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Full-length infectious clone of a pathogenic Australian isolate of chicken anaemia virus

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Chicken anaemia virus (CAV) causes both clinical and subclinical infection in chickens throughout the world. CAV-induced anaemia results from destruction of haemopoietic precursor cells in the bone marrow and infection is also associated with marked immunosuppression due to the depletion of thymocytes, particularly young T cells.[1,2] CAV is a distinct member of the *Circoviridae* family of viruses that are characterised by their small, single-stranded, circular DNA genome. In studies in the Netherlands, Japan and Northern Ireland, the full-length CAV genome has been cloned into plasmid vectors in a form that is infectious when transfected into in vitro cultured cells.[3-6] Such molecular clones are important tools for analysing the function of the virus's genes and for dissection of the pathogenesis of disease caused by CAV. The purpose of this study was to derive an infectious molecular clone of a virulent Australian isolate of CAV to further studies of the virus in Australia.

The CAV isolate used in this study (CAU269/7) was obtained from D O'Rouke and T Bagust (Faculty of Veterinary Science, The University of Melbourne) and was originally isolated from a commercial breeder flock in Australia.[7] The virus was cultured in the Marek's disease virus transformed lymphoblastoid cell line, MDCC-MSB1,[2] based on the method described by McNulty et al.[8] Inoculation of CAU269/7 into MDCC-MSB1 cells produced cytopathic effects consistent with that of other reported CAV isolates[9] and was characterised by the appearance of enlarged, misshapen cells within 40 h. Total cell degeneration was apparent within 96 h after infection. Infected cells were stained for the CAV-specific protein, VP3, in an indirect immunofluorescence assay (IFA) following the method of Renshaw et al[10] using the mouse derived monoclonal antibody JCU/CAV/1C1 (JCU TropBio, Townsville, Queensland). Infected cells showed strong fluorescence relative to mock-infected control cells.

To assess the pathogenicity of the Australian isolate CAU269/7 for chickens, culture medium (~$1x10^{5.5}$ $TCID_{50}$/mL) from CAV-infected cells was used to inoculate yolk sacs of fifteen CAV-free, 7-day-old, specific-pathogen-free (SPF) chicken embryos (SPAFAS, Parkville, Victoria). In parallel, fifteen 7-day-old chicken embryos were mock-infected with CAV-free culture medium. Thirteen days postinoculation (day 20; 1 day before hatch), five embryos from each group

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were euthanased and samples of heparinised peripheral blood were collected. No significant difference in packed cell volumes (PCV) were observed between the groups. Eight days later (day 7 posthatching), the PCV and total body weights of all remaining chicks were determined. At this time, infected chickens (n = 9; one dead from causes unrelated to CAV) had lower body weights (P = 0.049) and lower PCVs (P < 0.001) than the control group. All remaining chicks were euthanased at day 12 after hatching and samples of bone marrow were collected for analysis. Infected chicks (n = 5; four dead from CAV related infection) exhibited clinical signs associated with infection with CAV, including weakness, depression, anorexia, stunted growth, low PCV, pale bone marrow, thymus atrophy and haemorrhages in the subcutaneous tissues and skeletal muscles.[11] Body weights of infected chicks were significantly lower (P < 0.001) than those of uninfected chicks in the control group, which developed none of the clinical signs described above. In addition, CAV-infected cells, identified by IFA for VP3, were seen in bone marrow smears. These results were consistent with those observed by Yuasa and Yoshida,[12] with CAV having no detectable effect on the developing embryo but inducing a severe clinical disease after hatching.

To clone the genome of isolate CAU269/7, total DNA was isolated from CAV-infected MDCC-MSB1 cultures at 60 h postinfection using a proteinase K/SDS digestion and phenol/chloroform extraction protocol according to the method of Meehan et al.[5] For polymerase chain reaction (PCR) amplification of the CAV genome two pairs of oligonucleotide primers (C1 - 5' CTATCGAATTCCGAGTGGTTACTAT 3' and C7 - 5' CTTATTTGTGCCTTGCGCTA 3', C5 - 5' CAGTTTC-TAGACGGTCCTTC 3' and C25 - 5' CGGAATTC-GATTGTGCGGTGAACG 3') were synthesised. The sequences of these primers were based on the DNA sequence of the German CAECUX-1 CAV isolate.[6] Primer pairs (C1+C7 and C5+C25) were used to generate two overlapping PCR products that covered the entire 2.3 kb CAV genome. These were digested with *Eco*RI and *Xba*I restriction endonucleases and ligated into pGEM-4Z (Promega, Sydney) that had been digested with the same enzymes to derive a plasmid containing the full-length genome of CAU269/7, designated pCAU269/7 (Figure 1). *Eco*RI restriction endonuclease sites were introduced at either end of the genome (shown in bold in oligonucleotide sequence) by PCR mutagenesis to facilitate release of the complete CAV genome from the plasmid vector (Figure 1A). This site was not present in CAU269/7 but does occur in other isolates at this position. The DNA sequence of CAU269/7 was determined using a combination of vector specific (T7 and SP6) and CAV sequence specific primers. The complete sequence of each DNA strand was determined at least twice and submitted to GenBank (Accession No. AF227982).

