Identification of a genetic determinant of pathogenicity in chicken anaemia virus

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The molecular basis of pathogenicity of the chicken anaemia virus (CAV) needs to be clarified in order to develop a safe, live virus vaccine. In this study, several high- and low-pathogenic infectious DNA clones were obtained from field virus samples after 12 or 38 passages in MDCC-MSB1 cells. The high-pathogenic clones induced a low haematocrit, low weight gain and high mortality. Nucleotide sequence analyses identified one amino acid, at residue 394 of the VP1 capsid protein, as a major determinant of pathogenicity. To determine the role of this amino acid in pathogenicity, chimeric infectious DNA clones and point-mutated clones were used for chicken pathogenicity tests. These analyses clearly demonstrated that residue 394 of VP1 was crucial for the pathogenicity of CAV; all of the cloned viruses with glutamine at this position were highly pathogenic, whereas those with histidine had low pathogenicity. Low-pathogenic CAV, based on an infectious DNA clone, is a candidate for a genetically homogeneous and stable CAV live vaccine.

Introduction

Chicken anaemia virus (CAV) was first isolated in Japan and its pathogenic features were characterized (Taniguchi et al., 1982; Yuasa et al., 1979). CAV infection in chicken flocks occurs worldwide and can result in economically important clinical or subclinical disease in broiler chickens (McNulty, 1991; McNulty et al., 1991; Yuasa et al., 1987). CAV is classified in the virus family Circoviridae on the basis of its morphology and its single-stranded, circular genome (Studdert, 1993). The double-stranded replicative form (RF) of CAV DNA has been cloned and shown to produce an infectious virus following transfection to MDCC-MSB1 cells (Claessens et al., 1991; Noteborn et al., 1991). No antigenic differences have been recognized among CAV isolates by serum-neutralizing tests (Yuasa & Imai, 1986) and all isolates are suspected to belong to a single serotype (McNulty, 1991; Noteborn & Koch, 1995).

CAV infection causes mortality, severe anaemia, atrophy of the thymus and yellowish bone marrow in young chickens (Goryo et al., 1985; Otaki et al., 1987; Taniguchi et al., 1982; Todd et al., 1995; Yuasa et al., 1979; Yuasa & Imai, 1986). However, chickens become resistant to the disease by 1 month of age (Rosenberger & Cloud, 1989; Yuasa & Imai, 1986). The presence of a maternal antibody is protective in experimental infections with CAV (Yuasa et al., 1980). Therefore, the disease associated with CAV infection has been prevented by immunization of breeding flocks with live virus vaccines. As vertical transmission through the hatching egg is suspected to be the most important means of transmission of CAV infection (Chettle et al., 1989; Hoop, 1992), vaccine strains should have low pathogenicity even for young chickens.

This paper describes the generation of a low-pathogenic cloned virus by molecular cloning of CAV DNA from a low-passage CAV in cell culture and identification of the pathogenicity determinant in the genome of CAV.

Methods

+ Cells and viruses. The chicken lymphoblastoid cell line MDCC-MSB1 (Akiyama & Kato, 1974) was used for propagation and infectivity titration of CAV. The CAV pools AH9410 (AH) (Takagi et al., 1996) and A2 (Yuasa & Imai, 1986), which had respectively been passaged 12 and 38 times in MDCC-MSB1 cells, were used for the preparation of CAV RF-DNA and for pathogenicity tests.

+ Cloning of RF-DNA of CAV. DNAs were extracted by the Hirt procedure (Hirt, 1967) as described previously (Scott et al., 1999) from MDCC-MSB1 cells infected with the AH and A2 virus pools. The extracted DNAs were treated with PstI or XhoI to generate a linear RF-DNA and ligated into the cloning site of pBluescript SK+ (Stratagene). Recombinant plasmids containing CAV RF-DNA was selected by colony
Table 1. Construction of single amino acid-substituted clones and their pathogenicity. The linearized CAV genome and its genetic structure are shown at the top of the figure. The names of each clone are shown on the left and severity of pathogenicity (as determined by haematocrit) is shown on the right. AH-C140Q and AH-C364H were produced by replacement of the original XhoI–NcoI fragment with the XhoI–NcoI fragments of AH-C364 and AH-C140, respectively. A2-C15H was produced by oligonucleotide-mediated site-directed mutagenesis. Amino acid differences between the mutants and the parental clones are shown by the one-letter amino acid code.

