Site-directed mutagenesis of the VP2 gene of Chicken anemia virus affects virus replication, cytopathology and host-cell MHC class I expression

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Chicken anemia virus (CAV) is an immunosuppressive pathogen of chickens. To further examine the role of viral protein 2 (VP2), which possesses dual-specificity protein phosphatase (DSP) activity, in viral cytopathogenicity and its influence on viral growth and virulence, an infectious genomic clone of CAV was subjected to site-directed mutagenesis. Substitution mutations C87R, R101G, K102D and H103Y were introduced into the DSP catalytic motif and R129G, Q131P, R/K/K150/151/152G/A/A, D/E161/162G/G, L163P, D169G and E186G into a region predicted to have a high degree of secondary structure. All mutant constructs were infectious, but their growth curves differed. The growth curve for mutant virus R/K/K150/151/152G/A/A was similar to that for wild-type virus, a second cluster of mutant viruses had an extended latent period and a third cluster of mutant viruses had extended latent and eclipse periods. All mutants had a reduced cytopathogenic effect in infected cells and VP3 was restricted to the cytoplasm. Mutation of the second basic residue (K102D) in the atypical DSP signature motif resulted in a marked reduction in virus replication efficiency, whereas mutation of the first basic residue (R101G) attenuated cytopathogenicity, but did not reduce replication efficiency. Expression of major histocompatibility complex (MHC) class I was markedly downregulated in cells infected with wild-type CAV, but not in those infected with mutants. This study further demonstrates the significance of VP2 in CAV replication and shows that specific mutations introduced into the gene encoding this protein can reduce virus replication, cytopathogenicity and downregulation of MHC I in infected cells.

INTRODUCTION

Chicken anemia virus (CAV) is an immunosuppressive pathogen of chickens and a member of the genus Gyrovirus within the family Circoviridae (Pringle, 1999). CAV infection is ubiquitous in poultry flocks worldwide and significant production losses can occur as a consequence of both clinical and subclinical infections of commercial poultry (Fadley et al., 1994; Farkas et al., 1996; McNulty et al., 1988, 1989; von Bulow & Lesjak, 1987; Yuasa, 1983; Yuasa et al., 1983). Infections are of increasing concern because of their role in vaccination failure, reduced production performance and potentiation of other infectious diseases, including Marek’s disease (Barbour et al., 2002; Bisgaard, 1983; Caterina et al., 2004; Chettle et al., 1988; Engstrom et al., 1988; Markowski-Grimsmrud & Schat, 2003; McIlroy et al., 1992; McNulty, 1997; McNulty et al., 1988; Miller & Schat, 2004; Sommer & Cardona, 2003; Vielitz & Landgraf, 1988). In addition, as the CAV viral protein 2 (VP2) protein has functional homology to the homologous ORF2 of the group of viruses known collectively as TT viruses (TTV), CAV could be a model for investigations of these viruses, which have been detected in a high proportion of humans, but which are not cultivable and are of uncertain pathogenic significance.

CAV encodes three viral proteins on overlapping reading frames: VP1, the 52 kDa structural capsid protein; VP3, a 13.8 kDa virulence factor known to induce apoptosis in transformed cell lines; and VP2, a 28 kDa dual-specificity protein phosphatase (DSP) (Peters et al., 2002). The signature motif for the serine, threonine and tyrosine phosphatase activity has been identified in VP2 and key catalytic residues have been identified by expression and mutagenesis studies.

Mutagenesis of either of the cysteine residues to serines at positions 95 and 97 within the catalytic motif of VP2

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markedly reduced virus replication in cell culture (Peters et al., 2002). This suggests that VP2 protein phosphatase activity is not essential, but is required for efficient replication. The definition of a precise biochemical function for CAV VP2 as a DSP greatly facilitates the design of a mutagenesis strategy and provides a rational basis for the generation of mutants with altered VP2 function. However, it is likely that CAV VP2 is a multifunctional protein with a non-structural role in virus infection and replication. Expression studies by Noteborn et al. (1998) have shown that co-expression of both VP1 and VP2 was required for the generation of recombinant proteins capable of inducing neutralizing antibodies. Thus, VP2 may also have a role as a scaffolding protein during virion assembly and hence the effect of VP2 mutations that alter protein secondary structure could be to slow virus assembly.

The objective of this study was to examine the role of VP2 in viral cytopathogenicity and its influence on growth characteristics of the virus. Our previous studies of viruses with mutations affecting the two cysteine residues at the DSP catalytic site found that these mutations resulted in a marked reduction in replication efficiency and infectivity. Thus, sites that might be expected to only subtly modify DSP activity or to maintain DSP activity were targeted.

