Avian metapneumovirus SH gene end and G protein mutations influence the level of protection of live-vaccine candidates

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INTRODUCTION

Vaccines must demonstrate two key characteristics to be successful: they must be attenuated for disease and their administration must be able to induce an appropriate level of protection in the vaccinee. Protection from infection generally involves the induction of a suitable immune response from both the humoral and cellular arms. Much attention has focused on the molecular basis of attenuation in vaccine candidates, but there is little understanding of the factors that relate to the establishment of protective immunity other than the inclusion of appropriate epitopes in antigenic proteins. Avian metapneumovirus (AMPV) is responsible for major economic losses in domestic poultry throughout most of the world (Jones, 1996). The most severe clinical effects of infection are seen in turkeys, but there are significant economic losses in commercial chickens where it has also been implicated in swollen head syndrome. AMPV is a member of the family Paramyxoviridae, subfamily Pneumovirinae and genus Metapneumovirus (Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988), and is distinguished from members of the genus Pneumovirus by the order of its genes (3'-N-P-M-F-M2-SH-G-L-5') (Ling et al., 1992; Randhawa et al., 1996, 1997; Yu et al., 1992a, b) and the absence of non-structural protein genes (Randhawa et al., 1997). The only other member of the genus Metapneumovirus is the species Human metapneumovirus (van den Hoogen et al., 2002). Several AMPV vaccine candidates have been described, but most suffer from problems of either incomplete protection and/or reversion to pathogenicity (Catelli et al., 2006). The genetic basis for field strain attenuation and vaccine instability is not clear and an understanding of this is important for the rational design of vaccine candidates.

AMPV type A field strain #8544 was isolated in northern England in 1985 during a severe outbreak of turkey rhinotracheitis that rapidly spread across the whole country. When tested as a candidate vaccine, it proved to be highly protective against virulent challenge, but also caused severe post-vaccine disease in a number of cases (Williams et al., 1991a). Subsequently, strain #8544 was passaged extensively in Vero cells in our laboratory to yield an uncloned virus (named P20) that protected turkeys against virulent AMPV challenge (Williams et al., 1991b). However, on occasions, the putative P20 vaccine produced respiratory disease, typical of AMPV infection, during serial passage in turkeys. This was ascribed to a small subpopulation
of virulent virus in less than 1 in 10^5 infectious doses (Naylor & Jones, 1994). To remove the virulent subpopulation, stock P20 was plaque purified and 12 derivative viruses were prepared as candidate vaccines and, as anticipated, all were shown to be free of the virulent subpopulation.

Here, we describe the protection conferred by these 12 plaque-purified viruses and demonstrate that the loss of ability to confer protection from virulent challenge is associated with an alteration in the balance of protein expression, particularly the attachment (G) glycoprotein.

METHODS

Viruses. The vaccine candidate P20 was used to infect monolayers of Vero cells, and 12 individual plaques were picked and purified. These purified viruses, designated A–H and J–M, were grown in Vero cells and used to inoculate 1-day-old turkey pouls.

Clinical assessment of plaque-purified viruses. An established challenge model was used to assess the ability of the plaque-purified viruses to confer protection from pathogenic virus challenge (Naylor et al., 1997a; Williams et al., 1991a). One-day-old turkey pouls were divided into 13 groups, each comprising ten birds. Twelve groups received intraocular inoculations, each with a different virus (A–M), at a dose of 10^3.0 TCID_50 per poult. The final group was not inoculated. Clinical disease was assessed on a daily basis. At 3 weeks of age, half of the uninoculated control group was combined in one room, together with all of the birds from the inoculated groups and all were challenged by intraocular inoculation with virulent field virus strain #8544 at a dose of 10^3.0 ID_50 per poult. This ensured that all birds receiving a virulent challenge were in identical conditions and received the same challenge. For each bird, the clinical signs were assessed prior to its identification by wing band number and then scored on a daily basis until signs ceased, using a four-point scoring system, with a score of 0 representing no signs, 1 representing clear nasal exudates, 2 representing turbid nasal exudates and 3 representing swollen infraorbital sinus and/or frothy eye (Catelli et al., 2006). The disease severity for each group was expressed by summing the daily mean scores for the entire period of clinical disease to give a single cumulative score for each.