**Fig. 1.** Construction of single amino acid-substituted clones and their pathogenicity. The nucleotide sequence of the full-length RF-DNA was confirmed by detection of a 2.3 kb fragment by restriction enzyme digestion and agarose gel electrophoresis.

**Nucleotide sequence determination.** The nucleotide sequence of the full-length RF-DNA of CAV was determined for the selected AH- and A2-derived clones. Thermal cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing kit; Perkin-Elmer) was used as described by the manufacturer. Purified plasmid DNA prepared with a kit (Wizard Miniprep DNA Purification kit; Promega) was used as the template. Sequencing was performed in both directions with virus-specific primers based on the Cux-1 sequence (accession no. M55918; Noteborn et al., 1991). Insertion of full-length RF-DNA was confirmed by detection of a 2.3 kb fragment by restriction enzyme digestion and agarose gel electrophoresis.

**Preparation of molecularly cloned CAV.** Full-length CAV RF-DNA was excised from the recombinant plasmid by restriction enzyme digestion. DNA fragments were isolated with a kit (QIAquick PCR purification kit; Qiagen) and circularized by ligation. The ligated DNA solutions, containing about 10 ng CAV RF-DNA in 5 µl, were used to transfect 10^5–6 MDCC-MSB1 cells in 0.1 ml by the DEAE-dextran protocol, as described by the manufacturer (CellPhect transfection kit; Pharmacia Biotech).

**Quantification of CAV.** Infectivity titration of CAV was performed by the microtiter method, as described previously (Izai & Yuasa, 1990). Serial tenfold dilutions of the virus samples were prepared and 20 µl of each dilution was inoculated into each of two wells containing 4 x 10^4 MDCC-MSB1 cells in 200 µl. The results were read by examining the cytopathic effect (CPE) after seven subcultures.

Quantification of CAV grown in inoculated chicks was performed by competitive PCR as described previously (Yamaguchi et al., 2000). DNA was isolated from 10% (w/v) liver homogenates by phenol–SDS treatment and ethanol precipitation. In this CAV quantification, the number of molecules of CAV DNA was estimated by comparing the amount of PCR product generated from the wild-type template and that from the competitive template (33 nt deleted) by agarose-gel electrophoresis.

**Substitution of one amino acid in CAV cloned virus.** Two molecularly cloned viruses, AH-C140 and AH-C364, were selected from each of the low- and high-pathogenic clones for single amino acid substitution in the AH-derived clones. Substitution of a single amino acid, at VP1 residue 394, was achieved by exchanging the XhoI–NcoI DNA fragments of the clones. The resulting mutated clones were designated AH-C140Q and AH-C364H (Fig. 1). To achieve the amino acid substitution in the A2-derived clone A2-C15, the XhoI–NcoI fragment was subcloned into a plasmid and oligonucleotide-mediated site-directed mutagenesis was performed using a kit, as described by the manufacturer (Mutant-Super Express Km kit, Takara). After sequence analysis, the mutated XhoI–NcoI fragment was reintegrated into the original clone and the sites of mutation and ligation were verified by sequencing.

**Experimental inoculation.** One-day-old specific-pathogen-free (SPF) chicks were used for evaluation of the pathogenicity of CAV. SPF chicks were inoculated intramuscularly with 0.1 ml of the virus solution, or the cell culture medium as an uninoculated control group, and all groups were reared in a negative-pressurized isolator with a filtered air ventilation system. For evaluation of pathogenicity, the chicks were examined by haematocrit at 14 days post-inoculation (p.i.), body weight (BW) at 14 days p.i. and mortality during the experimental period. The mean BW in each group was calculated and the ratio to that of the uninoculated control group was determined. If chicks died before 5 days p.i., they were considered as accidental deaths and eliminated from the experiment. The severity of pathogenicity during each examination was expressed as +, ++ or − for mean percentage haematocrit (+, > 18%; ++, 18–27%; −, > 27%), mean BW ratio (+, < 0.8; ++, 0.8–0.9; −, > 0.9) and mortality (+, > 30%; ++, 10–30%; −, < 10%).