**METHODS**

**Analysis of CAV genome and design of sites for mutagenesis.**

Mutagenesis studies were carried out on an infectious genomic clone of CAV strain CAU269/7, pCAU269/7 (Brown et al., 2000). The entire genome of this infectious clone has been sequenced and the sequence submitted to Genbank (accession no. AF227982). Sites were targeted for mutagenesis on the basis of VP2 functional analysis (Peters et al., 2002, 2005) and protein structural analysis. The key residues C87, R101, K102 and H103 were identified within the protein loop associated with the DSP signature motif by comparison with known protein tyrosine phosphatase (PTPase) structures (Peters et al., 2002, 2005) (Fig. 1). Residues were also targeted in a region of VP2 predicted to have a high degree of secondary structure. VP2 protein structural predictions were made using software available through the Australian National Genomic Information Service interface. Protein modelling was used to derive tertiary structural predictions using the Swiss Pdb Viewer software available through the EXPASY (Expert Protein Analysis System) server of the Swiss Institute for Bioinformatics (www.expasy.ch/spdbv). Residues targeted for mutagenesis on the basis of structural analysis of VP2 were R129, R/K/K150/151/152G/A/A, D/E161/162G/G, L163P, R129G, Q131P, R/K/K150/151/152G/A/A, D/E161/162G/G, L163P, D169G and E186G. In the first stage, the reaction was incubated at 95°C for 2 min, followed by 15 cycles of 96°C for 40 s, 60°C for 60 s and 68°C for 40 s, with a final incubation at 68°C for 5 min. In the first stage, the reaction was incubated at 95°C for 2 min, followed by 30 cycles of 95°C for 40 s, 60°C for 60 s and 68°C for 40 s, with a final incubation at 68°C for 5 min. The first-stage template was removed by digestion with DpnI (Boehringer Mannheim) and the stage-one products were purified by gel electrophoresis and Qiaex II (Qiagen) gel extraction according to the manufacturer’s instructions. The second-stage PCR was incubated at 96°C for 2 min, followed by 15 cycles of 96°C for 40 s, 58°C for 60 s and 72°C for 60 s, with a final incubation at 72°C for 5 min.

The PCR products were digested with Stul and BsuI (both from New England Biolabs) and purified by gel electrophoresis as above. The

![Fig. 1. Model of VP2 structure obtained by threading of the VP2 sequence onto the solved muPTPA structure. β-Strand is shown as black arrows and α-helix as grey blocks. Features of significance in the muPTPA structure are the catalytic cleft formed by the convergence of the β-sheet and the WPD (protein loop found adjacent to the catalytic site in all receptor-PTPases) and P loops (protein loop) within the cleft. The positions of mutated residues in the VP2 model are annotated. The C95 residue is the catalytic cysteine for the protein phosphatase. The C97 residue is located within the protein phosphatase signature motif and has a differential effect on activity for phosphothreonine/phosphoserine as opposed to phosphotyrosine substrates.](image)

PCR was carried out in two stages. The first stage consisted of a set of two PCRs: one from an upstream flanking primer to the negative-sense mutagenesis primer and the other from a downstream flanking primer to the positive-sense mutagenesis primer. In the second stage, PCR products from the first stage were used as the template for a PCR using only the flanking primers. All PCRs comprised 100 μl containing 300 μM each of dATP, dCTP, dGTP and dTTP, 2 mM MgSO4, 0.2 μM of each primer, 10 μl 10× Platinum Pfu Taq DNA polymerase buffer, 2 U Platinum Pfu Taq DNA polymerase (Promega) and 0.1 μl template DNA.

The upstream flanking primer CAV.5, 5′-GGGAGCCGCAGG-GGC-3′, and downstream flanking primer CAV.4, 5′-TGCTCAG-GTGTGCAAGGC-3′, were used for the construction of R101G, R129G, Q131P, R/K/K150/151/152G/A/A, D/E161/162G/G, L163P, D169G and E186G. In the first stage, the reaction was incubated at 95°C for 2 min, followed by 30 cycles of 95°C for 40 s, 60°C for 60 s and 68°C for 40 s, with a final incubation at 68°C for 5 min. The first-stage template was removed by digestion with DpnI (Boehringer Mannheim) and the stage-one products were purified by gel electrophoresis and Qiaex II (Qiagen) gel extraction according to the manufacturer’s instructions. The second-stage PCR was incubated at 96°C for 2 min, followed by 15 cycles of 96°C for 40 s, 58°C for 60 s and 72°C for 60 s, with a final incubation at 72°C for 5 min.

The PCR products were digested with Stul and BsuI (both from New England Biolabs) and purified by gel electrophoresis as above. The
plasmid pCAU269/7 was also digested with StuI and BsmI to remove the region of 357 bp to be replaced with the mutated sequence and the remainder of the plasmid was purified by gel electrophoresis. The PCR product was then ligated to the plasmid. *Escherichia coli* strain DH5α was electrottransformed with the ligated plasmid and transformants were selected by culture at 37°C on Luria–Bertani agar containing 50 μg ampicillin ml⁻¹ (Sambrook et al., 1989). Clones were screened for the presence of the insert by PCR using the forward primer CAV.5 and reverse primer CAV.4 in a 100 nl reaction mixture containing 300 μM each of dATP, dCTP, dGTP and dTTP, 2 mM MgCl2, 0.2 μM of each primer, 10 μl 10× *Taq* DNA polymerase buffer (Promega), 2 μl Taq DNA polymerase (Promega) and 2 μl template DNA. The reaction was incubated at 96°C for 2 min, followed by 40 cycles of 96°C for 40 s, 63°C for 60 s and 72°C for 60 s, and a final incubation at 72°C for 5 min. The cloned DNA was sequenced using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Perkin Elmer) and primers CAV.5 and CAV.4.