Sequence difference confirmed using a new RNA extraction from the relevant virus followed by RT-PCR spanning the base(s) in question. The remaining eight cloned vaccine viruses were sequenced in all regions where any differences were found between strain #8544, vaccine P20 and purified viruses C, F, H and K. This included the putative mutation together with a flanking sequence of about 250 bases in both directions. Subsequently, the G gene of purified virus I was fully sequenced after detection of additional mutations at the start of the G gene.

Western blotting of cloned viruses. Protein expression of selected viruses was studied by Western blotting. Viruses F, K, G and L were grown in Vero cells and 10^3.5 TCID_50 of each was purified by ultra-centrifugation through a 25 % sucrose cushion followed by protein separation on 10 % SDS-PAGE gels. The proteins were electrophoresed onto nitrocellulose and incubated with serum raised during natural infection of turkeys with strain #8544. As a control, a separate gel was silver stained to confirm that similar amounts of material were present in each sample (not shown). The bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and diaminobenzidine as the substrate.

Generation of recombinant viruses. Recombinant viruses, based on the subgroup A AMPV strain CVL-14/1, were created by reverse genetics. Full-length cDNA copies of the virus genome were prepared with modifications to the sequence to insert start and stop enzyme recognition sites before the N gene initiation codon, in the L gene untranslated region and before the gene-start signals of the P, M, SH, G and L genes. The parental clone, designated F2, was modified by introducing the sequence of the SH gene-end and SH–G intergenic region from protective C and unprotective F and H viruses. This was achieved by PCR amplification of the region from the intergenic M2–SH to the intergenic region of the SH–G genes including restriction sites enabling it to be inserted into F2 digested with the same enzymes. Virus was rescued from these plasmids in the T7 RNA polymerase-expressing cell line BSR-T7 (Buchholz et al., 1999) following transfection with plasmids containing the full-length antigenome and the N, P, M2-1 and L genes in pCTIE4 (Novagen). Virus was propagated in BS-C-1 cells and cells infected at the third passage were used for RNA extraction.

Measurement of SH, G and SH–G readthrough mRNA levels. RNA was extracted from BS-C-1 cells infected with viruses C and F or recombinant virus using an RNeasy Midi kit (Qiagen). RNA (1 µg per lane) was analysed by Northern blotting using glyoxal gels along with serial dilutions of in vitro transcripts [generated using Megascript T7 or T3 kits (Ambion) and purified on 5 % acrylamide/urea gels] corresponding to the SH or G genes to allow quantification of RNA levels. Blots were probed with antisense RNA probes generated with a Maxi-script kit (Ambion) directed against the SH or G genes and containing 5 % of a probe generated against the Millenium markers (Ambion), which were included on each gel. Hybridized and washed blots were wrapped in cling film and exposed to an imaging plate for reading in an FLA5000 imager (Fuji). Analysis of 16-bit TIFF files was carried out with TOTALLAB 2003 (nonlinear dynamics) and Microsoft EXCEL to give the number of copies of each specific RNA (µg total RNA)^-1.

Use of a dicistronic minigenome to measure the effects of different SH gene-end regions on a downstream reporter. Overlap-extension PCR was used to alter the gene-end region of the first gene in a dicistronic minigenome, as described previously (Edworthy & Easton, 2005), to that of strain #8544 or of virus C, F or H sequence. The sequences in the minigenome constructs contained the desired SH gene end and the subsequent intergenic region sequence of the appropriate virus (Fig. 1). The minigenomes were rescued in a recombinant vaccinia T7 virus-based transfection system,
and chloramphenicol acetyl transferase (CAT) and luciferase (Luc) reporter expression were measured as described previously (Edworthy & Easton, 2005).