In Experiment A, for screening of low-pathogenic clones, groups of four to ten chicks were inoculated with the molecularly cloned viruses or virus pools containing 10^5–10^6 TCID₅₀ in 0.1 ml and reared for 14 days. Experiments A-1 and A-3 were screening tests with the AH- and A2-derived clones, respectively, and used four chicks in each group. Experiment A-2 was done to confirm the results of experiment A-1 with selected clones and used 10 chicks in each group.

In experiment B, experiments for the determination of the effect of a single amino acid substitution on pathogenicity, groups of 12 chicks were inoculated with the cloned virus containing 10^5 or 10^6 TCID₅₀ in 0.1 ml. Ten chicks in each group were reared for 21 days to evaluate pathogenicity and the remaining two chicks in each group were used for examination of virus growth by quantification of CAV DNA in liver at 7 days p.i. The liver homogenate from each inoculated chick was also used for direct PCR sequencing to confirm the sequence of the virus produced.

**Results**

**Preparation of molecularly cloned virus**

The transfected MDCC-MSB1 cells started to show characteristic CPE as a result of CAV growth by 4 days after transfection. Resulting from the transfection, 10 and two cloned viruses were obtained, respectively, from the AH and A2 virus pools. The cloned viruses were passaged twice or three times in MDCC-MSB1 cells before experimental use and they showed infectivity titres of 10^6.2–10^7.2 TCID₅₀/0.1 ml.
Table 1. Pathogenicity screening test of molecular cloned CAVs and CAV virus pools (expt A)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of chicks</th>
<th>Titre of inoculum (log₁₀ TCID₅₀ per 0·1 ml)</th>
<th>Haematocrit (severity)</th>
<th>BW ratio (severity)</th>
<th>Mortality (severity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt A-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>4</td>
<td>–</td>
<td>32·3% (−)</td>
<td>1·00 (−)</td>
<td>0/4 (−)</td>
</tr>
<tr>
<td>AH-C140</td>
<td>4</td>
<td>8·2</td>
<td>23·0% (+)</td>
<td>0·93 (−)</td>
<td>0/4 (−)</td>
</tr>
<tr>
<td>AH-C363</td>
<td>4</td>
<td>8·2</td>
<td>24·3% (+)</td>
<td>0·86 (−)</td>
<td>0/4 (−)</td>
</tr>
<tr>
<td>AH-C364</td>
<td>4</td>
<td>8·2</td>
<td>9·0% (++)</td>
<td>0·61 (++)</td>
<td>2/4 (++)</td>
</tr>
<tr>
<td>AH-C366</td>
<td>4</td>
<td>8·2</td>
<td>16·7% (+)</td>
<td>0·91 (−)</td>
<td>1/4 (+)</td>
</tr>
<tr>
<td>AH-C367</td>
<td>4</td>
<td>8·2</td>
<td>13·5% (+)</td>
<td>0·70 (++)</td>
<td>2/4 (++)</td>
</tr>
<tr>
<td>AH-C368</td>
<td>4</td>
<td>8·2</td>
<td>10·0% (+)</td>
<td>0·68 (++)</td>
<td>3/4 (++)</td>
</tr>
<tr>
<td>AH-C369</td>
<td>3</td>
<td>8·2</td>
<td>19·0% (+)</td>
<td>0·90 (−)</td>
<td>0/3 (−)</td>
</tr>
<tr>
<td>AH-C370</td>
<td>3</td>
<td>8·2</td>
<td></td>
<td>NE</td>
<td>3/3 (+)</td>
</tr>
<tr>
<td>AH virus pool</td>
<td>4</td>
<td>8·2</td>
<td>13·0% (++)</td>
<td>0·71 (++)</td>
<td>2/4 (++)</td>
</tr>
<tr>
<td>Expt A-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>10</td>
<td>−</td>
<td>31·9% (−)</td>
<td>1·00 (−)</td>
<td>0/10 (−)</td>
</tr>
<tr>
<td>AH-C140</td>
<td>10</td>
<td>8·2</td>
<td>20·6% (+)</td>
<td>0·93 (−)</td>
<td>0/10 (−)</td>
</tr>
<tr>
<td>AH-C363</td>
<td>10</td>
<td>8·2</td>
<td>11·8% (++)</td>
<td>0·76 (++)</td>
<td>4/10 (++)</td>
</tr>
<tr>
<td>AH-C368</td>
<td>10</td>
<td>8·2</td>
<td>14·5% (++)</td>
<td>0·78 (++)</td>
<td>0/10 (−)</td>
</tr>
<tr>
<td>AH-C369</td>
<td>10</td>
<td>8·2</td>
<td>18·2% (+)</td>
<td>0·97 (−)</td>
<td>1/10 (+)</td>
</tr>
<tr>
<td>Expt A-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>4</td>
<td>−</td>
<td>31·5% (−)</td>
<td>1·00 (−)</td>
<td>0/4 (−)</td>
</tr>
<tr>
<td>A2-C15</td>
<td>4</td>
<td>5·7</td>
<td>13·0% (++)</td>
<td>0·62 (++)</td>
<td>1/4 (+)</td>
</tr>
<tr>
<td>A2 virus pool</td>
<td>4</td>
<td></td>
<td>15·0% (++)</td>
<td>0·65 (++)</td>
<td>1/4 (+)</td>
</tr>
</tbody>
</table>

