-Ct option). Quantitative results were calculated automatically by including a reference panel of serial dilutions of a known amount of an RNA transcript produced by in vitro transcription of an N gene-containing plasmid (Guionie et al., 2007) in each RRT-PCR experiment.

**RESULTS**

A series of recombinant AMPVs based on the CVL/14 genome were generated. These included a virus containing only three amino acid alterations in the F gene (APVA), a...
virus in which the intergenic regions were altered to insert unique restriction sites between each gene (CASA) and three deletion mutants, based on the CASA genome, lacking the SH, G or M2-2 genes. All viruses were able to replicate in tissue culture. Virus stocks from the third passage after transfection were used to generate growth curves and to assess the ability of the recombinant viruses to generate syncytia. Growth curves generated in BSC-1 cells are shown in Fig. 2 and, for comparison, a non-recombinant AMPV CVL/14 stock was also used. Released virus and cell-associated virus were assessed separately and the general patterns for both were very similar. As can be seen, wild-type recombinant virus (APVA) produced slightly lower yields late in infection than the parental, non-recombinant virus (BSC), and the introduction of restriction sites into the intergenic regions impaired virus growth of CASA relative to that of APVA. The level of impairment differed at different time points and the biggest differences generally occurred early, during the first few days of growth. Over the period of the growth curve, yields of CASA virus were reduced relative to those of APVA by a median of 18-fold, with the final yield being reduced by approximately 10-fold. Compared with CASA, yields of dSH were reduced by a median of 10-fold and those of dM2-2 by a median of 1.6-fold, whilst dG was relatively unaffected.

The morphology of plaques produced by the recombinant AMPVs was similar for the CASA, dG and dM2-2 viruses (mostly small foci of infected cells), but the dSH virus almost exclusively generated larger, syncytial-type plaques (the percentage of syncytial plaques is shown in Table 1; typical cytopathic effects are shown in Fig. 3). In contrast to the parental virus, which had been plaque-purified extensively to remove syncytium-forming virus, APVA did produce some syncytial plaques, suggesting that mutations may have accumulated as the virus was passaged; however, no mutations in the F or SH genes were detected by direct sequencing of RT-PCR products (data not shown). These data show that the deletion of the SH gene was specifically responsible for the appearance of the syncytial-plaque morphology, but the observation that APVA produces a low level of syncytial plaques may indicate that factors

**Fig. 2.** Multi-step growth curves of the original stock virus grown in BSC-1 cells (■), wild-type recombinant virus (APVA; △), recombinant virus with restriction sites inserted into the intergenic regions (CASA; ▽) and CASA with deletions of the SH or G genes (dSH, ◊; dG, ×) or the second ORF of M2 (dM2-2; □). Mean log_{10} p.f.u. per well and 95% confidence limits (error bars) for three wells are shown. (a) Cell-associated virus; (b) virus in culture medium.

**Table 1.** Growth and plaque morphology of wild-type (BSC) virus grown in BSC-1 cells, the wild-type recombinant virus (APVA), recombinant virus with restriction sites inserted into the intergenic regions (CASA) and CASA with deletions of the SH or G genes (dSH and dG) or the second ORF of M2 (dM2-2)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Comments</th>
<th>Titre of virus stock</th>
<th>Plaques showing syncytial phenotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSC</td>
<td>Plaque-purified CVL/14</td>
<td>1.0 × 10^7</td>
<td>0.1</td>
</tr>
<tr>
<td>APVA</td>
<td>Wild-type, generated by reverse genetics</td>
<td>1.2 × 10^6</td>
<td>25.5</td>
</tr>
<tr>
<td>CASA</td>
<td>Restriction sites in intergenic regions</td>
<td>1.9 × 10^4</td>
<td>6.0</td>
</tr>
<tr>
<td>dM2-2</td>
<td>CASA without M2-2 ORF</td>
<td>6.0 × 10^4</td>
<td>7.0</td>
</tr>
<tr>
<td>dSH</td>
<td>CASA without SH gene</td>
<td>5.5 × 10^4</td>
<td>93.0</td>
</tr>
<tr>
<td>SHdPN</td>
<td>CASA lacking most of SH ORF</td>
<td>1.3 × 10^4</td>
<td>100.0</td>
</tr>
<tr>
<td>SHBV</td>
<td>CASA with subgroup B SH</td>
<td>1.76 × 10^4</td>
<td>2.4</td>
</tr>
<tr>
<td>SHCV</td>
<td>CASA with subgroup C SH</td>
<td>6.7 × 10^3</td>
<td>98.6</td>
</tr>
<tr>
<td>dG</td>
<td>CASA without G gene</td>
<td>1.9 × 10^4</td>
<td>3.0</td>
</tr>
</tbody>
</table>
other than the loss of a functional SH gene may also generate syncytia.