The organisation of the genome of Australian CAV isolate CAU269/7 was similar to other reported CAV isolates. It is composed of 2298 nucleotides and contains three overlapping open reading frames (ORFs) encoding the VP1, VP2 and VP3 proteins (Figure 1A) that have been identified in other CAV isolates.[3-6] CAU269/7 contained four almost-perfect 21 bp direct repeat sequences (nucleotides [nt] 144-237) upstream of a single transcriptional start site (position +1 in Figure 1A) and a polyadenylation signal (nt 2266). The small noncoding region of the CAV genome (nt 2185-2251) possesses a high GC content and contains sequences considered to be involved in the termination of transcription.[5] Others have reported difficulties

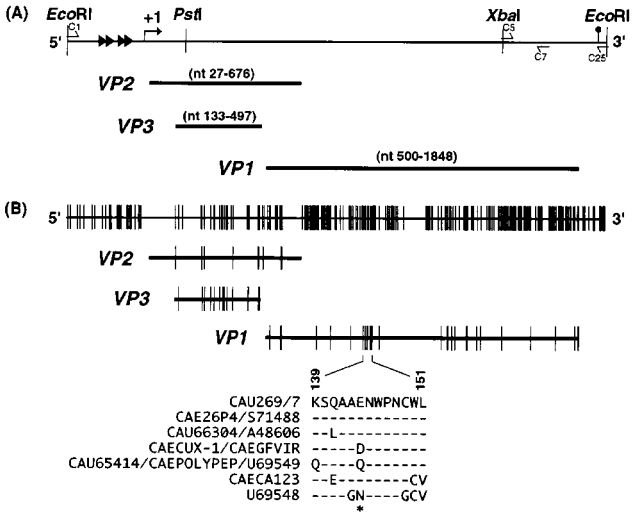

Figure 1 (A) Schematic representation of the linearised CAV genome. Sequences encoding open reading frames for the three known CAV proteins are indicated. Nucleotide positions are relative to the transcriptional start site, as indicated by an arrow and +1. The location of the promoter/enhancer repeat region is shown by four repeated arrow heads. Oligonucleotide primers employed in PCR amplification of the genome are indicated (arrows, C1-C7, C5-C25) as are the restriction sites used for cloning and orientation of the full-length cloned genome. (B) Synonymous and non-synonymous nucleotide differences between CAU269/7 and other reported CAV isolates. Each nucleotide position that varies in any of the sequenced CAV isolates is indicated on the genome by a line (top). Those changes that encode an amino acid change in VP1, 2 or 3 are indicated as lines on the relevant open reading frame (middle). Expanded at the bottom of the panel is an alignment of the 13 amino acid hypervariable region within VP1. The position that shows the most variability, residue 144, is highlighted (asterisk). The identities of the 11 distinct VP1 sequences used in the comparison were obtained from GenBank and are as follows: CAE26P4 (The Netherlands); S71488 (Australia); CAU66304 (Northern Ireland); A48606 (Northern Ireland); CAECUX-1 (Germany); CAEGFVIR (Northern Ireland); CAU65414 (Australia); CAEPOLYPEP (USA); U69549 (USA); CAECA123 (Japan); U69548 (USA).

in resolving the sequence in this region using standard chain termination sequencing chemistries.[5,6] We also experienced difficulties in determining the sequence of this region, despite the use of a range of standard approaches designed to resolve such sequences, and were unable to confirm the identity of 12 residues (nt 2193-2204). These residues do not vary between the known CAV isolates and so for the purposes of the analysis presented herein we have inserted the corresponding CAECUX-1 sequence into this region of CAU269/7.

CAU269/7 demonstrated an overall nucleotide sequence identity of approximately 95% with each of the seven other sequenced CAV isolates, indicating that this isolate was closely related to, but clearly distinct from, other isolates. This was a lower identity than any of the other isolates have with each other (96-99%). The amino acid identities of CAU269/7 with other sequenced isolates ranged from 96-99%, 98-99% and 94-98% for VP1, VP2 and VP3 proteins, respectively. The majority of nucleotide differences between the isolates were synonymous