NE, Not examined.

Experiment A

Eight and one molecularly cloned viruses obtained from the AH and A2 virus pools, respectively, were used for evaluation of pathogenicity to chicks together with the parental virus pools. The uninoculated groups showed no mortality and a normal haematocrit in each experiment. All of the inoculated groups showed various severities of pathogenicity as determined by haematocrit, mean BW ratio and mortality. Each group showed similar pathogenic severity in the three examinations. The pathogenic severity determined in experiment A-1 was confirmed in experiment A-2, especially for the haematocrit. Both the AH and A2 virus pools and some cloned viruses, such as AH-C364, AH-C367, AH-C368, AH-C370 and A2-C15, were scored as ‘+ + ’ for severity in most of the examinations and were judged as highly pathogenic. The other groups, such as AH-C140, AH-C363 and AH-C369, gave scores of ‘− ’ or ‘+ ’ for severity in all examinations and were judged as having low pathogenicity (Table 1).

Sequence differences among clones

Three clones from the low-pathogenic group (AH-C140, AH-C363 and AH-C369) and four clones from the high-pathogenic group (AH-C364, AH-C368, AH-C370 and A2-C15) were selected on the basis of the results of pathogenicity tests in experiment A. To search for sequence differences between the high- and low-pathogenic clones, nucleotide sequences of the entire genome were determined and compared. AH-C140 and AH-C364 had nucleotide sequences completely identical to those of AH-C363 and AH-C370, respectively, and four genetically different clones (accession nos AB046587–AB046590) existed among the AH-derived clones. Among the six AH-derived clones, three nucleotides differences were detected over the entire genome, each leading to amino acid differences, at VP1 residues 141, 394 and 444. Of the three amino acid residues, only residue 394 was correlated with the pathogenic phenotype, and that was glutamine in the high-pathogenic clones and histidine in the low-pathogenic clones (Fig. 2). Clones AH-C368 and AH-C369, which were respectively high- and low-pathogenic, showed only one amino acid difference, at position 394 in VP1.

The A2-derived clone, A2-C15 (accession number AB031296), was highly pathogenic and showed six nucleotide differences in the untranslated region and 10 amino acid differences in the VP1 coding region compared with AH-C140. The amino acid at VP1 residue 394 was glutamine.