Whilst the data showed that the loss of the entire SH gene generated an altered plaque morphology, it was possible that this was due to a change in the pattern of transcription from the genome resulting from the loss of a transcription unit. To address this, an additional deletion mutant was generated. This virus, designated SHdPN, contained a deletion of a 402 nt region within the SH gene, produced by excision of a PstI/NsiI fragment from the CASA genome. The deletion removed aa 27–160 of the 174 aa SH protein, but left the transcription-initiation and -termination signals intact. Observation of the plaque morphology showed that SHdPN also produced only large syncytia (Table 1; Fig. 3). As SHdPN contains the same number of transcription units as CASA, the altered phenotype must be due to the loss of the SH protein-coding region.

In order to investigate the potential for the SH genes from viruses in other AMPV subgroups to complement that from subgroup A, two further recombinant viruses were generated. In one, the SH gene of the subgroup A virus was replaced with that from a subgroup B virus. Recombinant virus isolated from this clone gave predominantly non-syncytial plaques (Table 1; Fig. 3). In contrast, a recombinant virus in which the subgroup A virus SH gene was replaced with that of a subgroup C virus almost exclusively generated syncytial plaques (Table 1; Fig. 3). The different abilities of the SH genes from different AMPV subgroups to complement the plaque morphology reflect the genetic distances between these viruses.

Prolonged serial passage of AMPV in mammalian cell culture results in loss of virulence in vivo. However, despite the loss of virulence, cell-culture-grown virus is able to infect turkeys, and virus can be detected in the trachea,

Fig. 3. Cytopathic effects observed in BSC-1 cells infected with wild-type and recombinant viruses. Plaques were visualized by immunostaining with anti-AMPV P protein mAbs and HRP-conjugated secondary antibodies, with aminothiolcarbazole as the substrate. The viruses were: the original stock virus grown in BSC-1 cells (BSC), the wild-type recombinant virus (APVA), recombinant virus with restriction sites inserted into the intergenic regions (CASA), CASA with deletions of the SH (dSH) or G (dG) genes, CASA carrying a deletion of the ORF of the SH gene (SHdPN), CASA deleted in the second ORF of M2 (dM2-2), and CASA with the SH gene substituted from a subgroup B (SHBV) or subgroup C virus (SHCV). Mock-infected cells are also shown (MI). Bars, 100 μm.
together with an associated antibody response. We used an ELISA to detect antibodies and quantitative RT-PCR to detect virus genome and mRNA in turkeys infected with the recombinant viruses. The results showed that, despite the poorer growth in cell culture, CASA virus elicits an antibody response almost as high as that seen for the recombinant virus APVA and is released at a similar level in the trachea (Table 2). With both CASA and APVA, all birds were positive both for antibody and, at day 6 post-infection, for virus in the trachea. Fewer birds infected with viruses deleted for the SH, G or M2-2 genes showed the presence of anti-AMPV antibody or virus secretion and, in the positive birds, the levels of antibody and shed virus were lower than in controls infected with non-deleted viruses. The most dramatic difference was seen with the dSH virus, where infection resulted in only half of the birds being antibody-positive, with the titres being approximately one-third of that of the relevant wild-type virus (CASA); no virus RNA was detectable in any bird (Table 2). In the case of the G and M2-2 gene-deleted viruses, some birds gave positive results in each assay; however, there was no correlation between the birds containing antibody and those showing the presence of virus RNA, e.g. with the G-gene-deleted virus, one turkey remained seronegative throughout the experiment, but secreted virus at 6 days post-infection, whilst no virus RNA was detected in one bird that was seropositive at 20 days post-inoculation. Similarly, for the M2-2-deleted virus, virus RNA was detected in two seronegative turkeys at 6 and 14 days post-infection, but could not be detected in two seropositive turkeys.

Comparison of the ELISA results obtained at 20 days post-inoculation with antigens derived from AMPV subgroups A, B and D revealed that, as observed previously with subgroup A field and vaccine viruses, antibodies induced by challenge with the APVA and CASA viruses were detected more efficiently with the homologous subgroup A antigen, as these sera produced limited cross-reactivity with subgroup B (mean ELISA ratios of 0.115 and 0.101, respectively) and subgroup D (mean ELISA ratios of 0.370 and 0.250, respectively). Similar differences in the performance of the ELISA antigens were observed in birds receiving the dM2-2, dSH and dG viruses, as none of the birds that were seropositive with the subgroup A ELISA antigen after these challenges were positive with the heterologous B and D ELISA antigens (data not shown).