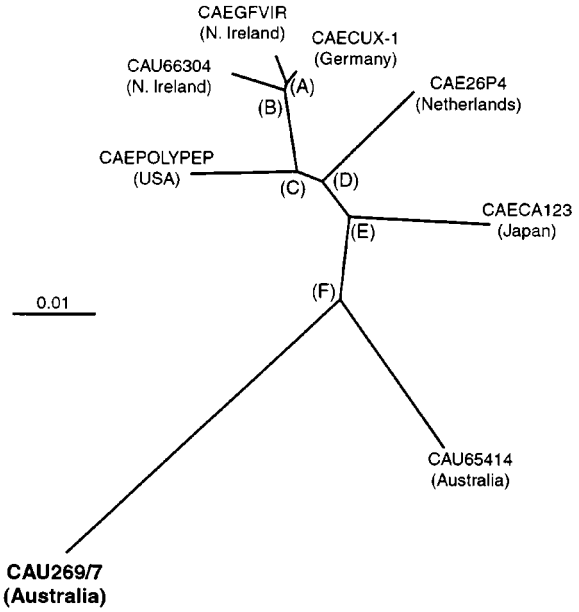

Figure 2. Phylogenetic tree inferred from the entire nucleotide sequences of all reported CAV isolates as calculated by the maximum likelihood method using the program EDNAML running on the Australian National Genomic Information Service. All branch lengths have significantly positive confidence limits (A, P=0.001; B, P=0.005; C, P=0.009; D, P=0.011; E, P=0.012; F, P=0.016).

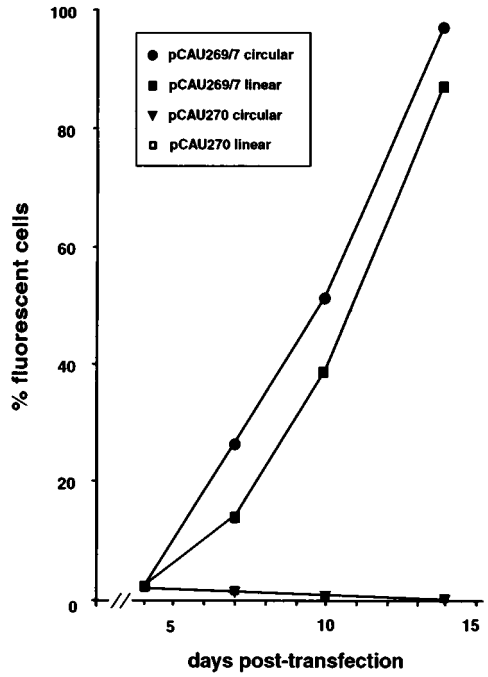

Figure 3. Expression of VP3 in MDCC-MSB1 cells following DNA transfection of both linear and circularised forms of the full-length CAV genomic clone, pCAU269/7. Transfected cells were passaged at 3-4 day intervals without freeze-thawing and stained for the CAV-specific protein, VP3, in an indirect immunofluorescence assay.

(Figure 1B). Most of the non-synonymous mutations, particularly in VP2 and VP3, were not consistently variable across isolates (data not shown). One 13-amino-acid region within VP1 (residues 139-151) contained a cluster of non-synonymous changes that did vary between isolates (Figure 1B). Naturally occurring amino acid changes at positions 139 and 144 have been shown to markedly affect the growth and spread of CAV isolates in cultured cells[10] highlighting a possible functional role for the hypervariable region. Residue 144 displays remarkable variability with four different amino acids found in this position (Figure 1B). It is likely that variability observed within residues 139-151 is a consequence of its biological significance and reflects the selection of variants in response to environmental/host cell changes and/or immune selection.

To more closely examine the evolutionary relationships of CAV isolates, a phylogenetic analysis was performed (Figure 2). While CAU269/7 is phylogenetically distinct from all other isolates, it appeared to be most closely related to another Australian isolate (CAU65414). Interestingly, these two Australian isolates have different sequences in the hypervariable region of VP1 (Figure 1B). This analysis also showed that CAU269/7 is more similar to the Japanese isolate than to those originating in Europe or the USA.

In order to determine the infectious nature of the cloned CAV DNA, the 2.3 kb CAV genomic fragment was released from pCAU269/7 by *Eco*RI digestion and transfected by electroporation into MDCC-MSB1 cells either in a linear form or as closed circular (re-ligated) DNA. Briefly, $4x10^6$ cells were washed twice with sterile PBS and resuspended in 700 µL PBS containing 10 µg DNA. Cells were pulsed with 400 V/375 µF then immediately transferred to 4.5 mL pre-warmed culture medium containing 10% foetal calf serum. Transfection of MDCC-MSB1 cells with the *Eco*RI-released insert of plasmid clone pCAU269/7 resulted in patterns of infection similar to those observed in virion-initiated infections. Usually 1 to 2 passages of transfected cells were required before virus-induced cell death was apparent. At several time points after transfection, cells were analysed for the presence of CAV by IFA. Cells transfected with *Eco*RI-digested pCAU269/7 reacted with the anti-VP3 monoclonal antibody, while cells transfected with control DNA, consisting of a truncated form of the full-length CAV genome, did not (Figure 3). Furthermore, medium from pCAU269/7 transfected cultures passaged onto fresh MDCC-MSB1 cultures produced cytopathic effects characteristic of CAV infection. Taken together, these data show that pCAU269/7 contains all the elements necessary for generating CAV infection in MDCC-MSB1 culture. This study has demonstrated the distinct sequence of an Australian isolate of CAV and has developed reagents suitable for future manipulation of CAV to generate attenuated variants suitable for vaccination.