The construction of K102D was as described for R101G, R129G, Q131P, R/K/K150/151/152G/A/A, D/E161/162G/G, L163P, D169G and E186G but, following mutagenesis, the PCR product was cloned initially into the pGEM-T-2 vector (Promega), the plasmid DNA was digested with *Stu*I and *Bsm*I and the CAV gene fragment was subcloned into the digested pCAU269/7.

Mutations C87R and H103Y were generated by full-circle, overlap extension mutagenesis in a single PCR. The reaction mixture was as described above, except that only the relevant mutagenesis primers were included. The reaction was incubated at 96°C for 2 min, followed by 1 cycle of 96°C for 40 s, 55°C for 60 s and 68°C for 5 min, then 40 cycles of 96°C for 40 s, 60°C for 60 s and 68°C for 5 min, with a final incubation at 68°C for 5 min. Template DNA was removed by digestion with *Dpn*I and the PCR product was purified by phenol/ chloroform/isoamyl alcohol (25:24:1, by volume) extraction and ethanol precipitation. The PCR product containing C87R and H103Y was cloned by 1 cycle of 96°C for 5 min. The cloned DNA was sequenced using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Perkin Elmer) and primers CAV.5 and CAV.4.

The number assigned to mutations refers to the VP2 amino acid sequence. Mutated bases are highlighted in bold.

**Table 1. Primers used for site-specific mutagenesis within CAV VP2 gene sequence**

<table>
<thead>
<tr>
<th>Mutation in CAV VP2</th>
<th>Positive-sense oligonucleotide†</th>
<th>Negative-sense oligonucleotide†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C87R</td>
<td>CTGCCGCGAACGCTCGGGTTCCACGGTAAAG</td>
<td>AACCGCGAGGTTGCGCACGCCACACAGCA</td>
</tr>
<tr>
<td>R101G</td>
<td>CTGCCGAAATCTCGGAAACACTGAG</td>
<td>CAGTGTTCGTCAGTTTCGCCAGA</td>
</tr>
<tr>
<td>K102D</td>
<td>CTGCCGCAATTCGAGAACACTGGCTTTC</td>
<td>GAAACCAGTTGCTCTGAATTGGCCAGCA</td>
</tr>
<tr>
<td>H103Y</td>
<td>CAGAAAATCTGGTTTGAATGCTGGCGCAGAC</td>
<td>GAAACGAGTATTTGCTGATTGGCCAGAC</td>
</tr>
<tr>
<td>R129G</td>
<td>CTGCCGACCCCCTCGGAGTACAGGG</td>
<td>CCGCTAGCCGGGCTGGCAGGATCGC</td>
</tr>
<tr>
<td>Q131P</td>
<td>CGAGTGACCGAACTGCAAGCTGCTAAAG</td>
<td>CGCITTACCGGTACTGCGAGG</td>
</tr>
<tr>
<td>R/K/K150/151/152G/A/A</td>
<td>CCGAAGCGCGCCGCGGCTGGTATAAG</td>
<td>ATACAGCGGCCGCGCTGGCGGTC</td>
</tr>
<tr>
<td>D/E161/162G/G</td>
<td>TAAGATGCGCAAGCGCGGCTGGCAGACC</td>
<td>TGCGAGGCGCTGTCGATCTC</td>
</tr>
<tr>
<td>L163P</td>
<td>GACGAGGCCGCAAGCCAGAGGAG</td>
<td>GGCCTCTGGTCTGGCGGCTGTC</td>
</tr>
<tr>
<td>D169G</td>
<td>GAGAGGGCGGTTTTACCGCTCTACG</td>
<td>GGCTAAAACCGGCCTCAGGGTC</td>
</tr>
<tr>
<td>E186G</td>
<td>GCGACTTGCGACGGATATAATTTC</td>
<td>TTATATCTCCGTCGAAAGTCGC</td>
</tr>
</tbody>
</table>

†Mutated bases are highlighted in bold.

**Transfection of mutated viral genomes into MDCC-MSB1 cells.** Transfection of MDCC-MSB1 cells with viral genome was used to generate mutant virus from genomic DNA that had been mutagenized in vitro. Plasmid DNA was digested with EcoRI (New England Biolabs) to release the CAV genome, which was purified by gel electrophoresis and extraction and resuspended in sterile 10 mM Tris (pH 8.0). MDCC-MSB1 cells were washed twice in RPMI 1640 medium (Sigma–Aldrich), resuspended at a final concentration of 10⁶ cells ml⁻¹ and 700 μl was added to 10 μg DNA in a microfuge tube on ice. Transfection was performed in a 0.4 cm gap electroporation cuvette in a Gene Pulser apparatus (Bio-Ray) set at 400 V, 900 μF, ∞ resistance and extension capacitance. The cells were incubated at room temperature for 5 min, then resuspended in 5 ml warm growth medium. The cells were grown as suspension cultures at 37°C in 5% CO₂ in RPMI 1640 medium supplemented with 2 mM glutamine (Sigma–Aldrich), 2 mM pyruvate (Sigma–Aldrich), 0.2% NaHCO₃, 50 μg ampicillin ml⁻¹, 50 μg gentamicin (Sigma–Aldrich) ml⁻¹ and 10% fetal calf serum (CSL).