Experiment B

One clone from the low-pathogenic group, AH-C140, and two clones from the high-pathogenic group, AH-C364 and
A2-C15, were selected and used for substitution of VP1 residue 394. No chicks died before 14 days p.i. in any group used for pathogenicity examinations. Clone AH-C140Q, which was created from AH-C140 by substitution of glutamine for histidine at VP1 residue 394, showed distinctly higher pathogenicity in all examinations compared with the parental clone (Table 2). On the other hand, clones AH-C364H and A2-C15H, which were created by substitution of histidine for glutamine, showed distinctly lower pathogenicity than the parental clones.

All clones with glutamine at the VP1 residue 394 were highly pathogenic and were scored as + + or + for severity,

Table 2. Determination of pathogenicity with CAV clones and their mutants (expt B)

<table>
<thead>
<tr>
<th>Group</th>
<th>Amino acid at VP1 residue 394</th>
<th>No. of chicks used for determination of:</th>
<th>Titre of inoculum (log10 TCID50 per 0.1 ml)</th>
<th>Pathogenicity examinations</th>
<th>Mean DNA content (log10 molecules/µl)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haematocrit (mean ± SD)</td>
<td>Mean BW ratio (severity)</td>
</tr>
<tr>
<td>Expt B-1</td>
<td></td>
<td></td>
<td></td>
<td>(severity)</td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>–</td>
<td>10</td>
<td>2</td>
<td>33.7 ± 1.5% (-)</td>
<td>1.0/0 (-)</td>
</tr>
<tr>
<td>AH-C140</td>
<td>H</td>
<td>10</td>
<td>2</td>
<td>6.2</td>
<td>23.2 ± 3.6% (+)</td>
</tr>
<tr>
<td>AH-C140Q</td>
<td>Q</td>
<td>10</td>
<td>2</td>
<td>6.2</td>
<td>15.2 ± 3.4% (+ +)</td>
</tr>
<tr>
<td>AH-C364</td>
<td>Q</td>
<td>9</td>
<td>2</td>
<td>6.2</td>
<td>10.4 ± 1.9% (+)</td>
</tr>
<tr>
<td>AH-C364H</td>
<td>H</td>
<td>10</td>
<td>2</td>
<td>6.2</td>
<td>26.0 ± 6.4% (+)</td>
</tr>
<tr>
<td>Expt B-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>–</td>
<td>10</td>
<td>2</td>
<td>33.8 ± 2.1% (-)</td>
<td>1.00 (−)</td>
</tr>
<tr>
<td>A2-C15</td>
<td>Q</td>
<td>10</td>
<td>2</td>
<td>5.5</td>
<td>16.8 ± 5.3% (+ +)</td>
</tr>
<tr>
<td>A2-C15H</td>
<td>H</td>
<td>10</td>
<td>2</td>
<td>5.5</td>
<td>31.1 ± 3.4% (-)</td>
</tr>
</tbody>
</table>

* CAV DNA content in 10% liver homogenates was quantified by competitive PCR. Two chicks were used for the quantification at 7 days p.i.
whereas the clones with histidine at this position were of low pathogenicity and were scored as — or + for severity in every examination (Table 2, Fig. 1). The mean number of CAV DNA molecules in the liver homogenate reached more than 10^{8} copies/μl at 7 days p.i. in all inoculated groups (Table 2). The CAV sequences detected in the liver homogenates of individual chicks were confirmed by direct PCR sequencing as which that was inoculated.

**Discussion**

We have demonstrated that a single amino acid, at position 394 in VP1, was crucial for the pathogenicity of CAV. Substitution of one amino acid in three cloned viruses could alter the pathogenicity of the virus distinctly. When the amino acid was glutamine, the virus showed high pathogenicity, and when it was histidine, the virus had low pathogenicity. There was no obvious difference between clones with glutamine and histidine at VP1 residue 394 in terms of virus yield, CPE kinetics or antigenicity by immunofluorescence tests in MDCC-MSB1 cells (data not shown).