RESULTS

Clinical assessment of plaque-purified virus from P20 vaccine stock

All of the viruses were attenuated for disease and no clinical signs were seen after inoculation with any of the purified viruses. After challenge with virulent virus, the progression of clinical signs was followed and details are given in Table 1. Only seven of the purified viruses generated high levels of protection, two were partially protective and three (viruses F, H and L) had similar levels of disease to the unvaccinated and challenged control group. Sera from all vaccinated groups were weakly positive prior to challenge, showing that the viruses had replicated to levels sufficient to induce an immune response (Table 1). There was no significant difference in antibody titres among groups receiving different viruses.

**Table 1.** Cumulative clinical scores of poults inoculated at 1 day of age with plaque-purified viruses and challenged at 3 weeks of age

Animals were bled to prepare sera for ELISA 1 day before challenge.

<table>
<thead>
<tr>
<th>Plaque-purified virus</th>
<th>Disease post-challenge</th>
<th>Description of clinical signs</th>
<th>Cumulative clinical score</th>
<th>Mean ELISA titre*</th>
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<tr>
<td>A</td>
<td>None</td>
<td>0.0</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Mainly clear nasal exudate</td>
<td>2.5</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>0.0</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Mainly clear nasal exudate</td>
<td>2.4</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>None</td>
<td>0.0</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Severe signs</td>
<td>7.2</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>None</td>
<td>0.0</td>
<td>6.8</td>
<td></td>
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<tr>
<td>H</td>
<td>Severe signs</td>
<td>8.2</td>
<td>7.0</td>
<td></td>
</tr>
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<td>J</td>
<td>None</td>
<td>0.0</td>
<td>6.4</td>
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<td>K</td>
<td>None</td>
<td>0.0</td>
<td>6.3</td>
<td></td>
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<tr>
<td>L</td>
<td>Severe signs</td>
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</tr>
<tr>
<td>None</td>
<td>None</td>
<td>10.8</td>
<td>5.4</td>
<td></td>
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</table>

*Log₂ ELISA titres pre-challenge. Titres >6.0 indicate significant APMV antibody titres. All groups showed low but significant antibody titres. Differences between protected and unproctected groups were not significant.
Nucleotide sequences of protective and non-protective viruses

The complete sequences of the genomes of the pathogenic field strain #8544 and the protective virus P20 derived from it were determined. As shown in Table 2, comparison of the consensus sequences of the #8544 and P20 genomes identified a total of nine base substitutions that had occurred during the attenuation process. Only three of these differences resulted in changes in amino acid sequence: two in the F protein and one in the M2-1 protein. When the two complete genomic sequences of the fully protective viruses C and K were determined, they were found to be identical to the P20 consensus sequence, indicating this to be the dominant sequence found in the population of viruses in the P20 stock. In contrast, the genomes of two (F and H) of the three viruses conferring negligible protection differed from those of the protective viruses only in the SH intergenic region where there were single point substitutions in the SH transcription termination (gene-end) sequence. A summary of the sequences in the SH gene-end, SH–G intergenic and G gene-start sequences is shown in Fig. 1.

Sequencing of the remaining eight plaque-purified viruses in regions spanning the 11 mutations that arose during the conversion of strain #8544 to P20 found them all to be identical to the P20 consensus sequence, indicating this to be the dominant sequence found in the population of viruses in the P20 stock. In contrast, the genomes of two (F and H) of the three viruses conferring negligible protection differed from those of the protective viruses only in the SH intergenic region where there were single point substitutions in the SH transcription termination (gene-end) sequence. A summary of the sequences in the SH gene-end, SH–G intergenic and G gene-start sequences is shown in Fig. 1.