**Mutant virus propagation.** Transfected cell cultures were serially passaged at a 1/10 dilution at 48 h intervals for ten passages. Infectivity was assessed every 48 h by examining the proportion of cells expressing CAV VP3 by indirect immunofluorescence staining (IFA) with mouse anti-VP3 monoclonal antibody (mAbVP3; Trobio) diluted 1/200 in 0.1% BSA/PBS/0.5% Tween 20 (BSA/ PBST) and secondary sheep anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC; Dako) diluted 1/100 in 0.1% BSA/ PBST. The presence of infectious mutant virus was further demonstrated by reinfection of cell cultures with cell-free preparations from the earliest passages of transfected cultures that had at least 50% of infected cells with CAV as determined by IFA. The culture was frozen and thawed three times and then clarified by centrifugation at 6000 g for 10 min. MDCC-MSB1 cells were then infected with the cell-free virus preparation. These infected cultures were assessed by IFA for VP3 expression. Western blotting of cell lysates using mAbVP3 was also used to confirm infectivity of mutated viruses.

DNA for CAV-specific PCR was purified from MDCC-MSB1 cells 48 h after infection using proteinase K and SDS lysis (Meehan et al., 1992) and phenol/chloroform extraction (Sambrook, 1989). The extracted DNA was digested with *Dpn*I to remove any residual transfected DNA. The mutated region was then amplified using the CAV.5 and CAV.4 primers and the PCR conditions described above for cloning screens for the presence of insert by PCR. The mutant genotypes were confirmed by sequencing of the PCR product.
Mutant viruses were titrated in 96-well trays in 200 μl volumes containing 5 × 10^5 MDCC-MSB1 cells ml^-1 using an adaptation of a method published previously by Goryo et al. (1987). Six duplicate tenfold dilution series of viral stocks were prepared, ranging from a final dilution of 0.05 to 0.5 × 10^-10, and inoculated onto cells. At intervals of 48 h, cells were passaged into fresh medium at a dilution of 1:4. Each well was scored for evidence of cytopathogenic effect (CPE) and cultures were serially passaged until no difference was detected between successive passages at the lowest dilution at which CPE was observed. The observation of CPE was confirmed by IFA with mAbVP3 of the end-point dilution.

**One-step growth curves of mutant viruses.** One-step growth curves were determined for all mutant viruses by infecting 10^6 cells at an m.o.i. of one (Flint et al., 2000). Samples of infected cell cultures were taken at 4 h intervals over a 60 h period commencing 8 h after infection. Pelleted cells and supernatant from each time point were individually titrated with six repetitions. Pelleted cells were lysed by three cycles of freeze–thawing and clarified prior to titration. The eclipse period (defined as the period between inoculation and the last time point sampled at which virus was not detectable extracellularly or intracellularly) and the latent period (defined as the time between inoculation and the last time point before extracellular virus was detected) were determined.

**Cytology.** In vitro cytopathology was assessed by phase-contrast microscopy of viable cultures and by IFA of fixed cells with mAbVP3 and counterstaining for 2 min with Hoechst stain.

Infected cells were stained 24 h after infection in separate reactions with mouse anti-chicken CD8 mAb–FITC conjugate and mouse anti-chicken CD4 mAb–FITC conjugate (both Southern Biotechnology) and with mouse anti-chicken major histocompatibility complex (MHC) I mAb–phycocerythrin (PE) conjugate (Southern Biotechnology), all diluted 1/100 in 0.1% BSA/PBS with incubation for 1 h for each staining reaction. The sensitivity and specificity of the MHC class I monoclonal antibody was determined using a dilution series of the antibody to stain MDCC-MSB1 cells. The dilution of antibody used in the study was the highest at which there was no reduction in the number of positive cells identified. The proportion of the cells stained for CD8 that were also stained for MHC I was determined using a flow cytometer (FacsCalibur; Becton Dickinson). Data were analysed using Cellquest software (Becton Dickinson). Sample populations of 10^8 cells were recorded on density plots, with the intensity of FITC staining on the x axis and the intensity of PE staining on the y axis. Quadrants established from plots of control cells were used to delineate positively and negatively stained populations.

**RESULTS**

**Analysis of CAV genome and design of sites for mutagenesis**

The substitutions R101G and K102D replaced the basic, charged residues predicted to be involved in coordination of the phosphotyrosine substrate within the catalytic cleft. Substitutions H103Y and C87R flanked the gyroivirus DSP signature motif and were of interest as they are highly conserved in TTV and CAV, both of which have DSP activity.