**DISCUSSION**

The use of reverse genetics for pneumoviruses has opened up the possibility of studying the roles of virus proteins in replication in cell culture and in vivo. A significant problem with in vivo analyses has been the requirement to use model systems rather than the natural host. Data from animal studies have shown that the details of recombinant

<table>
<thead>
<tr>
<th>Virus inoculated</th>
<th>None</th>
<th>CASA</th>
<th>dM2-2</th>
<th>dSH</th>
<th>dG</th>
<th>APVA</th>
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<tr>
<td>Mean ELISA ratio</td>
<td>0.004±0.008</td>
<td>1.028±0.216</td>
<td>0.402±0.434</td>
<td>0.172±0.167</td>
<td>0.337±0.430</td>
<td>1.351±0.131</td>
</tr>
<tr>
<td>No. ELISA positive turkeys/total</td>
<td>0/10</td>
<td>0/10</td>
<td>5/10</td>
<td>5/10</td>
<td>4/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Mean ELISA ratio of positives</td>
<td>0</td>
<td>0.781±0.251</td>
<td>0.321±0.084</td>
<td>0.830±0.115</td>
<td>1.351±0.131</td>
<td></td>
</tr>
<tr>
<td>Excretion at 3 days p.i.</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>No. positives</td>
<td>0</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Mean log_{10} copies per swab</td>
<td>6.18±0.48</td>
<td>5.19±0.27</td>
<td>5.39±0.85</td>
<td>6.97±0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excretion at 6 days p.i.</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. positives</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mean log_{10} copies per swab</td>
<td>5.79±0.29</td>
<td>4.85±0.02</td>
<td>5.04</td>
<td>5.86</td>
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<tr>
<td>Excretion at 10 days p.i.</td>
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<td>1</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>No. positives</td>
<td>0</td>
<td>4.95</td>
<td>4.82</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mean log_{10} copies per swab</td>
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<td>–</td>
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</table>

Table 2. Detection of anti-AMPV antibodies and virus genomic RNA in turkeys infected with wild-type recombinant virus (APVA), recombinant virus with restriction sites inserted into the intergenic regions (CASA) and CASA with deletions of the SH or G genes (dSH and dG) or the second ORF of M2 (dM2-2).

Antibody levels were measured by using an ELISA and are shown as the mean (±SD) ELISA ratios for all birds and for antibody-positive birds only. ELISA ratios were calculated after measurement of A_{405}, as (A_{405} of serum sample−A_{405} of reference negative turkey serum)/(A_{405} of reference positive turkey serum−A_{405} of reference negative turkey serum), so that a serum with the same reactivity as reference positive serum should exhibit a ratio equal to 1. Virus genomic RNA levels were determined at 3, 6, 10 and 14 days post-infection (p.i.) using quantitative RT-PCR and are expressed as the log_{10} of the mean of the values detected in virus-positive birds. Where appropriate, the SD of the levels is shown.
virus replication can differ in different systems. Here, we describe the replication characteristics of recombinant AMPVs in vitro and in the natural immunocompetent host.

A recombinant AMPV lacking the G gene showed no growth reduction in cell culture (Fig. 2), similar to reports for G gene-deleted HMPV, the other metapneumovirus (Biacchesi et al., 2004). This contrasts with data on deletion of the RSV G gene, which results in a significant reduction in virus yield (Techaarpornkul et al., 2001), suggesting that the metapneumoviruses are less dependent upon the presence of G protein for replication in cell culture than the pneumoviruses. However, in RSV, deletion of the G gene was shown to have a differential effect in different cell lines (Teng et al., 2001). Viruses lacking the G gene or M2-2 ORF both showed reduced frequency and yield of replication in turkeys, as measured by virus released into the trachea, and also elicited a reduced frequency of serum antibody response compared with the wild-type virus. This is comparable with the situation with HMPV, where replication in cell culture was not impaired by deletion of the SH or G genes. However, loss of the G gene resulted in reduced replication in hamsters and African green monkeys (Biacchesi et al., 2004, 2005).