Western blots of protective and non-protective viruses

Proteins prepared from Vero cells infected with selected representative plaque-purified viruses (protective G and K; non-protective F and L) were analysed by Western blotting using anti-AMPV serum prepared in birds infected twice by the respiratory route (Fig. 3). For all viruses, a strong band was seen at 55 kDa, which has been identified previously as the F protein (Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988). Protective viruses K and G both produced a band of approximately 85 kDa corresponding to the G protein (Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988). As shown previously, the heavily glycosylated protein was seen as a faint, diffuse band. Larger bands were also seen, which have been described as G protein multimers (Cavanagh & Barrett, 1988; Collins & Gough, 1988; Ling & Pringle, 1988). For non-protective viruses, the intensity of the G protein bands was much lower relative to the G protein bands of protective viruses. This suggested that expression of the G protein was reduced in the viruses that did not confer protection from infection compared with those that protected against challenge.

<table>
<thead>
<tr>
<th>Nucleotide position*</th>
<th>Genome location</th>
<th>Strain #8544</th>
<th>Vaccine P20</th>
<th>Amino acid alteration on attenuation</th>
<th>All protective clones</th>
<th>Virus F</th>
<th>Virus H</th>
<th>Virus L†</th>
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</thead>
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<tr>
<td>2941</td>
<td>F gene start</td>
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<td>U</td>
<td>U</td>
<td>U</td>
<td>C</td>
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<td>A</td>
<td>G</td>
<td>V→A</td>
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<tr>
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<td>U</td>
<td>E→K</td>
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<td>U</td>
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<tr>
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<td>M2-1</td>
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<td>C</td>
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<td>C</td>
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<td>M2-1</td>
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<tr>
<td>5929</td>
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<td>C</td>
<td>C</td>
</tr>
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<td>SH gene end</td>
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<td>None</td>
<td>A</td>
<td>U</td>
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<td>A</td>
</tr>
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<td>5949</td>
<td>SH gene end</td>
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<td>U</td>
<td>None</td>
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<td>U</td>
<td>A</td>
<td>U</td>
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<td>6358</td>
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<tr>
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<td>A</td>
<td>C</td>
<td>None</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>11624</td>
<td>L</td>
<td>A</td>
<td>G</td>
<td>None</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
</tr>
</tbody>
</table>

*Sequences are shown in the 3′→5′ genomic sense.
†Sequence changes in the G gene are not shown.
Effect of SH gene-end mutations on levels of SH–G dicistronic RNAs and G gene expression

The effects of the SH gene-end mutations on transcription of the downstream G gene were determined using RNA extracted from cells infected with the plaque-purified viruses. This was studied further using recombinant viruses containing the same mutations in the SH–G intergenic region.

Total cellular RNA was prepared from virus-infected cells, separated by electrophoresis, blotted and probed. The results of samples probed with plus-strand-specific SH and G gene probes are shown in Fig. 4(a, b). The SH gene probe showed that the wild-type (F2, equivalent to #8544) and protective virus SH gene ends (Fig. 4b, tracks C and rC) allowed only negligible readthrough into the G gene, as shown in Table 3. In contrast, a significant level of SH–G gene readthrough mRNA was seen for all non-protective vaccine SH gene ends (Fig. 4a, b, tracks F, rF and rH). The ratios of G gene transcripts to SH gene transcripts (SH\(^+\) dicistronic SH–G), as calculated from G- and SH-probed blots, are shown in Table 3. In general, the relative amount of G gene transcribed was lower in the non-protective viruses, but only to a marked degree for rF.