A region with a high degree of secondary structure was predicted towards the carboxyl-terminal end of VP2. Chou–Fasman plots of the region predicted an acidic region of z-helix, followed by a basic region of z-helix and b-sheet, and a second acidic region of z-helix. The secondary structure was subdivided by a series of proline residues. There were three predicted regions of amphipathic z-helix from residues 128 to 143, 160 to 171 and 174 to 187 and amphipathic b-sheet from residues 151 to 158. These secondary structure predictions were in agreement with those obtained using the Garnier, Osguthorpe and Robson algorithms. We predicted that the high degree of secondary structure correlated with a functional protein domain and mutations were designed to disrupt the structural organization of the region, thereby modifying any potential function. It is also possible that the predicted amphipathic helices may contribute to the properties of the protein phosphatase catalytic cleft and thereby to the activity of the enzyme.

The three-dimensional model obtained by threading VP2 onto mouse protein tyrosine phosphatase A (muPTPA) predicted an internal domain consisting of five b-strands, which would form the catalytic cleft of the protein. In both the muPTPA and VP2 models, this region had a concentration of basic residues aligned along the axis of the b-sheet. Flanking each side of the cleft are regions of z-helix. The two z-helices flanking the cleft towards the carboxyl terminus in VP2 were predicted to be acidic-faced, amphipathic helices. They formed a continuous face of negative charge across the surface of the protein. This acidic charge distribution was not found in the corresponding domain of the muPTPA model. The z-helix towards the amino side of the cleft was predicted by the Chou–Fasman algorithm to be positioned between residues 51 and 60, rather than between residues 71 and 76, as predicted by the threaded model. When the z-helix was positioned between residues 51 and 60 in the threaded model, the effect was to slightly raise the elevation of the first helix above the catalytic cleft and the loop of random coil that corresponds to the WPD loop of the muPTPA model was less exposed.

Within the region of predicted, basic amphipathic z-helix, R129G and R/K/K150/151/152G/A/A were constructed to neutralize the polar basic charge distribution in the secondary structure. The substitution Q131P was also introduced into the z-helix in this region to break the helix. An identical approach involved the introduction of L163P into a segment of basic b-strand to disrupt the conformation. The introduction of proline at positions 131 and 163 was predicted, using the GROMOS96 43BI parameter set, to increase the energy at both locations by 100-fold. These mutations would be expected to destabilize the secondary structure. The substitutions D/E161/162G/G, D169G and E186G were introduced with the objective of neutralizing the acidic charge distribution in the amphipathic helices.

**Transfection of mutated viral genomes into MDCC-MSB1 cells**

Four to five serial passages were required for wild-type virus at tenfold dilutions before an exponential increase was seen in the numbers of cells stained for VP3 expression by IFA, which was interpreted as active virus replication and infection rather than simply maintenance of transfected DNA.
constructs. The number of passages required to reach this point varied, but was in the range of five to eight passages for all mutated genomes. Mutant virus was harvested from the earliest passage at which an exponential increase in VP3 expression was observed.

**Replication competency and titre of mutant viruses**

Cell-free virus generated from each mutated genome was able to infect fresh cell cultures, as confirmed by IFA and Western blotting to detect VP3 and by CAV-specific PCR. Although replication-competent virus was generated from all mutant constructs, mutant virus Mut K102D produced maximal titres of only 10^{1.5} TCID_{50} ml^{-1}, despite repeated attempts to optimize the culture conditions. These titres were considered too low for propagation and further assessment of growth. For viruses Mut C87R, Mut R101G, Mut H103Y, Mut R129G, Mut Q131P, Mut D/E161/162G/G, Mut L163P and Mut E186G, initial titres were in the range 10^{3}–10^{4} TCID_{50} ml^{-1}. For these viruses, 400 ml infected culture was concentrated by ultracentrifugation and resuspended at 10^{4} TCID_{50} ml^{-1}.

**One-step growth curves**

One-step growth curves were determined for all viruses (Table 2). The eclipse and latent periods for wild-type CAU269/7 were found to be 24 h. The burst size for the wild-type CAU269/7 virus was 10^{6} TCID_{50} ml^{-1}. Infectious virus was present in the supernatant 8 h before it was found intracellularly and the plateau titre for intracellular virus was tenfold higher than that for extracellular virus. The eclipse period for all mutant viruses tested was approximately 40 h, with the exception of Mut R/K/K150/151/152G/A/A, for which the eclipse and latent periods were

24 h, as for wild-type virus, and Mut E186G, for which the eclipse period was 28 h and the latent period only slightly longer at 32 h. For viruses Mut C87R, Mut R101G and Mut D169G, the latent period was 40 h and infectious virus was detected in the supernatant before it was detected intracellularly. For viruses Mut R101G, Mut R129G, Mut Q131P, Mut D/E161/162G/G and Mut L163P, the latent period was in the range 44–60 h and the detection of extracellular infectious virus was delayed significantly, with virus initially being detected intracellularly. The burst size for viruses Mut H103Y, Mut Q131P, Mut D/E161/162G/G and Mut E186G was 10^{6} TCID_{50} ml^{-1}, equivalent to wild-type. The burst size for viruses Mut C87R, Mut R101G, Mut R129G, Mut R/K/K150/151/152G/A/A, Mut L163P and Mut D169G was 10^{5} TCID_{50} ml^{-1}, tenfold lower than for wild-type virus.