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An unusual haemolymphoid mass in the retroperitoneum of a dairy cow

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HE	Haematoxylin and eosin
MA	Myeloid/histiocyte antigen
α-SMA	Alpha-smooth muscle actin
Factor VIII-related antigen	Von Willebrand factor

Haemolymph and haemal nodes are lymphatic organs located in the blood circulation of ruminants and rats.[1-3] In contrast, accessory spleen and splenosis are referred to by the term ectopic splenic tissue.[4] This study focuses on the histological and immunohistological examination of a retroperitoneal mass from a cow with watery diarrhoea. The histology of the lesion was consistent with lymphatic or splenic tissue with extramedullary haematopoiesis, but our pathological findings did not completely agree with those of previous studies on haemolymph and haemal nodes and ectopic splenic tissue.[1-4] Here, we describe an unusual case with the histopathological features of a haemolymphoid mass and the systemic clinical signs.

After calving, a 29-month-old Jersey cow showed severe, progressive, weight loss with anorexia and excessive alopecia with roughening of the hair coat. Eighty days post-partum the cow developed watery diarrhoea and pyrexia. Despite treatment with procaine penicillin G for 4 days the clinical condition was unchanged, and the animal was euthanased. Serum cholesterol (total cholesterol 0.65 mmol/L; reference range 2.07 to 3.11 mmol/L) and albumin (2.2 g/dL; reference range 30.3 to 35.5 g/dL) concentrations were significantly lower than normal. Haematological findings were all within normal limits. Antibodies to bovine leukemia virus were not detected by the agar gel immunodiffusion test.[5] Results of bacteriological and parasitological examination of the faeces for *Mycobacterium*, *Salmonella*, *Cryptosporidium*, protozoan cysts and helminth eggs were negative.

At necropsy a soft, red and white mottled, encapsulated mass, 25 x 12 x 15 cm, was detected in the left adrenal area in the abdominal cavity (Figure 1). There was no direct connection between this mass and other organs. It had a hilus and was supplied arterially from the abdominal aorta. The cut surface bulged, and was homogeneous, fleshy, and grayish white with red mottling due to haemorrhage. No invasive or metastatic lesions were noted in other organs. The spleen and systemic lymph nodes did not show any gross changes.

The mass and other tissues were fixed in 10% phosphate buffered formalin and embedded in paraffin. Tissue sections (approximately 3 μm thick) were obtained using routine histological techniques and were stained with HE and Azan for histological investigation. Paraffin wax-embedded sections were also labeled by the avidin-biotin-peroxidase complex immunoperoxidase technique (BioGenex Laboratories, San Ramon, CA, USA). The following were used as primary antibodies: mouse monoclonal antibodies to CD3, CD79a, MA, α-SMA, vimentin (Dako Corporation, Carpinteria, CA, USA), desmin (Bio-Science Products, Emmenbrücke, Switzerland), Factor VIII-related antigen (BioGenex Laboratories, San Ramon, CA, USA), and polyclonal antibodies to S100 protein (Nichirei

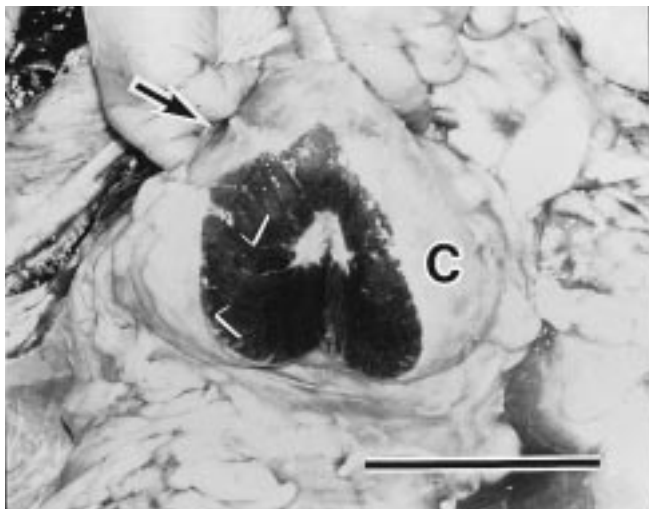

Figure 1. Gross appearance of the retroperitoneal mass cut open to show its atypical splenic pulp structure. Note the capsule (C), trabeculae (arrowheads), and hilus (arrow). Bar =13 cm.