To determine the effect of the SH gene-end mutations directly on the level of downstream gene protein expression, a reverse-genetics approach involving a dicistronic synthetic minigenome was used. A similar approach has been used previously to determine the effect of mutations in AMPV gene-start sequences (Edworthy & Easton, 2005). A minigenome was constructed containing two reporter genes, CAT and \(\text{luc}\), with the CAT gene positioned to be transcribed first from the synthetic genome. The wild-type gene-end, intergenic and second gene-start sequences were those found in strain #8544, and constructs containing the SH gene-end sequences from plaque-purified viruses C, F and H were also generated. The plasmids containing the

Fig. 2. Comparison of the sequences of the G genes of the P20 and plaque-purified L viruses. The nucleotide sequence of the G gene of P20 virus is shown with the amino acid sequence of the predicted G protein below. The nucleotide sequence of the G gene of virus L is shown above the P20 virus sequence and differences in its G protein sequence are shown above.

Fig. 3. Western blot analysis of AMPV proteins. Proteins from Vero cells infected with vaccine stock P20 and plaque-purified viruses F, G, K and L were purified by ultracentrifugation and separated by SDS-PAGE, blotted and probed with polyclonal antisera collected from turkeys infected with APV under experimental conditions. A similarly purified but uninfected cell preparation was also used. Molecular masses are indicated.
minigenomes under the control of the bacteriophage T7 promoter, together with plasmids expressing the AMPV N, P, L and M2-1 genes, also under the control of a T7 promoter (Naylor et al., 2004), were transfected into cells infected with a recombinant vaccinia virus expressing the T7 RNA polymerase.

The levels of expression of CAT protein (measured by ELISA) and Luc (in arbitrary units) were expressed as a ratio (Luc/CAT) for each minigenome. In Table 3 and Fig. 4(c), each ratio is given as a percentage of the ratio of the wild-type #8544 virus (which gave the highest ratio). This approach provided a relative measure of expression of the second gene compared with the first gene for the various sequences. The level of expression of the luc gene in protective virus C was almost identical to that of the parental virus. However, for the sequences from the two non-protective viruses, the level of expression of the luc gene was significantly reduced compared with the protective viruses, with the sequence from virus F causing the most reduction (43% of the control). This demonstrated that the alteration in the SH gene-end sequence that altered the level of readthrough mRNA at the SH–G gene junction also reduced the level of expression of the downstream gene. This was in agreement with the reduction in the level of G protein seen in the Western blots of the virus proteins (Fig. 3).

![Fig. 4. Analysis of the effect on transcription and gene expression of alterations in the SH gene-end sequence. (a) Northern blot of mRNA extracted from cells infected with the plaque-purified viruses C and F, or recombinant viruses with the SH gene end corresponding to #8544 (F2) or to clones C, F or H (rC, rF or rH, respectively). The blot was probed with a G gene-specific probe. The positions of molecular size markers are indicated on the right. For quantification purposes, various amounts of AMPV-specific RNA synthesized in vitro were included on the gel and are indicated as the log10 of the number of molecules. The positions of the SH, G and readthrough mRNAs are indicated. Also indicated is the position of the antigenome RNA, which was also detected by the negative-sense probe. (b) Northern blot similar to that described in (a) but probed with an SH gene-specific probe. (c) Graph showing the relative levels of expression of the luc gene compared with the CAT gene in a dicistronic AMPV minigenome assay. The minigenomes contained the SH gene-end and SH–G gene intergenic regions from the viruses indicated (see also Fig. 2). The levels of expression were determined as described previously (Edworthy & Easton, 2005).]
Table 3. Effect of different SH gene-end sequences on readthrough and downstream gene expression.

The quantification of mRNA was derived from Northern blots of RNA from virus-infected cells (Fig. 4a, b). The relative levels of expression of the luc gene compared with the CAT gene in a dicistronic minigenome construct are presented as the mean of data derived from Luc and CAT assays on plasmid rescue of dicistronic minigenomes, as shown in Fig. 4(c). Ratios are expressed as a percentage of that obtained with the wild-type strain #8544 SH gene-end sequence.