**CPE of mutated CAV and immunostaining of virus-infected MDCC-MSB1 cell cultures for VP3**

CPE was readily identified within 24–72 h of inoculation of MDCC-MSB1 cell cultures with wild-type CAU269/7 virus. Nuclear pyknosis and karyolysis was evident in infected cells. Irregular patterns of dark-staining densities, consistent with chromatin clumping and margination, were seen throughout the nuclei and bordering the nuclear membrane of infected cells. Hoechst staining of the nuclei was intense and clumping of the chromatin and nuclear fragmentation was evident by staining. There was cellular swelling, vacuolation of the cytoplasm and lysis of the cell membrane. Aggregates of VP3 were seen in both the nucleus and the cytoplasm by IFA.

Viruses mutated in VP2 replicated in MDCC-MSB1 cells, but induced only mild CPE. MDCC-MSB1 cells infected with viruses Mut R/K/K150/151/152G/A/A and Mut L163P

### Table 2. One-step growth curves of wild-type and mutant CAV

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Eclipse period (h)</th>
<th>Latent period (h)</th>
<th>Maximal titres (log_{10} TCID_{50})</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cell-associated Extracellular</td>
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<tr>
<td><strong>Cluster I</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Wild-type</td>
<td>24</td>
<td>24</td>
<td>6 5</td>
</tr>
<tr>
<td>Mut R/K/K150/151/152G/A/A</td>
<td>24</td>
<td>24</td>
<td>5 4</td>
</tr>
<tr>
<td><strong>Cluster II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mut C87R</td>
<td>40</td>
<td>40</td>
<td>4 5</td>
</tr>
<tr>
<td>Mut H103Y</td>
<td>40</td>
<td>40</td>
<td>6 5</td>
</tr>
<tr>
<td>Mut D169G</td>
<td>40</td>
<td>40</td>
<td>5 4</td>
</tr>
<tr>
<td>Mut E186G</td>
<td>28</td>
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<td><strong>Cluster III</strong></td>
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<tr>
<td>Mut R101G</td>
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<td>56</td>
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<td>Mut Q131P</td>
<td>40</td>
<td>44</td>
<td>5 6</td>
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<tr>
<td>Mut D/E161/162G/G</td>
<td>36</td>
<td>60</td>
<td>5 6</td>
</tr>
<tr>
<td>Mut L163P</td>
<td>40</td>
<td>60</td>
<td>5 4</td>
</tr>
</tbody>
</table>
are shown in Fig. 2 as examples of the characteristic cytopathology. Mild cellular swelling, mild cytoplasmic vacuolation and mild nuclear pyknosis were evident. CPE was more obvious in MDCC-MSB1 cell cultures infected with viruses Mut R129G, Mut Q131P, Mut R/K/K150/151/152G/A/A, Mut L163P or Mut E186G than in those infected with viruses Mut C87R, Mut R101G, Mut K102D, Mut H103Y, Mut D/E161/162G/G or Mut D169G. MDCC-MSB1 cell cultures infected with viruses Mut R129G, Mut Q131P, Mut R/K/K150/151/152G/A/A or Mut L163P were examined by immunostaining for VP3. Nuclei of cells infected with these mutant viruses stained uniformly with Hoescht stain, as in uninfected cells, and there was no evidence of nuclear fragmentation or chromatin clumping (Fig. 2). IFA for VP3 identified intensely stained, crescent-shaped aggregates of VP3 in the cytoplasm, but VP3 was not detected in the nucleus (Fig. 2).

MHC class I expression during virus infection

Whereas the majority of MDCC-MSB1 cells were CD4 

+ , a proportion were CD8 

+ and the effect of CAV infection on MHC class I expression was most pronounced in these cells. Thus, MDCC-MSB1 cells that had the phenotype CD8 

+ MHC class I 

+ , based on surface staining with fluorescently labelled antibodies and fluorescence-activated cell sorting (FACS) analysis, were examined specifically for the effect of viral infection on MHC class I expression. The intensity of MHC class I–PE surface staining in these cells followed a normal distribution (Fig. 3 and Table 3). Following CAU269/7 infection, the shape of the distribution changed markedly. A larger proportion of cells had lower levels of cell-surface expression of MHC class I and the geometric mean and median of the distribution were reduced (Table 3). The shape of the distributions and geometric means for MHC class I–PE staining intensity on cells infected with Mut R101G or Mut H103Y resembled the distribution for uninfected MDCC-MSB1 cells (Fig. 3). For cells infected with Mut Q131P, the shape of the distribution was intermediate between those of wild-type-infected and uninfected cells (Fig. 3). For this virus, there was a slight shift in the geometric mean to the left and a shoulder to the distribution, indicating a small proportion of cells with lower levels of surface expression of MHC class I.