We could obtain low-pathogenic clones successfully from the AH isolate. This is the first report of the isolation of low-pathogenic CAV clones from a non-attenuated virus pool. It was shown that the AH virus pool used in this study consisted of a mixed population of genetically and pathogenically diverse clones. Genetically and pathogenically diverse clones were also obtained from multiply passaged CAV isolates in MDCC-MSB1 cells by Scott et al. (1999) and Todd et al. (1995). Spontaneous mutation may occur during propagation in chickens and in cell culture, and most CAV isolates may consist of a genetically diverse mixed population. As there is no effective conventional method of virus selection for CAV, such as plaque cloning, recombinant DNA cloning and transfection methodologies are essential for biological characterization of CAV isolates.

There has been no report of a low-pathogenic CAV isolate obtained from field materials, as far as we know. All 28 CAV DNA sequences retrieved from DNA databases had glutamine as the putative amino acid corresponding to position 394 in VP1. Todd et al. (1995) attenuated CAV by multiple passage in MDCC-MSB1 cells and molecularly characterized the virus, but they could not define the genetic determinants of pathogenicity (Meehan et al., 1997; Todd et al., 1998). Their attenuated CAV clones possessed glutamine at this residue. There may be many genetic determinants of pathogenicity in the CAV genome, and the amino acid at VP1 residue 394 may be a crucial one.

Pathogenicity evaluation by examination of haematocrit was very reliable because the severity score was highly reproducible. The cloned viruses AH-C140 and AH-C364 were used three times in experimental inoculation to chicks, in experiments A-1, A-2 and B-1, and there were no significant differences in mean haematocrit among the experiments. Retardation of BW expressed as a ratio to the control group was also highly reproducible in each experiment and is an important factor for evaluation of the pathogenicity of CAV when the economic influence of this virus infection is considered.

Numbers of molecules of viral DNA in the livers of inoculated chicks seemed to be higher in the high-pathogenic clones (AH-C140Q and AH-C364) than in the low-pathogenic clones (AH-C140 and AH-C364H) at 7 days p.i. (Table 2). This should be confirmed statistically by using a number of chicks sufficient to define the pathogenicity mechanism of CAV. Previous papers have reported that the CAV VP3 protein induced apoptosis in cultured cells (Danen-Van Oorschot et al., 1997; Noteborn et al., 1994a; Pietersen et al., 1999) and revealed the existence of an enhancer/promoter region in the untranslated region (Noteborn et al., 1994b) that could be related to pathogenicity (Noteborn et al., 1998). The genetic determinant for pathogenicity defined in this study did not relate either to the VP3 protein or to the enhancer/promoter region but was related to the VP1 capsid protein. Amino acids in the virus capsid protein could be related to pathogenicity through the function of a receptor for target cells, such as erythroblastoid cells. Histidine belongs to the positively charged group and glutamine belongs to the polar but uncharged group, and this difference could influence the interaction between the virus and cells as a constituent of a receptor. Precise examination is necessary to define the function of this amino acid.

As the amino acid found in the low- and high-pathogenic clones was histidine (codon CAC) and glutamine (codon CAG orCAA), respectively, differentiation of low-pathogenic clones from high-pathogenic clones was possible by PCR with a primer corresponding to the low-pathogenic sequence (reverse primer: residues 2034–2013 of accession no. AB046587, 5’ TGTAGCTGTGCCGAACTTGTAG 3’) (data not shown). The naturally existing low-pathogenic cloned CAV could have advantages for development of a live vaccine. Because the virus genome was not modified by recombinant DNA techniques, it requires less complicated safety tests before field application. The naturally existing low-pathogenic clones in the AH virus pool may have been created by spontaneous point mutation and maintained in the virus pool without extinction. However, the genetic stability of the low-pathogenic clones obtained in this study must be assessed.

The methodologies applied in this study for the selection, identification and creation of low-pathogenic clones may be useful for the development of a genetically homogeneous and stable CAV live vaccine as well as for determination of the functions of CAV genes.

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