<table>
<thead>
<tr>
<th>Virus/SH–G sequence in minigenome</th>
<th>Fractional readthrough at SH gene end*</th>
<th>Ratio of G RNA to SH–SH–G RNA†</th>
<th>Luc/CAT ratio as a percentage of #8544‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>#8544</td>
<td>0.009</td>
<td>0.423</td>
<td>100</td>
</tr>
<tr>
<td>Virus C</td>
<td>0.012</td>
<td>0.347</td>
<td>99</td>
</tr>
<tr>
<td>rC§</td>
<td>0.007</td>
<td>0.466</td>
<td></td>
</tr>
<tr>
<td>Virus F</td>
<td>0.314</td>
<td>0.294</td>
<td>43</td>
</tr>
<tr>
<td>rF§</td>
<td>0.683</td>
<td>0.173</td>
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<tr>
<td>rH§</td>
<td>0.231</td>
<td>0.380</td>
<td>79</td>
</tr>
</tbody>
</table>

†Comparison of the fraction of G RNA relative to transcripts initiated at the upstream gene (SH + SH–G dicistronic RNA) determined from SH- and G-probed Northern blots.
‡Comparison of Luc (measured as relative light units) to CAT (measured as pg CAT) ratios, expressed as a percentage of the highest value.
§Virus on which the recombinant virus was based.

DISCUSSION

It is generally accepted that a small number of alterations in genome sequence can lead to dramatic differences in pathogenicity. This has led to the proposal that it will be possible to develop rationally designed vaccine candidates lacking pathogenicity determinants. However, another important aspect of a vaccine is its ability to establish protection from challenge. The parameters that affect the ability to confer protection have been little studied, but it will be necessary to clarify what they are for successful vaccine design. In our current study, analysis of 12 plaque-purified viruses derived from the original attenuated and protective P20 stock showed that all were still attenuated, but three failed to confer significant protection when tested as candidate vaccines (Table 1). The remaining plaque-purified viruses gave either complete or near-complete protection from challenge and presumably represented the majority of the protective virus in the original vaccine stock.

The complete genomic sequences of the parental pathogenic strain #8544 and the P20 vaccine derived from it were determined to locate the mutations associated with the loss of pathogenicity and the inability to protect from infection. This indicated that there were only nine nucleotide changes between the two viruses, only three of which resulted in amino acid changes, two in the F protein and one in the M2–1 protein, whilst of the remainder, four were silent mutations and two occurred in a non-coding region.

The complete genome sequences of two protective viruses, C and K, and two non-protective viruses, F and H, were determined. The protective viruses were identical in sequence to the P20 consensus sequence. In contrast, the non-protective viruses, F and H, both contained a single mutation, not present in the protective viruses, in the transcription termination signal of the SH gene (Fig. 1). The mRNA profile of the parental and selected plaque-purified viruses showed that the level of transcriptional readthrough at the SH–G gene junction in the non-protective viruses was considerably greater than that seen for protective viruses. In addition, a similar alteration in the frequency of SH–G readthrough was seen in recombinant viruses engineered to contain the altered SH gene-end sequence (Fig. 4a; Table 3). When the levels of gene expression for a series of dicistronic minigenomes containing SH gene-end sequences from either the protective or non-protective viruses was assessed, the level of protein expression from the downstream gene was significantly reduced compared with the control (Fig. 4c), indicating that the alteration in transcriptional readthrough led to a reduction in gene expression of the downstream gene, which was that for the G protein in the virus genome (Fig. 4b; Table 3).