DISCUSSION

This study and our previous study of mutants of the DSP catalytic motif of VP2 are the only studies to have examined VP2 as a target for mutagenesis. In mutagenesis experiments targeting VP3, replication-competent virus could not be recovered from mutant constructs (Danen-Van Oorschot et al., 1997, 1999, 2000). In our study, site-directed mutagenesis of VP2, based on a carefully designed strategy, was found to be an effective means of rapidly generating viruses with distinct phenotypes. The sites for mutagenesis were selected based on predictions of the protein secondary structure. The secondary structure predictions for VP2 were compared with a three-dimensional model of VP2 obtained by threading the VP2 sequence onto the solved muPTPA crystallographic structure. In general, there was a high degree of agreement between the structural predictions based on amino acid sequence alone and the model based on the muPTPA crystallographic structure, with the exception of the regions from residues 99 to 109 and from residues 116 to 123, which

Fig. 2. Cytopathology in MDCC-MSB1 cells infected with wild-type or representative mutated CAV. Upper panel, CPE as seen by phase-contrast microscopy. Lower panel, distribution of VP3 in infected cells as revealed by IFA with mAbVP3 (green, indicated by arrows) and counterstaining with Hoescht nuclear stain (blue). Mut R/K/K150/151/152G/A/A and Mut L163P are shown as examples in comparison with wild-type CAV.
were predicted to be α-helix using the Chou–Fasman algorithm and to be β-strand in the threaded model. The threading energy computed using the GROMOS96 43BI parameter set (DF, Hochschulverlag ETHZ; http://igc.ethz.ch/gromos) for residues 99–109 and 116–123 of the VP2 model was highly favourable for a β-strand conformation. On this basis, predictions of either α-helical or β-strand conformations would be equally energetically feasible for this region.

In general, DNA viruses require entry into the nucleus for replication to commence and therefore they tend to have longer latent periods than viruses replicating in the cytoplasm. CAV was found to have a lengthy latent period, as is found for other DNA viruses. Simple viruses such as CAV may require the host cell to enter the S phase of the cell cycle for the cell to be permissive for virus replication. As the number of actively dividing cells at the time of infection may influence the latent period, the cells were passaged 24 h prior to infection. We found that, for wild-type infection of MDCC-MSB1 cells, infectious virus was detected in the extracellular supernatant prior to intracellular virus, but that, ultimately, virus in the intracellular fraction was of higher titre than virus in the extracellular fraction. For many viruses, modifications to the virus capsid or envelope at the point of release from the cell confer infectivity on the virus particle and, for these viruses, infectious virus is found first in the supernatant. No such modifications have been identified for the CAV particle and this is unlikely to be a factor in CAV infectivity, as infectious virus is also found intracellularly at a later stage after infection. It is possible that infectious virus was present first in the supernatant because virus assembly and release coincide temporally with host-cell lysis.

Mutation of the VP2 gene conferred distinct growth characteristics on all viruses tested. All mutants could be grouped into one of three different types of growth curve, the first being the wild-type pattern. Virus Mut R/K/K150/151/152G/A/A had a growth curve similar to that of wild-type virus. The second cluster of viruses, Mut C87R, Mut H103Y, Mut D169G and Mut E186G, had eclipse and latent periods that were prolonged by about the same amount relative to wild-type. As for infection with wild-type virus, infectious virus was first detected extracellularly. The prolonged eclipse period may reflect greater reliance on active cell division than for wild-type virus. For the third cluster of viruses, Mut R101G, Mut R129G, Mut Q131P, Mut D/E161/162G/G and Mut L163P, the eclipse period was prolonged by an amount similar to most other mutants, but the latent period was

### Table 3. Expression of MHC class I on the cell surface of CD8<sup>+</sup> MHC class I<sup>+</sup> MDCC-MSB1 cells infected with wild-type or VP2-mutated CAU269/7

<table>
<thead>
<tr>
<th>Virus</th>
<th>Events*</th>
<th>Fluorescence intensity†</th>
<th>Coefficient of variance</th>
<th>Median intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCC-MSB1</td>
<td>9452</td>
<td>52.92</td>
<td>75.68</td>
<td>57.25</td>
</tr>
<tr>
<td>CAU269/7</td>
<td>8680</td>
<td>30.83</td>
<td>86.51</td>
<td>31.34</td>
</tr>
<tr>
<td>Mut R101G</td>
<td>8552</td>
<td>63.42</td>
<td>61.19</td>
<td>64.36</td>
</tr>
<tr>
<td>Mut H103Y</td>
<td>8768</td>
<td>54.42</td>
<td>64.41</td>
<td>57.25</td>
</tr>
<tr>
<td>Mut Q131P</td>
<td>8503</td>
<td>41.97</td>
<td>78.68</td>
<td>43.71</td>
</tr>
</tbody>
</table>

*Number of MDCC-MSB1 cells analysed by FACS.
†Geometric mean of fluorescence intensity from MHC class I–PE cell-surface immunostaining.
much more prolonged. For these viruses, infectious virus was first detected intracellularly, rather than extracellularly. The long latent period and the significant interval between infectious virus being found intracellularly and being released into the supernatant may reflect a reduced capacity for assembly and release of virions. The burst size for wild-type CAU269/7 and for viruses Mut H103Y, Mut Q131P and Mut D/E161/162G/G was 106 TCID50 ml−1.