In contrast to the situation in other pneumoviruses AMPV lacking the SH gene replicated significantly less well than the wild-type virus control and the other deleted viruses, both in cell culture and in turkeys. In all cases, the deleted AMPVs either failed (dSH virus) or were significantly impaired (dG and dM2-2 viruses) in producing virus in the trachea of infected birds (Table 2). The lack of production of virus RNA was mirrored by the poor immunogenicity of the viruses, with serum antibody generated in only approximately half of the infected animals. In the cases of the G- and M2-2-deleted viruses, where an infection was established, antibody levels were similar to those seen with wild-type virus. The presence of the antibody indicates that the viruses did indeed replicate to some extent in the birds. Whilst ELISA-positive, virus RNA-negative birds may reflect a situation where poorly replicating viruses are cleared, consideration of individual birds identified that were virus RNA-positive and ELISA-negative (data not shown). The most extreme examples of the lack of correlation between ELISA and PCR results were seen with birds infected with SH-deleted virus, where no virus genomic RNA was detected at any time, although some birds showed detectable serum antibody. The lack of correlation between a positive virus RNA result and a negative ELISA result could be related to the type of antibody measured in the assay, as ELISA titres have recently been shown not to correlate with the levels of virus-neutralizing antibody in AMPV-infected birds (Liman & Rautenschlein, 2007). However, ELISA has been shown to be more sensitive than virus neutralization (Eterradossi et al., 1995). It is possible that the discrepancies are due to virus replication in tissues other than the trachea, as suggested by Cook et al. (1999).

The subgroup specificity of the ELISA antibody responses elicited by recombinant subgroup A viruses, such as APVA and CASA, was consistent with that reported previously for wild-type subgroup A viruses, which are detected most efficiently with ELISA antigens belonging to the homologous AMPV subgroup (Eterradossi et al., 1995; Toquin et al., 2000). Most interestingly, whilst the SH and G genes are both highly subgroup-specific, sera from animals infected with subgroup A viruses lacking either the SH and G genes did not differ in the cross-reactivity with subgroup B and subgroup D antigens in an ELISA compared with sera from animals infected with a wild-type virus. Indeed, the sera raised in response to infection with the dSH and dG viruses were detected by the subgroup A ELISA antigen more efficiently, suggesting a higher level of subgroup specificity. This suggests that neither the SH nor the G protein alone is the molecular basis for the antigenic subgroup specificity in subgroup A AMPV.

The data presented here show clearly that the AMPV subgroup A or B SH protein regulates the cell–cell fusion process when present in a subgroup A genetic background. Whilst this is, in principle, similar to the situation with RSV, where the SH protein has been implicated in fusion, there are significant differences. In particular, for AMPV, the absence rather than the presence of the SH protein enhances fusion. It is not known whether the large syncytia in the SH-gene-deleted virus are due to the presence of the SH protein having an inhibitory effect on fusion or whether the SH protein is required for development of the focal type of cytopathic effect, and this will require further investigation. The effects of deletion of the SH gene on the processing and localization of the F protein are also unknown and could provide a means for the SH protein to influence the cell-fusion process. The failure of the SH protein from subgroup C to complement the missing subgroup A protein is likely to reflect the greater phylogenetic distance between subgroups C and A compared with that between subgroups B and A. This could result from a failure of subgroup C SH protein to interact with a subgroup A virus component or from the SH protein not being involved in regulation of fusion in subgroup C viruses, as appears to be the case for HMPV, to which subgroup C AMPV isolates are related most closely. The phenotypes of the various viruses are not due to alterations in the relative expression of the G gene, as we have shown that the relative expression of the G gene in dSH is not affected compared with wild-type virus. Similarly, the introduction of sequences into the intergenic regions in CASA results in only a very slight increase in the relative expression of the G gene (R. Ling & A. J. Easton, unpublished data).

The results described here indicate that, despite the reduced replication of a recombinant AMPV modified to facilitate genetic modification in cell culture, replication in birds and antibody response were relatively unaffected. Unfortunately, a consistent observation with AMPV is that extensive passage in tissue culture renders the virus
non-pathogenic, so it is not possible to investigate aspects of disease with recombinant viruses. However, a reverse-genetics approach is valuable for studying protective responses or generating vaccine candidates. In contrast to previous reports with recombinant pneumovirus infections, it has been possible to study this virus in its natural host and the data reported here indicate that, unexpectedly, the SH gene plays an important role in AMPV replication in tissue culture and in vivo. The data also show that even levels of recombinant virus replication that cannot be detected in the trachea can induce an antibody response. By using this system, it will be possible to explore further the role(s) of virus proteins in the natural host.

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