A Western blot of the P20 vaccine and the four plaque-purified viruses using polyclonal antiserum raised during infection of turkeys by a natural route under experimental conditions showed antibody responses primarily to the F protein, irrespective of whether the viruses were protective or not (Fig. 3). Assuming that there were similar responses in the inoculated birds, this indicates that, for AMPV, an antibody response to the F protein alone is not sufficient to protect birds from challenge. The detection of high levels of antibodies directed against the F protein after infection by a natural route is in contrast to the antibody profile seen for other similar viruses in mammals. However, the immune systems of mammals and birds are significantly different. It should be borne in mind that a previous study showed that virus stock P20 fully protected bursectomized turkeys where antibody was undetectable (Jones et al., 1992). Equally, turkeys with high levels of circulating antibody can be unprotected (Naylor et al., 1997b). This implies that viruses that do not protect are likely to have lost T-cell rather than B-cell epitopes. It is possible that the non-protective viruses may not be expressing sufficient levels of key T-cell epitopes on the G protein gene.

Whilst Western blotting of the proteins from selected protective and non-protective viruses only showed a weak diffuse band in the region corresponding to the reported size for the G protein, this was noticeably reduced in blots of non-protective viruses (Fig. 3). Taken together, these
data strongly suggest that an alteration in the balance of level of expression of virus proteins, in particular a reduction in the relative level of the G protein, can significantly reduce the potential protective efficacy of a vaccine candidate.

Alterations of transcription termination signals have been shown to have an effect on transcriptional readthrough, but did not result in any significant difference in the ability of the altered viruses to replicate in vitro or in vivo (Tran et al., 2004). An A → G substitution (genomic sense) at position 5937 (strain #8544 numbering) in the SH gene end inadvertently introduced into a recombinant AMPV has been shown to produce increased readthrough at the SH–G gene junction and reduced expression of G mRNA (R. Ling and A. J. Easton, unpublished data).

The conservation of strain #8544 sequences in plaque-purified virus L at positions 2941 and 11624 might lead to the expectation that it would be more virulent and more protective than the other clones. However, the G protein gene in virus L also contained extensive mutations, which may have resulted in the reduced level of protection to challenge as well as the absence of virulence (Fig. 2). The predicted protein sequence of the G protein of L contained more potential O-linked glycosylation sites than was the case for the G protein of the other plaque-purified viruses. The additional potential glycosylation sites were at positions 109 (I → T), 130 (I → S), 170 (I → T) and 217 (M → T), whilst two were lost at positions 45 (T → I) and 160 (S → P; Fig. 2). The origin of the G gene mutations in virus L is unclear, but it may have been present as a subpopulation in the original strain #8544 isolate or have been generated some time later during passage in tissue culture. A possible explanation for the A → G transitions in the genomes of negative-strand RNA viruses involving cellular adenine deaminase enzymes has been described (Bass et al., 1989).

Reduction of G gene expression or sequence variations in it therefore appear to reduce protection afforded by potential vaccine viruses. The importance of the G protein in generating a fully protective response has been demonstrated in respiratory syncytrial virus using a recombinant virus in which the G gene was deleted (Johnson et al., 2004). However, this contrasts with the data from human metapneumovirus (HMPV) where a virus lacking the G gene was shown to confer effective protection to African Green monkeys in challenge trials (Biacchesi et al., 2005). The reasons for the differences seen with HMPV are not clear, and it is possible that this reflects differences in the host species or in the challenge protocols in the different systems. In the current study, it was possible that the reduction in protection observed in protective viruses was due in part to a reduction in replication rates of non-protective viruses. Serology indicated that replication occurred, but this may not be a measure of the relative levels of in vivo replication when comparing protective and non-protective viruses.

The data presented here clearly show that the features required in an effective vaccine – the ability to replicate in the host without causing disease and the ability to confer protection from subsequent infection – are quite different. Also, the factors that determine whether a vaccine candidate can confer protection from disease can be very subtle and can be affected by as little as a single point mutation. The data also show that a critical factor for AMPV in determining the ability to confer protection is the balance in levels of G protein expression compared with other virus proteins, even when the other major antigen, the F protein, is expressed at effective levels. It will be of interest to explore further the potential effects of altering the balance of other virus proteins to determine whether this is a G protein-specific effect or whether it applies more generally to the overall balance of virus protein expression.

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REFERENCES