Therefore, it could be expected that these viruses could be grown to titres equivalent to wild-type under appropriate culture conditions. For viruses Mut C87R, Mut R101G, Mut R129G, Mut R/K/K150/151/152G/A/A, Mut L163P and Mut D169G, burst size was approximately tenfold lower than that for wild-type.

Virus Mut K102D grew in cell culture following transfection. However, reinfection with cell-free virus resulted in only very low levels of infectivity, even with repeated passaging, suggesting that the mutation resulted in inefficient virus replication and infectivity, as seen for viruses containing the C95S and C97S mutations (Peters et al., 2002, 2005). Both the C95S and C97S mutations are known to either reduce or abrogate VP2 PTPase activity in vitro (Peters et al., 2002, 2005). Interestingly, there are two basic residues within the signature motif CXCCXXXRK of CAV VP2 DSP, at positions R101 and K102. As the mutation R101G does not significantly affect replication efficiency, it seems likely that it might not induce a reduction in DSP activity. Alternatively, it might be postulated that the attenuation observed for the R101G mutation may involve modification of only the serine/threonine protein phosphatase (S/T PPase) activity of the DSP, which does not appear to be linked as closely to reduced replication efficiency because, even though the C95S mutation abrogates S/T PPase activity and the C97S mutation enhances this activity, viruses with either of these mutations have marked reductions in replication efficiency (Peters et al., 2002, 2005).

VP3 alone can induce apoptosis in transformed cell lines, but not in normal cells. VP3 is rapidly compartmentalized into the nucleus of transformed cells, whereas it remains localized in the cytoplasm of untransformed cells (Danen-Van Oorschot et al., 1997). Deletion of the nuclear transport signals in VP3 blocks its capacity to induce apoptosis in transformed cells. Nuclear transport of VP3 in transformed cell lines is dependent on phosphorylation of threonine 108. During infection with VP2 mutants, VP3 was distributed only in the cytoplasm and, in some cases, in focal cytoplasmic aggregates or granules, paralleling the difference observed between transformed and normal cells (Noteborn, 2004). It is possible that VP2 mutations alter the trafficking of VP3 into the nucleus that normally occurs in transformed cell lines. Thus, VP2 may regulate the phosphorylation state of VP3 and therefore the induction of apoptosis in a cell. As virus replication occurs within the nucleus, it is possible that compartmentalization of VP3 in the cytoplasm may result in prolongation of the latent period.

Downregulation of MHC class I cell-surface expression was found in CD8+ MDCC-MSB1 cells after infection with CAU269/7. MHC class I expression is typically upregulated in an adaptive antiviral response (Joyce, 2001). Although MDCC-MSB1 cells are functionally and phenotypically distinct from untransformed lymphocytes in vivo, the virus-specific downregulation of MHC class I suggests an interaction with host-cell messenger pathways that may also occur in untransformed lymphocytes and is thus a potential viral mechanism for suppression of cytotoxic lymphocyte responses.

VP2 mutations R101G and H103Y significantly abrogated the virally induced downregulation of MHC class I and mutation Q131P partially abrogated the effect. This confirmed that MHC class I downregulation was a virus-specific effect and suggests that VP2 is a mediator of MHC class I downregulation. The substitutions R101G and H103Y had a predicted location in the peptide loop containing the DSP signature motif, whereas substitution Q131P had a predicted location in a segment of α-helix adjacent to the catalytic cleft. As many protein phosphatases have interactions with lymphocyte signalling pathways, it is possible that VP2 DSP activity may have been a mediator of the MHC class I downregulation.

Mutations introduced into the region of VP2 predicted to have a high degree of secondary structure either neutralized acidic or basic charged residues or introduced prolines into a sequence that was predicted to form an α-helix. Although functional roles for this region are unknown, it was hypothesized that a high degree of secondary structure would correlate with a functional role. Subtle alterations to conformation may potentially change affinities or specificities for binding with other proteins. Although the effect of these mutations on VP2 function is not known, the phenotypic effect, both in CPE and growth characteristics, suggests a structure–function relationship. The differences in growth characteristics between these viruses suggest that the mutations had different effects on VP2 function.

Thus, the studies reported here have identified a previously unrecognized effect of CAV on lymphocytic cells and have also demonstrated the effect of mutations in VP2 on replication, CPE and MHC class I downregulation in a lymphocytic cell line.

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inclusion body hepatitis viruses in their offspring. Vet Res Commun 26, 397–405.


